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A variant of the hyperthermophile *Archaeoglobus fulgidus* adapted to grow at high salinity

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Abstract

A variant of *Archaeoglobus fulgidus* VC-16 was isolated from cultures obtained after a stepwise transfer from media containing 1.8–6.3% NaCl by a plating-independent, selected-cell cultivation technique, using a laser microscope. This variant, *A. fulgidus* VC-16S, had a higher growth rate throughout the salt range of the parental strain, but was also able to grow in media containing NaCl up to 6.3%, whereas the parental strain could not grow above 4.5% NaCl. Diglycerol phosphate (DGP), only encountered in the Archaeoglobales, was the major solute accumulated under supra-optimal salinities, whereas at supra-optimal growth temperatures di-*myo*-inositol phosphate was the predominant solute. The accumulation of compatible solutes during growth of variant VC-16S was lower than in the parental strain within 1.8–4.5% NaCl, but the levels of compatible solutes, including DGP, increased sharply in the variant at higher salinities (5.5 and 6.0%). This variant represents, at this time, one of the most halophilic hyperthermophiles known, and its ability to grow at high salinity appears to be due to the massive accumulation of DGP.

Keywords: Hyperthermophile; *Archaeoglobus fulgidus*; Compatible solute; Diglycerol phosphate; Optical tweezers

1. Introduction

Archaeoglobus fulgidus is an anaerobic hyperthermophilic archaeon that utilises sulfate as a terminal electron acceptor [1,2]. The type strain of the species, designated VC-16, was isolated from marine hydrothermal vents of the Island of Vulcano, Italy [1], but other strains have also been isolated from marine sediments [3] and oil field water in the North Sea and Alaska [4,5]. Two other species of the genus *Archaeoglobus*, *A. profundus* and *A. veneficus*, have been described which are also slightly halophilic hyperthermophiles isolated from abyssal geothermal areas [6,7].

Like most halophilic organisms *A. fulgidus* VC-16 accumulates organic compounds in response to salt stress [8]. However, the major organic solute accumulated by this organism is diglycerol phosphate (DGP), which has only been found, thus far, in the genus *Archaeoglobus*. DGP has a high protective effect on several proteins isolated from different sources, even at low concentrations (100 mM) [9,10]. Di-*myo*-inositol-1,1′(3,3′) phosphate (DIP) also accumulates in *A. fulgidus*, but in response to growth at elevated temperatures [8].

In this work, *A. fulgidus* VC-16 was adapted to high salt concentrations by a stepwise transfer in medium with increasing NaCl concentrations. From a medium containing 6.3% NaCl, a variant of *A. fulgidus* VC-16 was isolated by a plating-independent, selected-cell cultivation technique, based on the use of laser microscope 'optical tweezers' [11,12]. This variant, being able to grow at higher salinities, could be a good candidate for the production of higher levels of DGP than the wild-type strain; therefore, the pattern of solute accumulation in the new variant VC-16S was examined in detail as a function of growth temperature, medium salinity, and growth phase.

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2. Materials and methods

2.1. Strains and culture conditions

A. fulgidus strain VC-16 (= DSM 4304^T) and variant VC-16S were routinely maintained in the medium described by Stetter et al. [1], except that Na_2SO_4 was replaced by the same concentration of $Na_2S_2O_3$ and sodium hydrogen carbonate was replaced by 20 mM PIPES disodium salt. The total sodium ion concentration of the medium was 80 mM and the total chloride ion concentration was 39.4 mM, but NaCl was not included in the formulation of the medium. In this study the medium was supplemented with NaCl to the required levels.

The organisms were cultured in 2-l or 5-l fermentation vessels with continuous gassing using a gas phase composed of N_2 and CO_2 (80:20 v/v) with stirring at 80 rpm. Cell growth was monitored by counting with a Neubauer counting chamber (Weber, UK). Unless otherwise stated, cultures were grown until the late exponential phase. Cells were harvested by centrifugation (10 000 × *g*, 4°C, 15 min), and washed twice with the growth medium without carbon sources, PIPES, or resazurin. The cell pellet was resuspended in water to a final concentration of approximately 10 mg protein ml^{-1}. The effect of salinity on the accumulation of organic solutes by *A. fulgidus* strains VC-16S and VC-16 was investigated in cultures grown in media containing NaCl to a maximum of 6.0%.

