A mathematical model for growth and osmoregulation in halophilic bacteria

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Many molecular details of the ecophysiology of halophilic bacteria that use compatible solutes to maintain osmotic equilibrium have been examined. We ask whether the details are consistent and complete enough to predict growth and osmoregulation in these bacteria by integrating this information in a mathematical model. Parameterized for the halophilic organism *Halomonas elongata*, the model predicts the substrate and salt dependence of growth, the uptake of potassium and ectoine and the synthesis of ectoine. It is shown that salt (NaCl) dependence of growth can be modelled by substrate inhibition kinetics. Osmoregulation is known to involve accumulation of both ectoine and potassium glutamate in *H. elongata*. Using published and newly determined parameters, osmoregulatory models using either direct turgor or two-step (turgor and potassium) signalling are compared. The results are consistent with a role for potassium as a second messenger for hyperosmotic stress. Simulations of osmotic upshifts show a transient overregulation of the intracellular solute levels, as has been previously observed in experiments. A possible adaptive value of this overregulation as 'pre-emptive' behaviour in an environment with frequent dry periods leading to steadily increasing osmolarity is proposed. As a result of growth parameter estimation, a maximum P:O value of 2 for *H. elongata* can be inferred. In conclusion, the model developed here reproduces essential aspects of growth and osmoregulation in halophilic bacteria with a minimal set of assumptions.

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INTRODUCTION

Oceans cover the major part of earth's surface; it is therefore not surprising that many micro-organisms are adapted to salt in their environment (35.3 g l⁻¹ in seawater; Grant, 2004). Maintaining the osmotic equilibrium between cytoplasm and environment is a major challenge to organisms in saline habitats. Some halophilic Bacteria and Archaea accumulate KCl while excluding Na⁺ ions from the cytoplasm. Since their cytoplasmic enzymes have adapted to the intracellular accumulation of KCl that compensates the osmolarity in their particular environment, these organisms are usually confined to a narrow range of salinities (Galinski, 1995; Ventosa *et al.*, 1998; Oren, 1999; Grant, 2004). A more flexible and widespread strategy is the accumulation of small, highly soluble molecules, termed compatible solutes (Brown, 1976). This strategy allows rapid adaptation to changes in osmolarity by adjusting the intracellular solute pools, and

tolerance of a wide range of salt concentrations. A large variety of different compatible solutes have been described (Galinski, 1995; Ventosa *et al.*, 1998; Oren, 1999; Grant, 2004), including non-reducing sugars, polyols, quaternary amines and other zwitterions, amino acids and their derivatives.

Adjustment of the intracellular solute pool involves uptake or synthesis of compatible solutes when extracellular osmolarity is rising and export of compatible solutes when extracellular osmolarity decreases. The first response to osmotic upshifts and the resulting efflux of cellular water is uptake of K⁺ and synthesis of counter-ions, e.g. glutamate in *Escherichia coli* (Morbach & Krämer, 2002). Following this fast response, cells start to accumulate compatible solutes by uptake or *de novo* synthesis, replacing K⁺-glutamate (Kempf & Bremer, 1998). Some of the stimuli which trigger activation of solute uptake systems have been revealed, and sensing of K⁺ concentration in the cytoplasm appears to play an important role (Wood, 2006). It has also been postulated that K⁺-glutamate acts as a second

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messenger (Kempf & Bremer, 1998; Lee & Gralla, 2004). However, details of the osmosensing mechanisms are not well understood. Mechanical properties of the cytoplasmic membrane, ionic strength, osmolality, macromolecular crowding and degree of hydration have all been suggested as potential stimuli for osmosensors (Poolman & Glaeser, 1998; Wood, 1999, 2006; Poolman *et al.*, 2002).

Osmotic downshifts necessitate the reduction of intracellular solute pools to prevent influx of water that could lead to the bursting of the cells. As a 'safety valve', mechanosensitive channels open if the turgor pressure rises beyond a certain threshold, forming a pore that allows the unselective efflux of water and small molecules like compatible solutes (Morbach & Krämer, 2002; Poolman *et al.*, 2002). The activity of these channels is also used to harvest compatible solutes from productive strains using alternating osmotic shifts in a process called 'bacterial milking' (Sauer & Galinski, 1998).

The bacterium *Halomonas elongata* DSM 2581^T was first isolated from solar salterns (Vreeland *et al.*, 1980) and can be classified as extremely halotolerant since it is able to grow over a very broad range of salt concentrations (<1 to 25 %, w/v, in minimal media) and as moderately halophilic because its growth optimum is near the salinity of seawater (~0.5 mol NaCl l⁻¹). *H. elongata* is a relatively well-studied member of the family *Halomonadaceae*, belonging to the γ -Proteobacteria (Vreeland, 1999). It can adapt to different osmotic conditions by synthesizing the compatible solute ectoine and its derivative hydroxyectoine (Severin *et al.*, 1992). It is assumed that ectoine synthesis is triggered by the initial accumulation of K⁺-glutamate after osmotic upshifts (Kraegeloh & Kunte, 2002). The pathway of ectoine synthesis from aspartate (Peters *et al.*, 1990), and specific uptake systems for ectoine (Grammann *et al.*, 2002) and potassium (Kraegeloh *et al.*, 2005), have been described.

We have chosen *H. elongata* as model organism, because it has the broadest salt tolerance of the better-studied organisms, allowing the investigation of salt effects over a wide range of salinities.

In the present work, we describe the development of a mathematical model for the growth of halophilic micro-organisms in continuous culture, including the regulation of uptake and synthesis of compatible solutes. We show that the salt dependence of the growth rate can be described by substrate inhibition models and that the regulation of accumulation of compatible solutes can be modelled as a two-step process with potassium acting as a second messenger.

METHODS

Bacterial strain and growth conditions. *Halomonas elongata* DSM 2581^T (Vreeland *et al.*, 1980), obtained from the DSMZ (Braunschweig, Germany), was used in all experiments in this study.

All batch cultures were grown aerobically at 30 °C with glucose as sole carbon and energy source and in various NaCl concentrations (0.17 to 3.57 mol l⁻¹). Batch cultures were first grown in 30 ml of complex medium K3, K12 or K20 (Severin *et al.*, 1992) (numbers indicate the NaCl concentration in %, w/v), to allow the cells to accommodate to the desired NaCl concentration (accommodation culture). In a second step, 5 ml (or less) of adaptation culture was transferred to 100 ml MM63 minimal medium (Larsen *et al.*, 1987) (pre-culture). NaCl concentrations in pre-cultures were the same as in the main cultures. The main cultures were inoculated with up to 5 ml of pre-culture and grown in 100 ml MM63 in 250 ml baffled shake flasks with side arms for measuring turbidity. Batch cultures for determining growth rate as a function of salt concentration were set up with the glucose mineral salt medium described as G10 by Severin *et al.* (1992). GXX media with NaCl concentrations (XX) from 1 to 25 (%), w/v) were used, which varied only in NaCl concentration.

