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INVESTIGATION OF THE INFLUENCE OF NaCl CONCENTRATION ON *HALOBACTERIUM SALINARUM* GROWTH
Microcalorimetry and transmission electron microscopy

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Microcalorimetry was used to study the influence of NaCl concentration on *Halobacterium salinarum* growth. From the thermogenic curves and thermokinetic parameters of *H. salinarum* growth in different concentrations of NaCl, it was found that the optimum NaCl concentration for *H. salinarum* growth was not a wide range from 3.5 mol L^{-1} to NaCl saturation (about 5.2 mol L^{-1}), as is generally acknowledged, but just around 230 g L^{-1} (approximately 3.9 mol L^{-1}). And when external NaCl concentration was above 230 g L^{-1}, the growth metabolism of *H. salinarum* decreased constantly with the increasing of NaCl concentration. These have never been described before. Further investigation by transmission electron microscopy revealed that *H. salinarum* growing in approaching NaCl saturation underwent plasmolysis, which interpreted the novel finding of microcalorimetry perfectly. Our work shows that microcalorimetry may reveal more and newer details about microbial growth than the existing methods do. These details are significant to understand biological processes.

Keywords: *Halobacterium salinarum, microcalorimetry, NaCl concentration, plasmolysis, transmission electron microscopy*

Introduction

Hypersaline environments worldwide, such as salt lakes and saltern crystallizer ponds, are inhabited by a group of microorganisms known as halobacteria (family *Halobacteriaceae*). As a kind of extremophiles, halobacteria have many unusual features and have become the subject of considerable scientific research in recent times [1].

Among the various features of halobacteria, the most striking feature is their absolute requirement for high concentrations of NaCl [2]. Many of the early studies on halobacteria were focused on the influences of environmental factors on halobacterial growth, and it has been found that the NaCl concentration is the most important factor in determining halobacterial growth [1]. Most halobacteria require more than 2.5 mol L^{-1} NaCl for growth and grow best at NaCl concentrations approaching saturation [1, 2]. Take *Halobacterium salinarum* (a halobacteria species) for instance. It is generally acknowledged that *H. salinarum* grow best at 3.5 mol L^{-1} – NaCl saturation (about 5.2 mol L^{-1}) and no *H. salinarum* growth occurs below 3 mol L^{-1} NaCl [1, 2]. In addition, it has also been found that the NaCl requirement for halobacterial growth is specific and NaCl cannot be replaced by other solutes [1, 2].

Since 1960s, the cellular and molecular basis of the 'NaCl requirement' has been the subject of in-depth studies till now. It has been shown that a stepwise reduction of the NaCl concentration causes the structural deformations of halobacterial cells, which undergo lysis ultimately [3]. Further studies revealed that halobacteria possess an S-layer cell wall, whose main constituent is a high molecular mass glycoprotein. This glycoprotein is highly negatively charged (contains a large relative number of acidic amino acid residues), and is stabilized only in the presence of a high sodium cation concentration [1, 2]. Thus it is generally assumed that halobacteria require high concentrations of NaCl primarily to maintain their glycoprotein cell wall structure. The positive charges on sodium prevent the negative charges from repelling each other and lysing the halobacterial cell. Without an environment with a high sodium cation concentration, the cell wall glycoproteins are destabilized and the cell wall weakens until the cell disintegrates [1, 2].

Microcalorimetry as a technique has been widely used in life sciences because of its high sensitivity, high accuracy and automaticity [4]. Microcalorimetry has a great advantage over studies of complex living systems, and provides a particularly useful tool for the characterization of the microbial growth processes [5]. In our previous work, we have applied microcalorimetry to study the halobacterial growth and got

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satisfactory results [6]. In this study, microcalorimetry was used together with transmission electron microscopy (TEM) to investigate the influence of NaCl concentration on *H. salinarum* growth. Some novel phenomena were discovered, which may help us better understand the influence of NaCl concentration on halobacterial growth.

Experimental

Organism and culture conditions

H. salinarum NRC817, firstly isolated from a solar saltern near Alicante, Spain, was kindly provided by Prof. Inmaculada Meseguer (Universidad Miguel Hernández, Spain).