The effect of the growth temperature on the accumulation of solutes by *A. fulgidus* was examined between 65 and 89°C. The effect of the growth phase on the accumulation of solutes was investigated in media containing 1.8 and 4.5%. For these investigations strain VC-16S was cultured in a 5-l fermentation vessel. At appropriate time intervals, samples (500 ml) were collected and cells were harvested as described above.

2.2. Extraction of intracellular solutes

The intracellular solutes were extracted with boiling 80% ethanol as previously described by Martins and Santos [13]. Freeze-dried extracts were dissolved in D_2O and analysed by nuclear magnetic resonance (NMR). Intracellular solutes were quantified by ^{31}P-NMR, using potassium phosphate as internal concentration standard. Glutamate levels were determined with an enzymatic assay [14].

The protein cell content in the samples used for solute analysis was determined by the Bradford assay after cell lysis by sonication (B. Braun-Biotech) and centrifugation to remove cell debris.

2.3. NMR spectroscopy

NMR spectra were acquired in a Bruker AMX300 spectrometer using a 5-mm inverse detection probe head at 30°C, with presaturation of the water signal, 60° flip angle and a repetition delay of 40 s. Chemical shifts were referenced with respect to sodium 3-trimethylsilyl[2,2,3,3-^{2}H]-propionate.

^{31}P-NMR spectra were recorded at 121.50 MHz, with proton broad-band decoupling using a repetition delay of 30 s and a 90° flip angle. The intensity of the resonances was compared with that of the signal due to a known amount of potassium phosphate added to the sample. Chemical shifts were referenced to external 85% H_3PO_4.

3. Results

3.1. Isolation of A. fulgidus strain VC-16S

A stepwise transfer of *A. fulgidus* VC-16 in media with increasing NaCl concentrations led to the appearance of populations with different cell volumes. From medium containing 6.3% NaCl, a single, enlarged cell was selected and isolated. The diameter of single cells increased significantly: in medium containing 1.8% NaCl the diameter was about 1 μm, in 4.5% NaCl medium it was about 2 μm, and in medium with 6.3% NaCl it was up to 4 μm. The latter cell dimension corresponds to an increase in cell volume of about 70-fold in the NaCl range examined. This high-salt-adapted culture of *A. fulgidus* VC-16 was designated *A. fulgidus* strain VC-16S.

3.2. Growth profile of A. fulgidus strain VC-16S

Strain VC-16S grew in media containing NaCl up to about 6% (0.8–6.8% total salts in the medium), with optimum growth at approximately 1.8% NaCl (Fig. 1). Growth within 48 h was not observed at 6.5 and 7.0% NaCl. At the optimum growth temperature, the doubling times of strain VC-16S were similar in media containing 0.9–4.5% NaCl, but at lower (0.0% NaCl) or higher salinities (5.5 and 6.0% NaCl) the doubling times increased sharply. The doubling times of *A. fulgidus* VC-16 were approximately two-fold higher than those obtained for

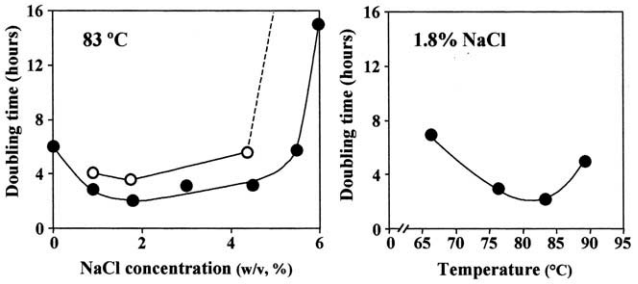

Fig. 1. Doubling times for growth of *A. fulgidus* VC-16 and *A. fulgidus* VC-16S as a function of the NaCl concentration in the growth medium (left panel) and the growth temperature (right panel). Solid symbols refer to *A. fulgidus* VC-16S and open symbols refer to *A. fulgidus* VC-16. The dashed line suggests that no growth was observed within 48 h for *A. fulgidus* VC-16 at NaCl concentrations of 5.5 and 6.0%.