Continuous cultures were grown in a 2-litre Biostat M fermenter (Braun), which was run as a chemostat or turbidostat with 1.5 l VVM medium at 30 °C, 0.15 l min⁻¹ aeration and 1000 r.p.m. stirring speed. The turbidostat mode was used at higher dilution rates to prevent accidental washout of the culture. Steady-state biomass density was controlled online using a flow-through cell (thickness 0.1 cm) fitted to a Novaspec II photometer (Pharmacia Biotech). VVM medium consisted of (g l⁻¹): glucose (10), MgSO₄·7H₂O (6.5), NH₄Cl (3), KCl (1), CaCl₂·2H₂O (0.01), KH₂PO₄ (0.5), FeSO₄·7H₂O (0.005), yeast extract (Difco) (0.1), trace element solution (Claus *et al.*, 1983) (1 ml l⁻¹) and vitamin solution VA (Imhoff & Trüper, 1977) (1 ml l⁻¹). NaCl was added to adjust salt concentration.

Analytical methods. Optical density was measured in a Novaspec II photometer (Pharmacia Biotech) at 600 nm against air. From steady states of continuous cultures, samples of 1.5 ml were rapidly taken and immediately frozen in liquid nitrogen. The thawed cell suspensions were centrifuged (5 min at 15 000 g) to determine the remaining substrate concentration in the supernatant. To estimate dry mass, further samples of exactly 1.5 ml were centrifuged directly and the pellet was dried for 48 h at 100 °C. For further analysis of biomass composition, about 150 ml cell suspension from selected steady states was harvested by centrifugation (15 min at 27 000 g) and freeze-dried. The inorganic salt content of this biomass was determined by incinerating 100 mg freeze-dried biomass for 1 h at 600 °C in order to correct the dry mass for differences in salt content. Ectoine was extracted from 30 mg freeze-dried cell material using a modified Bligh & Dyer method and analysed by HPLC (Wohlfarth *et al.*, 1990).

The concentration of glucose in samples of culture medium was determined by a photometric enzyme assay that couples glucose consumption to NADPH production (kit from R-Biopharm). Protein content was determined using bicinchoninic acid (Smith *et al.*, 1985) with a kit from Pierce.

Computational methods. The model consists of a set of ordinary differential equations that were solved numerically using algorithms for stiff systems (Klopfenberger–Shampine and Rosenbrock formulae; Shampine & Reichelt, 1997). Nonlinear regression was carried out using a least-squares method (Seber & Wild, 1989). The coefficients of determination (*R*² value) for nonlinear fits were calculated with *EzyFit* (free Matlab toolbox available at: <http://www.fast.u-psud.fr/ezyfit/>) based on the equation

$$R^2 = 1 - \frac{SS_{reg}}{SS_{tot}} = 1 - \frac{\sum_i [d_i - f(x)]^2}{\sum_i (d_i - \bar{d})^2} \quad (1)$$

with sums of squared differences between data *d_i* and fitted curve *f(x)* (*SS_{reg}*) and between data and arithmetic mean $\bar{d}$ (*SS_{tot}*).

The model. The model is based on the assumption of a well-mixed environment (e.g. chemostat) and thus spatial homogeneity. The state variables are biomass density (B), substrate concentration (S), and the cytoplasmic contents and extracellular concentrations of ectoine (E_{cyt} , E_{ex}) and potassium (K_{cyt} , K_{ex}); each variable's dynamics is described by an ordinary differential equation (Table 1). Since intracellular ectoine and potassium are components of the cell, their amount is not expressed as a concentration (amount per volume) but as a content (amount per biomass B), in accordance with experimental practice. An overview of the model processes and variables is provided in Fig. 1.

Biomass growth. The observed growth rate of micro-organisms depends on the temperature, the availability of carbon and energy sources, the presence of inhibitory compounds, and many other factors. We consider the dependence of growth on substrate and salt concentration. Therefore, we model growth g as a combination of two kinetic factors, one factor, $\gamma(S)$, for the dependence on the carbon and energy substrate, and another factor, $\xi(N)$, for salt dependence, with the maximum specific growth rate, g_{max} , as a common factor:

$$g = g_{\text{max}} \gamma(S) \xi(N) \quad (2)$$

Dependence of growth on the carbon and energy substrate was assumed to follow standard Monod kinetics:

$$\gamma(S) = \frac{S}{k_s + S} \quad (3)$$

The constant k_s represents a Michaelis–Menten type half-saturation constant and the usual μ_{max} is missing because it is part of g_{max} .

Salt is usually regarded to be an inhibitor of growth rather than a substrate, but even non-halophiles require a certain amount of salt to grow, and in this sense salt can be considered as a growth substrate. Therefore, in addition to other candidate models, we tested standard models of substrate inhibition of enzymes (Tables 2a and 2b) for their suitability to describe the salt dependence of growth, $\xi(N)$, by fitting these models to data from our growth experiments with *H. elongata* covering a wide range of salt concentrations. The model of Yano and Koga (Yano & Koga, 1969; Schröder *et al.*, 1997) assumes that substrate molecules can inhibit enzymes by binding to different sites. We tested this model for the case of two inhibitory sites (using the full version 'YanoKoga-F' and the reduced 'YanoKoga-R'). For only one inhibitory site, the YanoKoga models reduce to the well-known inhibition model of Haldane (Andrews, 1968) ('Haldane'). In addition to the enzyme kinetic models, we evaluated other published models such as that of Luong (1987), where growth becomes completely inhibited at the salt concentration N_{max} . The second parameter, n , determines the steepness of the growth rate decline from the maximum rate ('Luong'). Edwards (1970) introduced a model that assumes an exponential decrease in growth rate due to

Fig. 1. Schematic overview of the model. The figure illustrates dependencies between the system's state variables as described by the differential equations (Table 1). The regulation arrows correspond to the two-step osmoregulation models [Table 3, $\Delta\Pi/K(-d)$]. Substrate uptake (*) is part of the growth process rather than modelled explicitly.

substrate inhibition ('Edwards'), based on an analogous model for product inhibition previously published by Aiba *et al.* (1968). In addition, we also tested the so-called Arrhenius-type model (McMeekin *et al.*, 1993) that was previously used for modelling the combined effect of temperature and water activity (a_w) on growth

Table 1. Overview of mass balance equations

The contributions by different processes are separated into columns for easier interpretation. Symbols are explained in the text. E_{cyt} and K_{cyt} are expressed as amount per biomass (i.e. content).

	Growth/synthesis	Uptake	Leakage	Inflow	Outflow
$dB/dt =$	gB	$-(c_{\text{up},E}u_E + c_{\text{up},K}u_K)B$			$-DB$
$dE_{\text{ex}}/dt =$		$-u_E B$	$l_E B$	DE_r	$-DE_{\text{ex}}$
$dK_{\text{ex}}/dt =$		$-u_K B$	$l_K B$	DK_r	$-DK_{\text{ex}}$
$dS/dt =$	$-(Y_B^{-1}g + Y_E^{-1}p)B$			DS_r	$-DS$
$dE_{\text{cyt}}/dt =$	$p - gE_{\text{cyt}}$	u_E	$-l_E$		
$dK_{\text{cyt}}/dt =$	$-gK_{\text{cyt}}$	u_K	$-l_K$		

Table 2(a). Substrate inhibition models examined for their ability to describe the salt dependence of growth of *H. elongata*: rate expressions for the models that were examined

See text for further description.