The medium used were based upon that described by Rodriguez-Valera *et al*. [7] and contained salts in approximately the same proportions as found in seawater except for varied concentrations of NaCl. The medium contained per liter: $MgCl_2{\cdot}6H_2O$ 16 g, $MgSO_4{\cdot}7H_2O$ 25 g, $CaCl_2{\cdot}6H_2O$ 1.3 g, KCl 5.0 g, $NaHCO_3$ 0.25 g, NaBr 0.63 g, yeast extract 5.0 g and NaCl in different amounts (130, 150, 170, 190, 230, 260, 290 g, corresponding roughly to 2.2, 2.6, 2.9, 3.2, 3.9, 4.4, 5.0 mol L^{-1} respectively). The medium were sterilized by autoclaving at 121°C for 20 min.

Instrumentation

A TAM air Isothermal Microcalorimeter, manufactured by Thermometric AB Corporation of Sweden, was used to measure the heat output of *H. salinarum* growth in different concentrations of NaCl. This isothermal microcalorimeter is an eight-channel twin instrument. Normally, 20 mL reaction vessels made from glass or stainless steel are used. The thermal power detection limit is stated to be ±2 μW [4]. The microcalorimeter was thermostated at 37°C. The voltage signal was recorded by a computer. For details of the performance and structure of the instrument, see the Instruction Manual of TAM air Isothermal Microcalorimeter and [4].

A H-8100 transmission electron microscope, manufactured by Hitachi Ltd., was used to examine the morphology of *H. salinarum* cells growing in different concentrations of NaCl.

Microcalorimetry

The heat flow rate of the system can be measured *vs*. time. The heat flow–time dependence of bacterial growth was performed. The metabolic thermogenic curves of *H. salinarum* growth in different concentrations of NaCl were determined using the ampoule method. A 20 mL glass ampoule was cleaned and sterilized. In the beginning of the experiment, *H. salinarum* was inoculated in the prepared medium, initially containing $1{\cdot}10^6$ cells mL^{-1}, and the cells were suspended in the medium. Then 5 mL bacterial suspension was put into the ampoule and a lid was sealed onto it. The temperatures of the calorimeter system and the isothermal box were controlled at 37°C. Meanwhile, a computer was used to record the thermogenic curves of *H. salinarum* growth continuously.

Preparation of samples for transmission electron microscopy

H. salinarum was cultured in the medium of different concentrations of NaCl. Cultures of *H. salinarum* were incubated in 20 mL glass ampoules at 37°C. All above were the same as in microcalorimetry. Logarithmic phase cultures were used. The cells were collected by centrifugation and washed twice with the saline (the salt concentration of the saline was the same as which of the medium). A drop of the cell suspension was placed on a grid and left for 1.5–2 min. Excess cell suspension was removed by touching the grid to filter paper, and the grid was retained for transmission electron microscopy.

Results

Thermogenic curves

The power-time curves of *H. salinarum* growth in different concentrations of NaCl are showed in Fig. 1. They show highly characteristic bacterial growth patterns including the lag phase, the logarithmic phase, the stationary phase and the death phase. It is obvious that the NaCl concentration has influenced the metabolism of *H. salinarum* growth.

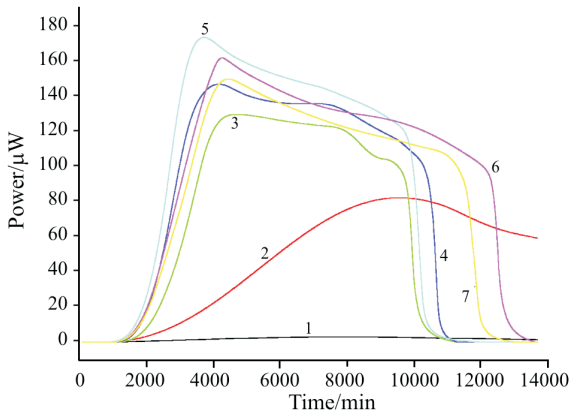

Fig. 1 The power-time curves of *H. salinarum* growth in different concentrations of NaCl: 1 – 130 g L^{-1}, 2 – 150 g L^{-1}, 3 – 170 g L^{-1}, 4 – 190 g L^{-1}, 5 – 230 g L^{-1}, 6 – 260 g L^{-1}, 7 – 290 g L^{-1}