A. fulgidus VC-16S in media with the same concentration of NaCl, and reached lower cell densities at optimum conditions than strain VC-16S. Moreover, strain VC-16 did not grow in media containing 5.5 or 6.0% NaCl within 48 h, while variant VC-16S reached the stationary phase within the same period of time in medium containing 6.0% NaCl (Fig. 1). Lag phases were not observed in any of the conditions examined. Strain VC-16 and variant VC-16S grew between 65 and 89°C, with the optimal temperature for growth in the vicinity of 83°C. The highest cell density attained during growth under optimum conditions of temperature and salinity was around 2×10^8 cells ml^{-1}, but strain VC-16 normally reached slightly lower cells densities than the variant.

3.3. Identification of major intracellular solutes in *A. fulgidus* VC-16S

The ^{31}P-NMR spectra of ethanol extracts of *A. fulgidus* strain VC-16S showed resonances assigned to DGP, DIP and an isomer of di-*myo*-inositol-phosphate (DIP″) that is not fully characterised. In addition to resonances due to these solutes the ^{1}H-NMR spectrum revealed the presence of α-glutamate and vestigial levels of acetate. Resonances were assigned by comparison with the proton and phosphorus chemical shift values reported previously [8]. The assignment of DGP was also confirmed by spiking the sample with the pure compound.

3.4. Effect of salinity and temperature on the accumulation of compatible solutes

DGP was the major solute accumulated by *A. fulgidus* VC-16S under all growth conditions examined, except when the medium was not supplemented with NaCl, when α-glutamate was the only solute detected in vestigial levels (0.01 μmol mg protein^{-1}) (Table 1). The total pool of solutes increased concomitantly with higher levels of NaCl in the medium, this increase in the total solute being attributed primarily to higher levels of DGP. At the highest NaCl concentration used (6.0%), the total amount of solutes reached 3.3 μmol mg protein^{-1}, and DGP corresponded to about 85% of the total organic solute pool (Table 1, Fig. 2). The level of DGP increased 17-fold between the optimum and the maximum NaCl concentration for growth. The levels of glutamate peaked to 0.71 μmol mg protein^{-1} in cells derived from growth in medium with 5.5% NaCl, which corresponded to 39% of the total organic solute pool, thereafter decreasing to only 9% of the total compatible solute pool at the highest salinity for growth. At the optimum temperature, DIP was only detected in vestigial concentrations within the salinity range examined, whereas the levels of DIP″ varied between 4% and 8% of the total pool in response to an increase in the salinity.

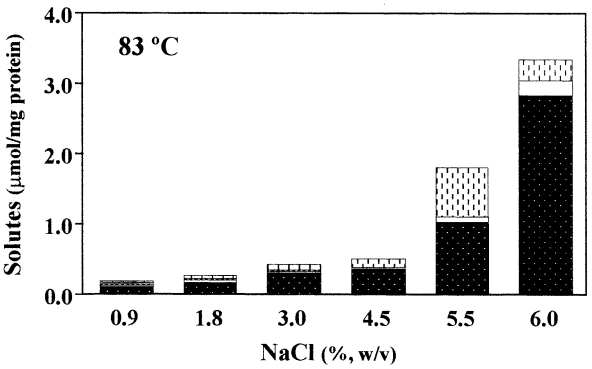

Fig. 2. Effect of NaCl concentration on the accumulation of solutes by *A. fulgidus* VC-16S. Samples were withdrawn in the late exponential phase of growth and solutes were quantified by ^{31}P-NMR. DGP (black dotted), DIP″ (white), DIP (checkered) and α-glutamate (stippled).