Model name	Equation	No. of parameters	Reference
Haldane	$g_N = g_{\max, N} \frac{N}{k_N + N + N^2/k_I}$	3	Andrews (1968)
YanoKoga-F	$g_N = g_{\max, N} \frac{N}{k_N + N + N^2/k_1 + N^3/k_2^2}$	4	Yano & Koga (1969); Schröder <i>et al.</i> (1997)
YanoKoga-R	$g_N = g_{\max, N} \frac{N}{k_N + N + N^3/k_2^2}$	3	Yano & Koga (1969); Schröder <i>et al.</i> (1997)
Edwards	$g_N = g_{\max, N} \frac{N}{k_N + N} \exp(-N/k_I)$	3	Edwards (1970)
Luong	$g_N = g_{\max, N} \frac{N}{k_N + N} \left(1 - \frac{N}{N_{\max}}\right)^n$	4	Luong (1987)
Arrhenius-type	$\log(g_N) = C(T) + C_1 a_w + C_2 a_w^2$	3	McMeekin <i>et al.</i> (1993)
Hyperbolic	$g_N = g_{\max, N} \frac{N}{k_N + N} \frac{1}{k_I + N}$	3	This work

Table 2(b). Substrate inhibition models examined for their ability to describe the salt dependence of growth of *H. elongata*: parameter values for the only two models that resulted in good fits with well-defined parameters

The $\pm$ value indicates the span of the 95 % confidence intervals.

Model name	$g_{\max, N}$ (h^{-1})	k_N (mol l^{-1})	k_I (mol l^{-1})	k_2 (mol l^{-1})
Edwards	1.72 ± 0.80	0.91 ± 0.55	0.86 ± 0.55	–
YanoKoga	0.88 ± 0.24	0.51 ± 0.24	–	0.96 ± 0.19

rates of food-related micro-organisms. We fitted this model ('Arrhenius-type') assuming constant temperature and calculating a_w from the salinity.

Moreover, we tested our own model with a multiplicative hyperbolic inhibition term, because the free cytoplasmic water content decreases hyperbolically with salt concentration ('Hyperbolic'), and it is plausible that growth rate declines with the free cytoplasmic water content.

Osmoregulation. Osmoregulation, the proper adjustment of intracellular solute pools in response to a certain salinity, requires some mechanism of osmosensing, such as sensing of physical membrane stress, or concentration of solutes or macromolecules (macromolecular crowding) (Kempf & Bremer, 1998; Wood *et al.*, 2001; Morbach & Krämer, 2002). While the actual stimulus is unknown, all these properties are coupled so we chose turgor pressure as a proxy for osmoregulation in our model for simplicity. It is a direct measure of the difference between the internal and external osmotic pressure: $\Delta\Pi = \Pi_{\text{cyt}} - \Pi_{\text{ex}}$. The osmotic pressure of a non-ideal solution can be approximated using the Van't Hoff relation (Potts, 1994):

$$\Pi \approx RT \sum_i c_i$$

(Π , osmotic pressure; R , gas constant; T , absolute temperature; c_i , molar concentration of solute i , used to approximate the activity of a compound).

Using this relation, the osmotic pressure of both compartments can be calculated as follows:

$$\Pi_{\text{cyt}} = RT \left(O_{\text{cyt}}^0 + \frac{E_{\text{cyt}} + (1+v)K_{\text{cyt}}}{\bar{V}_{\text{cyto},f}^{wa}} \right) \quad (4)$$

$$\Pi_{\text{ex}} = RT \sum_i c_{i,\text{ex}} \quad (5)$$

where O_{cyt}^0 is the basal osmolarity (concentration of osmotically active solutes) of the cytoplasm (without ectoine and K^+ -glutamate), E_{cyt} and K_{cyt} denote the intracellular concentration of ectoine and potassium, v the relative concentration of counter-ions that are coaccumulated with K^+ to balance the charge and $\sum_i c_{i,\text{ex}}$ the summed concentrations of all extracellular osmolytes. The effect of K^+ accumulation is multiplied by a factor $(1+v)$ assuming that a certain concentration v of counter-ions accumulates (e.g. glutamate $^-$). The term $\bar{V}_{\text{cyto},f}^{wa}$ corresponds to the volume of free cytoplasmic water as described below (see Cellular water content).

Since the actual mechanism of osmoregulation is unknown, we evaluated the potential of hypothetical mechanisms of osmoregulation to explain available data on solute changes upon osmotic shocks, starting from the simplest possible mechanism (Table 3). Generally, osmoregulation begins with the perception of a stimulus, which is followed by the production of a signal. The intensity of perception, σ , of a given stimulus (turgor or potassium, respectively) is described by the following saturating function ($\sigma_i \in [0;1]$):

$$\sigma_i = \begin{cases} 1 - \exp\left(a s_i \left(\frac{X}{X_{th}} - 1\right)\right); & \sigma_i > 0 \\ 0; & \text{otherwise} \end{cases} \quad \text{for } i = K, E \quad (6)$$

with sign $a = \pm 1$, sensitivity s_i , stimulus X and threshold X_{th} . This function works as a switch returning a saturating positive response value for input values passing a threshold, where a determines if the response is triggered by input values above ($a = -1$) or below ($a = 1$) the threshold.

The signal response is represented by ω , the level of a hypothetical intracellular signal molecule. Three hypothetical mechanisms were investigated that differ in the stimulus that triggers accumulation of ectoine (turgor or potassium) and in the characteristic of the signal

Table 3. Different approaches to osmosensing and regulation tested in the model

The stimulus functions σ_i ($i=E, K$) are described by equation (6). ω_p , osmoregulatory signal; τ_p , time constant of dynamic regulatory signal.

Name	Description	Stimulus (X)		Sign factor (a)		Signal
		$\sigma_K(X)$	$\sigma_E(X)$	K	E	$i=E, K$
$\Delta\P/\Delta\P$	K^+ and ectoine depend on turgor; direct signal	$\Delta\P$	$\Delta\P$	1	1	$\omega_i=\sigma_i$
$\Delta\P/K$	K^+ depends on turgor but ectoine on K^+ ; direct signals	$\Delta\P$	K_{cyt}	1	-1	$\omega_i=\sigma_i$
$\Delta\P/K\text{-d}$	K^+ depends on turgor but ectoine on K^+ ; delayed signals	$\Delta\P$	K_{cyt}	1	-1	$\frac{d\omega_i}{dt} = \frac{1}{\tau_i}(\sigma_i - \omega_i)$

function (direct or delayed) (Table 3). (1) The regulatory mechanism ' $\Delta\P/\Delta\P$ ' assumes a direct regulation of potassium and ectoine accumulation by turgor pressure alone. (2) In the regulatory mechanism ' $\Delta\P/K$ ', a two-step regulation is assumed where turgor pressure regulates the potassium content, which in turn stimulates ectoine accumulation. (3) In the regulatory mechanism ' $\Delta\P/K\text{-d}$ ', the osmoregulatory signals (ω_K, ω_E) are produced at a rate proportional to σ and degraded at a constant rate. Hence, the level of the signal follows the intensity of the stimulus with a delay that depends on a time constant τ_p .

The above-described mechanisms only regulate the accumulation of compatible solutes. Adaptation to decreasing salt concentrations is assumed to rely on the dilution of the cytoplasm resulting from cell growth or – in the case of a sudden drop in salt concentration – the opening of mechanosensitive channels. This is in line with observations of the cellular potassium content in *H. elongata*, which remains elevated well above the required level for several hours after an osmotic upshift (Kraegeloh & Kunte, 2002).