Thermokinetic parameters and equations

In the logarithmic growth phase, the heat output of cell growth is exponential [5, 8],

$$P_t=P_0\exp(kt)$$

or

$$\ln P_t=\ln P_0+kt \quad (1)$$

where P_t is the heat output power at time t, P_0 the heat output power at time zero, k the growth rate constant and t the experimental time. The growth thermogenic curves of the logarithmic growth phase correspond to Eq. (1). Therefore, using the data $\ln P_t$ and t taken from a growth thermogenic curve to fit a linear equation, one can obtain the growth rate constant (k) and the growth thermokinetic equation. The generation time (t_G), which is (ln2)/k, can be calculated. The maximum heat output power (P_m) and the time corresponding to P_m (t_m) can also be obtained from the growth thermogenic curve. The thermokinetic parameters and equations of *H. salinarum* growth in different concentrations of NaCl are shown in Tables 1 and 2, respectively. All of the experimental results have a good reproducibility and correlationship.

Table 1 The growth rate constants (k) and the growth thermokinetic equations of *H. salinarum* growth in different concentrations of NaCl

c_{NaCl}/ g L^{-1}	Thermokinetic equation	k/ 10^{-3} min^{-1}	R
130	–	0	–
150	$\ln P=-2.266+1.634\cdot10^{-3}t$	1.634±0.013	0.993
170	$\ln P=-3.261+2.549\cdot10^{-3}t$	2.549±0.020	0.996
190	$\ln P=-3.462+3.032\cdot10^{-3}t$	3.032±0.011	0.996
230	$\ln P=-3.384+3.261\cdot10^{-3}t$	3.261±0.008	0.997
260	$\ln P=-3.298+3.008\cdot10^{-3}t$	3.008±0.010	0.997
290	$\ln P=-3.224+2.916\cdot10^{-3}t$	2.916±0.012	0.996

Table 2 The thermokinetic parameters of *H. salinarum* growth in different concentrations of NaCl

c_{NaCl}/ g L^{-1}	k/ 10^{-3} min^{-1}	t_G/ min	P_m / μW	t_m/ min
130	0	–	2.8	–
150	1.634	424.2	81.8	9549
170	2.549	271.9	129.1	4660
190	3.032	228.6	146.3	4155
230	3.261	212.6	173.0	3709
260	3.008	230.4	161.3	4254
290	2.916	237.7	149.3	4436

Relationship between k and the concentration of NaCl

The relationship between the growth rate constant (k) and the corresponding concentration of NaCl (c_{NaCl}) is shown in Fig. 2. We can see that when the concentration of NaCl was below 230 g L^{-1} (130–230 g L^{-1}), the growth rate constant (k) increased with the increasing of NaCl concentration. However, when the concentration of NaCl was above 230 g L^{-1} (230–290 g L^{-1}), the growth rate constant (k) decreased as NaCl concentration increased.

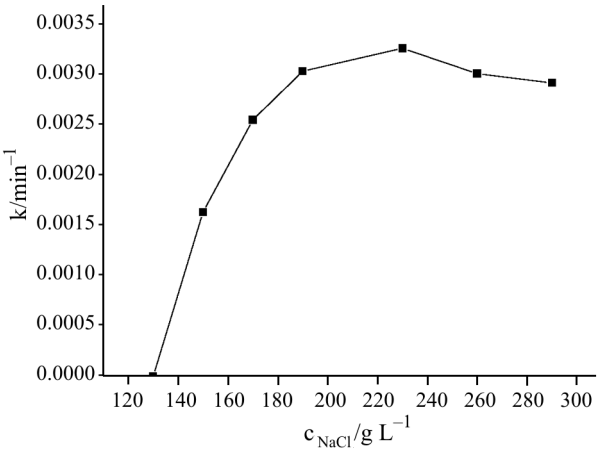

Fig. 2 Plot of k *vs.* c_{NaCl}

Relationship between P_m and the concentration of NaCl

Figure 3 shows the maximum heat output power (P_m) *vs*. the corresponding concentration of NaCl (c_{NaCl}). As shown in Fig. 3, when the concentration of NaCl was below 230 g L^{-1} (130–230 g L^{-1}), the maximum heat output power (P_m) increased as NaCl concentration increased. But when the concentration of NaCl was above 230 g L^{-1} (230–290 g L^{-1}), the maximum heat output power (P_m) declined with the increasing of NaCl concentration.