Cellular water content (water accessible volume). The first event upon changes in osmolarity is an alteration of cell volume due to osmotic water fluxes (Wood, 1999). Usually, adjusting the cytoplasmic solute pool restores the volume. Besides this transient volume change, a permanent reduction of the cell's volume with higher salinities has been observed (Migueluez & Gilmour, 1994; Cayley *et al.*, 2000). This decrease is primarily based on a decreasing amount of free cytoplasmic water, while the amounts of periplasmic water and permanently bound cytoplasmic water (e.g. in protein hydration shells) are independent of osmolarity (Cayley *et al.*, 2000). An estimate of the amount of free cytoplasmic water is needed to calculate the concentrations of solutes in the cytoplasm. According to Cayley *et al.* (2000), a hyperbolic function describes the decrease of overall cellular water volume with increasing osmolarity, which is described by the following equation:

$$\bar{V}_{\text{cell}}^{\text{wa}} = \frac{\alpha_1}{N + \alpha_2} + \bar{V}_{\text{cell}, \min}^{\text{wa}} \quad (7)$$

where α_1 and α_2 are parameters of the hyperbolic fit (Fig. 2), N is the salt concentration and $\bar{V}_{\text{cell}, \min}^{\text{wa}}$ is the minimum cellular water volume. In accordance with findings of Cayley *et al.* (2000), we assumed the amounts of periplasmic ($\bar{V}_{\text{peri}}^{\text{wa}}$) as well as permanently bound cytoplasmic water ($\bar{V}_{\text{cyto}, b}^{\text{wa}}$) to be 1/5 of $\bar{V}_{\text{cell}, \min}^{\text{wa}}$ each; hence the volume of free cytoplasmic water is given by

$$\bar{V}_{\text{cyto}, f}^{\text{wa}} = \bar{V}_{\text{cell}}^{\text{wa}} - \bar{V}_{\text{peri}}^{\text{wa}} - \bar{V}_{\text{cyto}, b}^{\text{wa}} \approx \frac{\alpha_1}{N + \alpha_2} + \frac{3}{5} \bar{V}_{\text{cell}, \min}^{\text{wa}} \quad (8)$$

Synthesis of compatible solutes. Production of compatible solutes is an essential component of the survival strategy of bacteria under

salt stress, yet the kinetics of compatible solute synthesis in *H. elongata* or other microbes is largely unknown. Therefore, we assumed that the kinetics of all enzymes involved in ectoine synthesis can be lumped into the kinetics of a single enzyme. Further, we assume that standard Michaelis–Menten kinetics holds:

$$p = p_{\max} \frac{S}{K_E^p + S} \omega_E \quad (9)$$

For a description of the parameters see Table 6.

Active transport of compatible solutes (uptake). Under normal conditions, uptake of potassium and ectoine will be uphill – up the concentration gradient. Thus, active, i.e. energy-dependent, transport will be required. The primary transport system for potassium in *H. elongata* is TrkI, which shows Michaelis–Menten-type kinetics (Kraegeloh *et al.*, 2005). Thus the uptake rate of potassium can be described by

$$u_K = u_{\max, K} \frac{K_{\text{ex}}}{K_K^u + K_{\text{ex}}} \omega_K \quad (10)$$

For a description of the parameters see Table 6.

Ectoine is taken up by the TeaABC system in *H. elongata* (Grammann *et al.*, 2002). Since cells growing under constant conditions maintain a

Fig. 2. Cellular water accessible volume $\bar{V}_{\text{cell}}^{\text{wa}}$. Solid line, hyperbolic least-squares fit to the data; dashed line, free cytoplasmic water (see text). Fitted parameters (equation 7): $\alpha_1 = 0.61 \mu\text{mol (mg protein)}^{-1}$, $\alpha_2 = 0.45 \text{ mol l}^{-1}$, $\bar{V}_{\text{cell}, \min}^{\text{wa}} = 1.69 \mu\text{l (mg protein)}^{-1}$. Data (○) taken from Migueluez & Gilmour (1994).

constant level of compatible solutes, the kinetics of ectoine uptake can be derived from the whole-cell growth kinetics of an ectoine-synthesis-deficient mutant, whose growth is limited by ectoine uptake (Grammann *et al.*, 2002). The uptake rate of ectoine is then – analogous to equation 10 – given by:

$$u_E = u_{\max,E} \frac{E_{ex}}{K_E^u + E_{ex}} \omega_E \quad (11)$$

For a description of the parameters see Table 6.

Passive transport of ectoine (leakage). Mutants of *H. elongata* lacking a functional TeaABC uptake system accumulate ectoine in the medium. It is therefore believed that the main function of TeaABC is the recovery of ectoine leaked into the medium (Grammann *et al.*, 2002), since wild-type cultures do not show this extracellular accumulation. How ectoine leaks through the cytoplasmic membrane is not yet known. Since this translocation is down the concentration gradient it is likely to be passive, i.e. not energy dependent. Such passive transport is most simply modelled as a diffusion process where the leakage rate is proportional to the concentration difference.

$$\ell = \lambda \Delta E = \lambda (E_{cyt} - E_{ex} \bar{V}_{cyto,f}^{wa}) \quad (12)$$

The volume of free cytoplasmic water ($\bar{V}_{cyto,f}^{wa}$) converts the extracellular concentration to an intracellular content.

Mechanosensitive channels. The mechanosensitive channels in the cytoplasmic membrane work like safety valves that open when the turgor pressure exceeds a critical value, thus allowing a rapid efflux of water and solutes. We modelled the opening of mechanosensitive channels as a step increase of permeability λ from a low value λ^0 to a high value λ^1 when the critical turgor pressure $\Delta\Pi_{crit}$ is exceeded:

$$\lambda = \begin{cases} \lambda^0, & \Delta\Pi < \Delta\Pi_{crit} \\ \lambda^1, & \Delta\Pi \geq \Delta\Pi_{crit} \end{cases} \quad (13)$$

Energetics of biomass formation and ectoine synthesis.

Formation of biomass requires a carbon source, and usually also an energy source, which for chemo-organo-heterotrophic organisms is often the same substrate, especially in minimal media. Also, the synthesis of compatible solutes requires a carbon source, and may or may not require an energy source. For example, the synthesis of ectoine from glucose and ammonia (net reaction: Glucose + 2 NH₄⁺ → ectoine + 4 H₂O + 2 H⁺) would be energetically neutral (Oren, 1999; Maskow & Babel, 2001) if *H. elongata* used the same pathways as *E. coli* to produce aspartate, the precursor of ectoine (*E. coli* uses the Embden–Meyerhof–Parnas pathway for glycolysis, then PEP-carboxylase for synthesis of oxaloacetate, and glutamate dehydrogenase for ammonia assimilation when ammonia is present at high concentration; pathways taken from the KEGG database <http://www.genome.jp/kegg>), which is the case in all experimental studies we have undertaken or taken from the literature. The pathway of ectoine synthesis from aspartate is known in *H. elongata* and involves three enzymes (Peters *et al.*, 1990). The measured yield of 1 mol ectoine per mol glucose (Maskow & Babel, 2001) confirms that ectoine synthesis is energetically neutral or near neutral if glucose is used as the carbon source. Note that the cost of ammonia uptake might be higher when it is limiting; e.g. at low ammonia concentrations, *E. coli* uses the GOGAT pathway, spending 1 mol ATP per mol ammonia taken up. Moreover, ectoine synthesis from glucose carries the opportunity cost of allocating glucose for ectoine synthesis instead of growth.