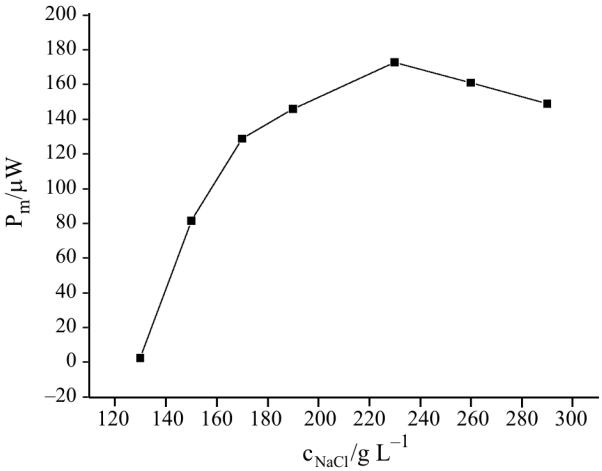

Fig. 3 Plot of P_m *vs.* c_{NaCl}

Relationship between t_m and the concentration of NaCl

The time corresponding to P_m (t_m) is also an important parameter as to growth metabolism of microbes. As shown in Fig. 4, when the concentration of NaCl was below 230 g L^{-1}, the time corresponding to P_m (t_m) decreased as NaCl concentration increased. On the contrary, when the concentration of NaCl was in the range of 230–290 g L^{-1}, the time corresponding to P_m (t_m) increased as NaCl concentration increased.

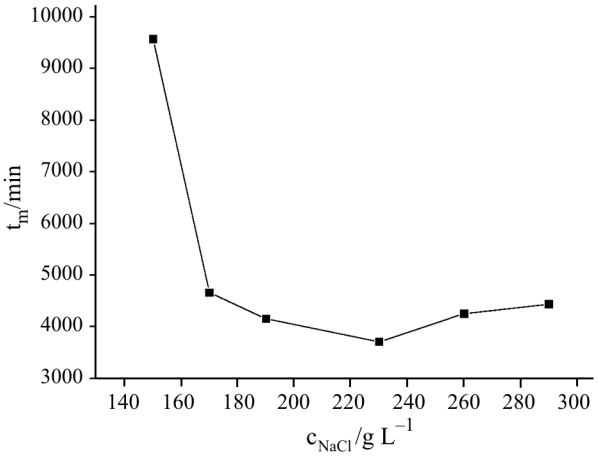

Fig. 4 Plot of t_m *vs.* c_{NaCl}

Transmission electron microscopic observations

The transmission electron microphotographs of *H. salinarum* are shown in Figs 5 and 6. Obvious morphological differences are found between *H. salinarum* cells growing in different concentrations of NaCl. At 230 g L^{-1} NaCl the *H. salinarum* cells were slender uniform rods (Fig. 5d). With the NaCl concentration lowered from 230 g L^{-1} to 150 g L^{-1}, the rods assumed firstly irregular and finally spherical shapes (Figs 5b, c). At 130 g L^{-1} NaCl no intact cells remained. There were only some much smaller vesicles visible with cell wall fragments around them (Fig. 5a). At the highest NaCl concentration studied (290 g L^{-1}, approximately 5.0 mol L^{-1}), the cells were regular rods with the cytoplasm shrinking and the plasma membrane contracting from the cell wall (Figs 5e and 6). It seems evident that plasmolysis occurred in these *H. salinarum* cells. This phenomenon has not been reported before.

Discussion

According to the thermogenic curves and thermokinetic parameters of *H. salinarum* growth in different concentrations of NaCl (from 130 g L^{-1} to nearly saturated NaCl concentration), it can be seen that when external NaCl concentration was below

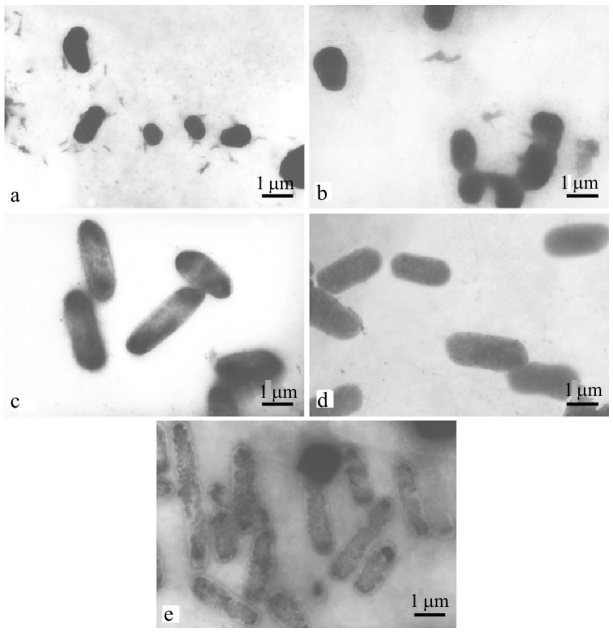

Fig. 5 Transmission electron microphotographs of *H. salinarum* cells growing in different concentrations of NaCl; a – cells growing in 130 g L^{-1} NaCl, b – cells growing in 150 g L^{-1} NaCl, c – cells growing in 170 g L^{-1} NaCl, d – cells growing in 230 g L^{-1} NaCl, e – cells growing in 290 g L^{-1} NaCl. A salt crystal was also present. Bar, 1 μm

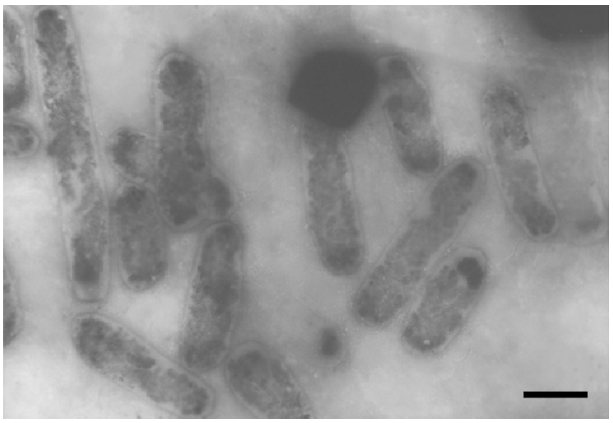

Fig. 6 Higher magnification of Fig. 5e

230 g L^{-1}, the growth metabolism of *H. salinarum* declined gradually with the decreasing of NaCl concentration, till ceased completely at 130 g L^{-1} NaCl. This is in agreement with the previous report [1, 2, 9]. But when external NaCl concentration was above 230 g L^{-1}, the growth metabolism of *H. salinarum* appeared to descend constantly as NaCl concentration increased, which has never been described before. The microcalorimetric experiment was repeated 4 times and the results were identical.

In order to interpret this novel finding, we examined the morphology of *H. salinarum* cells growing in different concentrations of NaCl using a transmission

electron microscope. It was found that with the external NaCl concentration gradually reduced from 230 g L^{-1}, the rod-shaped organisms appeared firstly irregular and then spherical. Ultimately they disintegrated into much smaller membrane vesicles. These observations are consistent with the conclusion obtained by microcalorimetry, and support the theory of 'NaCl maintain cell wall' [1, 2]. It was also found that *H. salinarum* cells growing in 290 g L^{-1} NaCl underwent plasmolysis. This phenomenon has not been reported previously, and cannot be explained by the theory of 'NaCl maintain cell wall'. Plasmolysis is the phenomenon of the cytoplasm shrinking away from the cell wall due to the loss of internal water through osmosis [10]. A wide range of bacteria (excluding halobacteria) have been documented to undergo plasmolysis in hypertonic environments [11]. The metabolism of plasmolyzed cells is usually retarded [10] and our microcalorimetric results confirmed it. Thus the results of transmission electron microscopy, together with the results of microcalorimetry, show that *H. salinarum* growing in nearly saturated NaCl undergo plasmolysis indeed.