In contrast to ectoine synthesis, the formation of biomass from glucose requires energy that has to be generated by glucose

catabolism; thus the biomass yield per unit of glucose is lower than the ectoine yield. Therefore, the yield of total dry mass (Y_X) increases with the fraction of ectoine in the dry mass according to

$$Y_X = (Y_E - Y_B) \cdot \hat{E} + Y_B \quad (14)$$

where Y_E and Y_B denote the yield of ectoine and (ectoine-free) biomass synthesis, respectively, and $\hat{E} = BE_{cyt}/(B + BE_{cyt}) = E_{cyt}/(1 + E_{cyt})$ is the fraction of ectoine in the cell mass. Using equation (14), Y_B can be determined by measuring total yields as a function of the ectoine fraction (Fig. 3).

As the ectoine uptake system TeaABC belongs to the TRAP family of secondary transporters we assume that it is also a secondary transporter. Although the coupling ion still needs to be determined, the energetic cost of uptake can be estimated by calculating the difference in electrochemical potential $\Delta\mu$ over the plasma membrane. Since ectoine bears no net charge, its electrochemical potential is $\Delta\mu = RT \log(a_{cyt}/a_{ex})$ (Adam *et al.*, 1977). It follows that for an assumed ratio of intracellular to extracellular activity (i.e. ~concentration) of 10⁶:1 at 298 K, the potential difference is about 34.3 kJ mol^{−1}. Hence, the uptake of one molecule of ectoine requires the energy of less than one molecule of ATP ($\Delta G' = -48$ kJ mol^{−1}; Thauer *et al.*, 1977), which is small compared to the cost of one molecule of glucose for *de novo* synthesis. Thus, transport costs for ectoine were neglected in the model. The uptake of potassium ions was assumed to be energetically about neutral (and thus also neglected), since the concentration difference is much less than for ectoine and uptake of cations is facilitated by the membrane potential.

Balance equations. Table 1 provides an overview of the model. The mass balance equations are assembled from the above-described building blocks. B , E_{ex} , K_{ex} and S flow out of the chemostat with dilution rate D . E_{ex} , K_{ex} and S also enter the system at rate D when present in the feed at concentrations E_r , K_r and S_r (reservoir concentrations), respectively. Intracellular compounds are diluted by biomass growth.

Fig. 3. Growth yield Y_X of *H. elongata* plotted against the fraction of ectoine in the dry weight. Equation (14) was fitted to experimental data (▲) by linear regression ($r=0.998$). The growth yield for ectoine-free biomass (Y_B) can be derived from the intersection of the fitted curve with the y-axis. Growth yields were determined by continuous-culture experiments from biomass density (B) and consumed substrate: $Y_X = B/\Delta S$, where $\Delta S = S_r - S$, i.e. the amount of substrate used.

RESULTS

Evaluation of salt inhibition models

The specific growth rates observed in batch culture clearly show a growth optimum of *H. elongata* slightly above 0.5 mol l⁻¹ NaCl (Fig. 4). Consistent with the classification as a halophilic organism, growth rate decreases sharply at lower salt concentrations, while cells still grow at 3.6 mol l⁻¹, showing the broad salt tolerance of *H. elongata*.

Fig. 4 also shows the nonlinear least-squares fits of the tested models to the combination of two independent datasets. The Arrhenius-type model leads to a 'bell-shaped' function that only roughly fits to the experimental data ($R^2=0.869$) and predicts far too high a specific growth rate at zero salinity. Both the Haldane ($R^2=0.893$) and Hyperbolic ($R^2=0.768$) models did not fit well and parameters were poorly defined, with confidence intervals ranging over several orders of magnitude. Surprisingly, the fitted curves for the models Edwards and Luong ($R^2=0.966$) were identical, as were the parameters $r_{\max}$ and K_N ; however, the parameters $N_{\max}$ and n of the Luong model were poorly defined and unrealistically high. In fact, for high $N_{\max}$ and n , the inhibition term of the Luong model can be approximated by the exponential term of the Edwards model since

$$\left(1 - \frac{N}{N_{\max}}\right)^n \xrightarrow{N_{\max}, n \rightarrow \infty} \exp\left(-n \frac{N}{N_{\max}}\right) \xrightarrow{K_I \equiv \frac{N_{\max}}{n}} \exp\left(-\frac{N}{K_I}\right) \quad (15)$$

The YanoKoga-F model fitted the data well ($R^2=0.966$), but the parameter K_I was ill defined. We removed the K_I term and found that the reduced model ('YanoKoga-R', Table 2a) fitted the data equally well with all parameters well defined. The YanoKoga models were also tested for more than one inhibitory site, but this did not improve the fit (data not shown).

The models Edwards and YanoKoga-R gave the best fits of all tested models and were also the only two with well-defined parameters (Table 2b). To compare these unrelated models we calculated the second-order Akaike's information criterion (Burnham & Anderson, 2002):

$$AIC_c = 2k + n \log\left(\frac{SS_{reg}}{n}\right) + \frac{2k(k+1)}{n-k-1} \quad (16)$$

where SS_{reg} is the residual sum of squares (equation 1), n the number of available data points and k the number of model parameters. The difference in AIC_c is only 0.22 for the two models and the relative likelihood resulting from this difference is 52.75 % for YanoKoga-R versus 47.25 % for Edwards. Neither of the two models can be preferred based on our dataset for *H. elongata*.

Determination of model parameters

Growth kinetics. The dependence of growth rate on glucose concentration was determined by measuring glucose concentrations in steady-state continuous cultures at different dilution rates (Table 4).

Since we assume that the influence of glucose and salt on growth is independent [see equation (2)], we can multiply the expressions for glucose and salt dependence:

$$g(S, N) = g_S(S) \frac{g_N(N)}{g_N(\hat{N})} = g_{\max, S} \gamma(S) \frac{g_{\max, N} \xi(N)}{g_N(\hat{N})} \quad (17)$$

where g_S , g_N are the independently fitted functions for glucose- and salt-dependent growth kinetics, respectively, and $g_N(N)$ is a reference growth rate, e.g. the optimal growth rate predicted by the salt dependence model at

Fig. 4. Salt dependence of specific growth rate. Curves represent predictions calculated by least-squares fit of the different models: YanoKoga (purple), Edwards and Luong (red; identical curves), Haldane (green), Hyperbolic (yellow) and Arrhenius-type (blue). The models were fitted to the combination of the two independent sets of experimental data from batch cultures of *H. elongata* grown in MM63 (▲) or in GXX (△) medium. Separately fitting to either dataset led to similar results. See Table 2 for descriptions of the different models. Specific growth rates were determined from exponential-phase growth. Data points represent individual measurements.

Table 4. Steady-state concentration of glucose in continuous cultures (concentration in the feed, $S_f=55.5$ mmol l⁻¹)

Specific growth rate was fitted to these data using Monod kinetics [$g_S = g_{\max, S} \gamma(S)$, equation (3)] with values $g_{\max, S}=0.60$ h⁻¹, $k_s=8.48$ mmol l⁻¹ ($R^2=0.977$).

Dilution rate D (h ⁻¹)	0.30	0.36	0.38	0.40	0.43	0.47	0.50
Glucose concentration (mmol l ⁻¹)	7.7	13.0	15.4	18.1	22.7	28.8	34.1

$\hat{N}=510 \text{ mmol l}^{-1} \text{ NaCl}$, used here to normalize the growth rate. This normalization makes the term for the salt dependent kinetics, $\xi(N)$, in equation (17) dimensionless. Now we can obtain equation (2) by defining

$$g_{\max} = \frac{g_{\max,S} g_{\max,N}}{g_N(\hat{N})} \quad (18)$$

Protein content. Measurements of protein content in dry biomass from continuous cultures revealed a linear decrease of protein content with rising salt concentration (Table 5). We used the fitted linear function for protein content to convert the protein specific data usually found in the literature to biomass specific data.

Kinetics of solute accumulation. The maximum specific rate of ectoine synthesis ($g_E^{\max}$) was estimated from an osmotic upshift experiment by Kraegeloh & Kunte (2002). Assuming full upregulation of ectoine synthesis immediately after the osmotic upshift, we calculated a value of $1.25 \mu\text{mol (mg B)}^{-1} \text{ h}^{-1}$ from the initial slope (first 30 min) of the ectoine increase.

Grammann *et al.* (2002) measured the growth rate of a strain that is deficient in ectoine synthesis (KB 1) under ectoine-limiting conditions. Since all intracellular ectoine in this strain must be taken up from the medium via the TeaABC transporter, it is possible to estimate ectoine uptake kinetics from the whole-cell growth kinetics determined in this experiment.

The ectoine permeability λ_E was assumed to be negligible since in wild-type cultures the steady-state extracellular concentration of ectoine is below the detection limit (Grammann *et al.*, 2002). The only way to determine this ectoine permeability is by monitoring the extracellular accumulation of ectoine in a strain deficient in ectoine uptake. The kinetics of the TrkI transporter for potassium uptake has been published recently (Kraegeloh *et al.*, 2005); parameters are listed in Table 6.

Determining the yield of ectoine (Y_E) and biomass (Y_B). Using equation (14), the yield coefficients for ectoine (Y_E) and biomass without ectoine (Y_B) can be derived from yield coefficients for total biomass (Y_X) measured at different ectoine contents (i.e. different salt concentrations). We determined the yield of ectoine to

Table 5. Protein content of biomass (ectoine-free dry weight) in continuous culture at different salt concentrations

Data were fitted to a linear function: protein content = $753 \mu\text{g (mg B)}^{-1} - 0.06 \mu\text{g (mg B)}^{-1} \text{ l mol}^{-1} \times (\text{salt concentration}) \text{ mol l}^{-1}$ ($R^2=0.951$).

Salt concentration (mol l ⁻¹)	0.51	1.71	2.57	3.42
Protein content	680	530	460	370
[$\mu\text{g (mg dry wt)}^{-1}$]				

be $0.82 \text{ g Ect (g Glc)}^{-1}$, i.e. $1.04 \text{ mol Ect (mol Glc)}^{-1}$ (Fig. 3), showing less than 5% deviation from the theoretical value of $1.00 \text{ mol Ect (mol Glc)}^{-1}$ that was discussed above.

The biomass yield Y_B depends on the amount of substrate that is used to form biomass and the amount catabolized to provide the necessary energy for biomass formation. It is described by the equation $Y_B = (c_{\text{assim}} + c_{\text{dissim}})^{-1}$, where c_{assim} is the amount of substrate that is assimilated to form new biomass and c_{dissim} is the amount of substrate that is dissimilated to provide the biochemical energy driving this process. The first can be estimated from the assimilation equation of the substrate, simplifying the synthesis of biomass to a single reaction that produces a hypothetical 'biomass molecule'. Using glucose as substrate and assuming a biomass composition of $\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}$ – for aerobically growing cells (Stouthamer, 1979) – a similar composition has been observed in *H. elongata* (Sauer, 1995), but the value reported in Stouthamer (1979) better represents ectoine-free biomass – leads to the following assimilation stoichiometry:

From this stoichiometry, c_{assim} can be derived to be $0.175 \text{ mol Glc C-mol}^{-1}$ or $7.1 \text{ mmol Glc (g B)}^{-1}$. The amount of c_{dissim} depends on the ATP yield v_{ATP} of glucose oxidation via glycolysis, citric acid cycle and oxidative phosphorylation and the Y_{ATP} value: $c_{\text{dissim}} = (v_{\text{ATP}} Y_{\text{ATP}})^{-1}$. To calculate the theoretical growth yield according to these equations, a standard Y_{ATP} value of 10 g mol^{-1} can be assumed, together with an ATP yield v_{ATP} of 26 or 38 mol ATP (mol Glc)⁻¹, depending on the P:O quotient of the respiratory chain, assumed to be 2 or 3, respectively. The observed Y_B of $0.466 \pm 0.005 \text{ g B (g Glc)}^{-1}$ (Fig. 3) corresponds to a P:O quotient of ~ 1.6 .

Prediction of solute contents

In our model the process of osmoregulation should preserve turgor pressure (slightly higher pressure inside to allow growth) by adjusting the intracellular content of potassium and ectoine. The minimally required ectoine content (E_0) for maintaining turgor pressure can be predicted using the following equation, which can be derived from equation (4) by setting $\Pi_{\text{cyt}} = \Pi_{\text{th}} + \Pi_{\text{ex}}$:

$$E_0 = \bar{V}_{\text{cyto},f}^{\text{wa}} \left((\Delta\Pi_{\text{th}} + \Pi_{\text{ex}}) / RT - O_{\text{cyt}}^0 \right) - (1 + v) K_{\text{th}} \quad (19)$$

The terms $\Delta\Pi_{\text{th}}$ and K_{th} correspond to the trigger values of the stimulus functions σ_i (Table 3). E_0 depends on the salt concentration (N) since both the extracellular osmotic pressure (Π_{ex}), and the cytoplasmic water volume $\bar{V}_{\text{cyto},f}^{\text{wa}}$ are functions of external salinity. The predicted E_0 , assuming constant turgor pressure, is compared with measurements in Fig. 5.

Table 6. Parameter values used for model simulations

Parameters were determined or assumed in this study if not stated otherwise.