The finding of plasmolysis in *H. salinarum* indicates that the protoplast of *H. salinarum* can be regarded as an osmotic system and alternations in external NaCl concentration will trigger the water movement across the cytoplasmic membrane. It implies that when external NaCl concentration decreases, water tends to enter the *H. salinarum* cell, leading to an enlargement of cell volume. However, as we have found, cell volume of '230 g L^{-1}' and that of '170 g L^{-1}' did not differ much (Fig. 5). All these suggest that the cell wall restricted continued expansion of the protoplast, and as has been documented [12], this would result in an outward-directed turgor (Fig. 7). It seems likely that besides electrostatic repulsion between cell wall glycoproteins, the turgor exerted on the cell wall was also responsible for the structural transformations and disintegration of *H. salinarum* upon NaCl concentration decreasing.

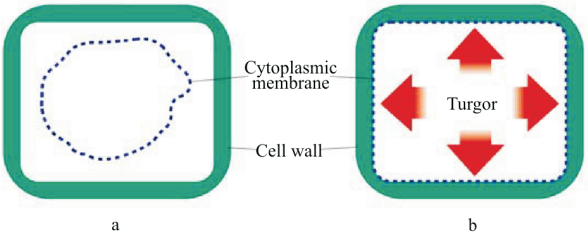

Fig. 7 The presumed physiological states of *H. salinarum* cells growing in different concentrations of NaCl; a – *H. salinarum* cells growing in approaching NaCl saturation undergo plasmolysis, b – A stepwise reduction of NaCl concentration causes the protoplast to swell, but its continued expansion is restricted by the cell wall, which generates the turgor pressure

Turbidimetry is the most commonly used technique in measuring microbial growth [13]. In previous studies involving halobacteria, the measurements of halobacterial growth were done mainly by turbidimetry [14, 15]. But problems have been found in following halobacterial growth using optical density as halobacteria generally produce massive amounts of carotenoid pigments that can interfere with spectrophotometric measurements [1, 14, 15]. It is known that the absorption peaks of these carotenoid pigments are close to that of halobacterial cells [15]. It is also known that carotenoid production varies with salt concentration, media composition, and at different stages of growth [1, 15]. These variations make absorbance measurements for halobacterial growth unreliable [14, 15]. However, this problem can be avoided when halobacterial growth is monitored by microcalorimetry. Microcalorimetry can directly determine the biological activity of a living system and provide a continuous measurement of heat production, which will not be disturbed by cellular pigments. Furthermore, the detector system is very sensitive and high accuracy can be obtained using microcalorimetry [16]. In this study, microcalorimetry has been applied to monitor *H. salinarum* growth and highly accurate results with good reproducibility have been obtained. Microcalorimetry has thus proven to be a particularly practical tool to monitor the growth processes of halobacteria, and other pigment-producing microbes as well.

The thermogenic curves of bacterial growth completely describe growth metabolism processes [17]. Under the same conditions, the thermogenic curves of every kind of bacteria all have good reproducibility and outstanding characteristics. Once environmental factors varied, the metabolic process will be influenced, hence the bacterial growth thermogenic curves changed remarkably. Due to this, microcalorimetric studies of bacterial growth may reveal subtle details not observable by other techniques [18]. In this study, by comparing the thermogenic curves of *H. salinarum* growth in different concentrations of NaCl and analyzing the thermokinetic information contained, we have found that the optimum NaCl concentration for *H. salinarum* growth was not a wide range from 3.5 mol L^{-1} to NaCl saturation (about 5.2 mol L^{-1}), as is generally acknowledged [1, 2], but just around 230 g L^{-1} (approximately 3.9 mol L^{-1}). And when external NaCl concentration increased gradually from 230 g L^{-1}, the growth metabolism of *H. salinarum* decreased constantly till external NaCl approached saturation. We have not found any description of this phenomenon in previous literature. In fact, it was difficult to discover using the conventional microbiological techniques (e.g. turbidimetry). Further investigation by transmission electron microscopy revealed that *H. salinarum* growing in approaching NaCl saturation under-

went plasmolysis, which interpreted the novel finding of microcalorimetry perfectly. Our study shows that microcalorimetry is a promising tool for studying microbial growth and it may reveal more and newer details about microbial growth than the existing methods do. All of these are very significant to understand biological processes.

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