Parameter	Symbol	Value	Unit	Source
Growth kinetics (parameters for YanoKoga and Monod model)				
Maximum specific growth rate	$g_{\max}$	1.41	h^{-1}	This study
Substrate Monod constant	k_S	8.48	mmol l^{-1}	This study
Salt Monod constant	k_N	508	mmol l^{-1}	This study
Salt inhibition constant	k_2	957	mmol l^{-1}	This study
Solute accumulation				
Ectoine maximum synthesis rate	$p_{\max}$	1.25	$\mu\text{mol (mg B)}^{-1} \text{h}^{-1}$	Derived* from Kraegeloh & Kunte (2002)
Ectoine synthesis Michaelis–Menten constant	k_E^p	8.48	mmol l^{-1}	Assumed to $=k_S$
Ectoine maximum uptake rate	$u_{\max,E}$	0.43	$\mu\text{mol (mg B)}^{-1} \text{h}^{-1}$	Derived* from Grammann <i>et al.</i> (2002)
Ectoine uptake Michaelis–Menten constant	k_E^u	0.027	mmol l^{-1}	Derived* from Grammann <i>et al.</i> (2002)
Potassium maximum uptake rate	$u_{\max,K}$	7.6	$\mu\text{mol (mg B)}^{-1} \text{h}^{-1}$	Kraegeloh & Kunte (2002)
Potassium uptake Michaelis–Menten constant	k_K^u	1.1	mmol l^{-1}	Kraegeloh & Kunte (2002)
Ectoine permeability	λ_E^0	0	h^{-1}	Assumed
Ectoine permeability if MSC† open	λ_E^I	60	h^{-1}	Assumed
Potassium permeability	λ_K^0	0	h^{-1}	Assumed
Potassium permeability if MSC open	λ_K^I	60	h^{-1}	Ajouz <i>et al.</i> (1998)
MSC critical turgor pressure	$\Delta\Pi_{\text{crit}}$	0.5	MPa	Assumed
Osmoregulation				
Minimum water accessible cell volume	$\bar{V}_{\text{cell,min}}^{\text{wa}}$	1.687	$\mu\text{l (mg protein)}^{-1}$	Derived* from Miguelez & Gilmour (1994)
Cell volume fitting parameter 1	α_1	0.608	$\mu\text{l (mg protein)}^{-1}$	Derived* from Miguelez & Gilmour (1994)
Cell volume fitting parameter 2	α_2	449	mmol l^{-1}	Derived* from Miguelez & Gilmour (1994)
Potassium stimulus function sensitivity	s_K	10	–	Assumed
Ectoine stimulus function sensitivity	s_E	10	–	Assumed
Potassium signal function time constant	τ_K	0.2	h	Assumed
Ectoine signal function time constant	τ_E	0.4	h	Assumed
Cytoplasmic basal osmolarity	O_{cyt}^0	50	mmol l^{-1}	Assumed
Ratio K^+ : counter-ions	ν	1	–	Assumed
Turgor pressure regulatory threshold	$\Delta\Pi_{\text{th}}$	0.04	MPa	Assumed
K_{cyt} regulatory threshold	K_{th}	0.90	$\mu\text{mol (mg B)}^{-1} \text{h}^{-1}$	Derived* from Kraegeloh & Kunte (2002)
Stoichiometric factors				
Biomass growth yield	Y_B	84	$\text{mg B (mmol Glc)}^{-1}$	Determined
Ectoine yield	Y_E	1	$\text{mmol Ect (mmol Glc)}^{-1}$	Determined
Cost of ectoine uptake	$c_{\text{up},E}$	3.23	$\text{mg B (mmol Ect)}^{-1}$	Determined
Cost of potassium uptake	$c_{\text{up},K}$	1.07	$\text{mg B (mmol K)}^{-1}$	Determined
Protein content fit, slope	–	0.06	$\mu\text{g (mg B)}^{-1} \text{l mol}^{-1}$	Determined
Protein content fit, y -intercept	–	753	$\mu\text{g (mg B)}^{-1}$	Determined
Growth conditions				
Substrate concentration in feed	S_r	50	mmol l^{-1}	VVM medium composition
Ectoine concentration in feed	E_r	0	mmol l^{-1}	VVM medium composition
Potassium concentration in feed	K_r	17	mmol l^{-1}	VVM medium composition
Medium osmolarity (without S, E, K)	$\sum_i c_{i,\text{ex}}$	83	mmol l^{-1}	VVM medium composition
Dilution rate	D	0.1	h^{-1}	Assumed

*See text for further information.

†MSC, mechanosensitive channel.

Fig. 5. Intracellular ectoine content: experiment and model prediction. Data points are ectoine contents measured in continuous cultures of *H. elongata* as described in Methods. Curves represent the ectoine content E_0 needed to maintain osmotic equilibrium predicted by equation (19) with $\nu=1$ (solid curve) and $\nu=0.5$ (dotted curve). For other parameters see Table 6.

Constant salinity

A first test of the suitability of the model was the simulation of a continuous culture under constant environmental conditions. Simulations were carried out with all regulation types listed in Table 3 and also for both YanoKoga and Edwards kinetics. Only the results for YanoKoga are discussed explicitly, because the differences in outcome between the two kinetics were negligible. For a list of all parameter values used, see Table 6.

All of the different regulation models tend to a steady state after about 15 h (Fig. 6). The development of both biomass and substrate concentration was similar for each regulation model. In all cases, the intracellular ectoine content initially dropped below E_{opt} due to growth-dependent dilution of the cytoplasm, leading to a decrease of $\Delta\Pi$ and hence activation of the osmoregulatory machinery (Fig. 6b). This in turn led to potassium uptake, and the further response was different for the different models. In the simplest model $\Delta\Pi/\Delta\Pi$, the ectoine content decreased further to a very low level after the biomass had already reached its steady state (Fig. 6b1). In the other model simulations, ectoine and potassium reached a steady state close to the optimum values K_{th} and E_{opt} (Fig. 6b2 and b3). Since cells keep growing in the (dynamic!) steady state, a small deviation from the optimal solute contents remains. The delayed signal response model ($\Delta\Pi/K-d$) showed damped oscillations of solute contents initially (Fig. 6b3), but reached the same steady state as the corresponding model with instantaneous signal response ($\Delta\Pi/K$, Fig. 6b2).

Changing salinity

The main purpose of compatible solutes is to allow flexible adaptation to changing osmolarities, so we tested the model's behaviour under situations of sudden osmotic shifts. Starting with a system in steady state as described in the previous

section, the NaCl concentration was shifted from 1000 to 2000 mmol l^{-1} for simulation of an osmotic upshift and from 2000 to 1000 mmol l^{-1} for osmotic downshift, respectively.

Osmotic upshift. After the sudden increase in osmolarity, the system entered a new steady state after a transient period of up to 20 h duration (Fig. 7a). In simulations of the osmoregulatory mechanism $\Delta\Pi/\Delta\Pi$, the osmotic balance was mostly restored by accumulation of potassium instead of adaptation of the ectoine content (Fig. 7a1), as in the simulations with constant salinity (Fig. 6b1). Ectoine content was very low before the osmotic shift and rose only by a small amount afterwards. In contrast, the two models using a two-step regulation mechanism reached a close to optimal steady state after an adaptation phase. In both cases, potassium initially accumulated to a high level, restoring the osmotic equilibrium (Fig. 7a2 and a3). Following this, ectoine accumulated, enabling potassium content to decrease again until it reached the new steady state. This process took about 12 h in the model with direct signal response ($\Delta\Pi/K$) and about twice as long (~ 24 h) in the delayed signal response model ($\Delta\Pi/K-d$). Overshooting, i.e. an accumulation of ectoine above E_{opt} (and a concomitant decrease of potassium content below K_{th}), occurred in both two-step models but was much more pronounced in the delayed signal response model, because of the longer duration of the adaptation process.

Osmotic downshift. Following a sudden decrease in NaCl concentration from 2000 to 1000 mmol l^{-1} , the solute pools of both potassium and ectoine decreased until osmotic equilibrium was restored (Fig. 7b). Again, the one-step model ($\Delta\Pi/\Delta\Pi$) showed a large deviation from the optimum solute contents before and after the downshift (Fig. 7b1). In both two-step models, without opening of mechanosensitive channels (MSCs), the solute contents showed two phases of adaptation to lower salinities (Fig. 7b2 and b3). After the initial decrease in both solute contents (approx. 4 h after the downshift), potassium increased again to reach a steady state a little above the optimum value while ectoine decreased further, restoring the steady state corresponding to Fig. 6(b) after about 17 h. If MSCs were opening in the models, their opening due to the high post-shift $\Delta\Pi$ led to an initially steep decrease of the solute contents (Fig. 7b1–b3). After the turgor pressure fell below the threshold value $\Delta\Pi_{crit}$ for channel opening, the solute content changes were similar to the simulations without MSCs. Although the new steady state was reached only 2 h sooner than without MSCs, the high turgor pressure immediately after downshift relaxed much faster until falling below $\Delta\Pi_{crit}$ (turgor pressure not shown).

DISCUSSION

Salt as an inhibitory substrate

While the exact mechanisms of growth stimulation and inhibition due to salt remain unclear, it is likely that with

Fig. 6. Simulations of a continuous culture ($D=0.1 \text{ h}^{-1}$) with 1000 mmol NaCl l⁻¹ using several osmoregulation mechanisms. (a) Concentrations of substrate (solid), total biomass ($B+E$, dashed) and ectoine-free biomass (B , dotted); the result is almost identical for all the osmoregulatory mechanisms (the figure shows the simulation of $\Delta\Pi/K$). (b) Intracellular content of ectoine (thick solid lines) and potassium (thin solid lines); dashed lines show the assumed optimum level for potassium K_{th} (thin dashed) and predicted optimum level (thick dashed) for ectoine (E_{opt} ; see equation 19). Panels (b1)–(b3) correspond to simulations of different models as denoted in the respective panels and described in Table 3. Note that the optimum level of ectoine decreases in parallel with the substrate concentration because the substrate is also an osmolyte.

increasing salt concentration different mechanisms combine to different extents to give rise to the overall observed growth dependence. The fact that only substrate inhibition models, i.e. the Edwards and YanoKoga models, could be fitted successfully suggests that salt can be regarded as a substrate for the growth of *H. elongata* with inhibitory effects, using ‘substrate’ here in the broader sense of any compound required for growth, whether it is metabolized or not. The inhibition at high concentration might be explained by shielding of ionic groups of enzymes or transport systems through ion-pairing effects. In fact, the YanoKoga model is a model for inhibition of enzyme activity by formation of enzyme–inhibitor complexes. In models of bacterial growth, the cell is often regarded as one ‘meta-enzyme’ that produces biomass from substrate, and the growth rate functions therefore are often based on models for enzyme activity (like the Monod model, which is equivalent to the Michaelis–Menten model for enzymes).

While a dependence of growth rate on free cytoplasmic water could be expected at higher salinities, the Hyperbolic model, which is based on the hyperbolic decrease of free cytoplasmic water with increasing salinity, could not be fitted to the data. We conclude that the decrease of free cytoplasmic water does not directly cause the decrease in growth rate.

How much ectoine is ‘required’?

By ‘required’ we refer to the minimal ectoine content needed to maintain turgor pressure. This content, which can be calculated with equation (19), is in reasonable agreement with experimental data (Fig. 5), although cells accumulate a limited amount of ectoine even if this would not be expected because of sufficiently low external osmolarity. A possible explanation is that ectoine might not be needed to maintain turgor pressure at lower

Fig. 7. (a) Simulation of an osmotic upshift from 1000 to 2000 mmol NaCl l⁻¹ at 10 h. (b) Simulation of an osmotic downshift from 2000 to 1000 mmol NaCl l⁻¹ at 10 h. Intracellular content of ectoine (thick solid lines) and potassium (thin solid lines); dashed lines show the assumed optimum level for potassium K_{th} (thin dashed) and predicted optimum level (thick dashed) for ectoine (E_{opt} see equation 19). Dotted curves show the content of ectoine and potassium for the model with active mechanosensitive channels. Panels correspond to simulations of different models as denoted in the respective panels and described in Table 3.

medium osmolarity, but be accumulated for its direct protective properties (Galinski, 1993). This higher ectoine content should come with an increased turgor pressure in cells at low medium osmolarity, as has indeed been observed for *E. coli* (Cayley *et al.*, 2000).

Prediction of the P:O ratio of the respiratory chain of *H. elongata*

We calculated the theoretical growth yield (Y_B) for aerobically growing cells to be 0.51 or 0.57 g B (g Glc)⁻¹, assuming a

P : O quotient of 2 or 3, respectively. Since we experimentally determined a yield of $0.47 \text{ g B (g Glc)}^{-1}$ for *H. elongata*, we conclude that the highest P : O ratio in the respiratory chain of *H. elongata* is unlikely to be more than 2. The observed P : O ratio of ~ 1.6 might result from a branched respiratory chain, with one branch having a P : O ratio of 2 and another having a P : O ratio of 1, or it could be due to a Y_{ATP} slightly below the regularly assumed value of 10.

Osmoregulation can be modelled as a two-step process

We tested different hypothetical regulation mechanisms for their suitability to describe and explain the process of osmoregulation in *H. elongata*. The simplest model, where the accumulation of K^+ and that of ectoine is triggered directly by turgor pressure, fails to reproduce the observed solute levels (K_{th} , E_{opt}). This model leads to preferential accumulation of potassium because its uptake rate is higher than the production rate of ectoine and there is no feedback regulation that reduces the potassium concentration back to the experimentally observed steady-state value, which seems to be independent of the salinity (Kraegeloh & Kunte, 2002). The other two regulation models that we tested assume a two-step process where the potassium concentration is directly regulated by salinity-dependent properties (i.e. turgor pressure) and potassium in turn triggers the accumulation of ectoine. Both models show a steady-state value for ectoine that is close to the optimum value. There remains only a small deviation caused by the growth of the cells that permanently dilutes the cytoplasm. In a chemostat in steady state, cells keep growing in exponential phase and this apparently requires a permanent, though weak, upregulation of solute accumulation.

In the simulations of the regulation with delayed response ($\Delta\Pi/K$ -d), the overshoot of solute concentrations is much higher and it takes longer to reach the steady state. This is caused by the additional time delay (the second regulatory step itself already causes a delay) of signal accumulation. However, the results of the two two-step models do not differ qualitatively and the additional parameters of $\Delta\Pi/K$ -d have not yet been determined experimentally. Thus the simpler model $\Delta\Pi/K$ should be preferred. The overshoot is not an artefact but has also been observed in experiments (Kraegeloh & Kunte, 2002). Also, the osmoadaptation after an osmotic upshift takes several hours, although accumulation of solutes itself is faster. In its natural habitat (e.g. solar salterns), *H. elongata* is subjected to fluctuations of osmolarity due to evaporation and rain. The overproduction of compatible solutes could be a pre-emptive strategy that would pre-adapt the cells to further increases of osmolarity due to continuing evaporation.

Concluding remarks

We have developed and validated a comprehensive mathematical model of growth and osmoregulation in

halophilic bacteria based on a minimal set of simple assumptions. The response of the model to changes in external osmolarity is in line with experimental observations. The simplest assumptions that can reproduce all the essential aspects of growth and osmoregulation of halophiles are that growth depends on substrate concentration according to Monod kinetics and independently on salt concentration according to substrate inhibition kinetics, and that osmoregulation using compatible solutes follows a two-step mechanism, with turgor pressure regulating potassium uptake and potassium concentration in turn regulating ectoine production. We expect that the model will be straightforward to adapt and parameterize for other compatible-solute-producing halophiles since all the processes described in this model are common to all of them and are modelled in a simple, generic way.

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