Raising the growth temperature from 66 to 89°C, at the

Table 1
Quantification of organic solutes in the parental strain and the variant using ^{31}P-NMR[a]

A. fulgidus	Conditions		Solutes (μmol mg protein^{-1})				
	Temp. (°C)	NaCl (%)	DGP	DIP	DIP″	α-Glutamate[b]	Total pool
VC-16	83	1.8	0.19	0.02	0.03	0.02	0.26
	83	4.5	0.68	ND	0.05	0.08	0.81
VC-16S	66*	1.8	0.07	0.01	ND	0.05	0.13
	76*	1.8	0.09	0.01	0.01	0.02	0.12
	83	0.0	ND	ND	ND	0.01	0.01
	83	0.9	0.10	0.02	0.01	0.03	0.16
	83	1.8	0.17	0.02	0.02	0.04	0.25
	83	3.0	0.31	0.01	0.02	0.08	0.41
	83	4.5	0.37	ND	0.02	0.11	0.50
	83	5.5	1.02	ND	0.07	0.71	1.81
	83	6.0	2.81	ND	0.23	0.29	3.34
	89	1.8	0.14	0.22	0.06	0.06	0.48
	89	4.5	0.45	0.05	0.09	0.06	0.64

ND: below the detection limit.
[a]Values are the mean of two to four replicates, but in the cases labelled with an asterisk, the determination was performed only once. Replicate determinations varied less than 20%.
[b]Glutamate concentrations were determined by an enzymatic assay.

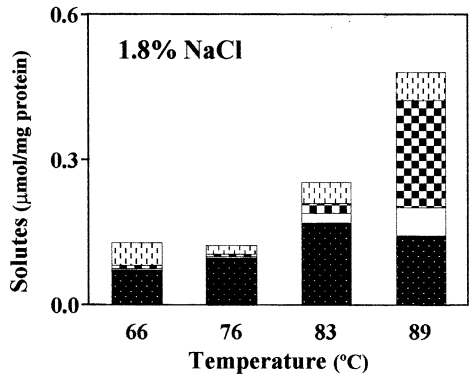

Fig. 3. Effect of growth temperature on the accumulation of solutes by *A. fulgidus* VC-16S. Samples were withdrawn in the late exponential phase of growth. DGP (black dotted), DIP″ (white), DIP (checkered) and α-glutamate (stippled).

optimum salinity, resulted in a four-fold increase in the total pool of compatible solutes (Fig. 3). At supra-optimal temperatures DIP became the major solute, constituting 46% of the total organic solute pool at 89°C.

The combination of moderate osmotic stress (4.5% NaCl) and heat stress (89°C) resulted in an increase of 30% in the total level of solutes. Under these conditions the proportions of DGP and DIP were drastically altered as compared with growth at 89°C and at the optimal salinity. The pool of DGP was nine-fold higher than that of DIP when the two stresses were combined. Conversely the pool of DIP was 1.6 times higher than that of DGP when the organism grew under heat stress (89°C) at the optimum salinity (Fig. 4).

The same compatible solutes were used for osmoadaptation by the parental strain and the variant; however, strain VC-16 accumulated higher amounts of compatible solutes in response to salt stress within the range of salt concentrations tolerated by both organisms (up to 4.5% NaCl). For example, the total organic solutes reached 0.81 μmol mg protein^{-1} in medium containing 4.5% NaCl, whereas the total solute pool in strain VC-16S was only 0.50 μmol mg protein^{-1}. Nevertheless, at higher salinities

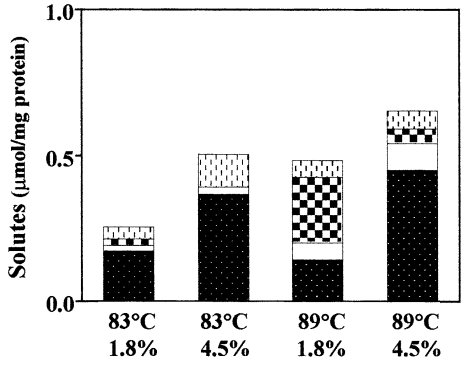

Fig. 4. Effect of combining high salinity and supra-optimal growth temperature on the accumulation of solutes by *A. fulgidus* VC-16S. Samples were withdrawn in the late exponential phase of growth. DGP (black dotted), DIP″ (white), DIP (checkered) and α-glutamate (stippled).

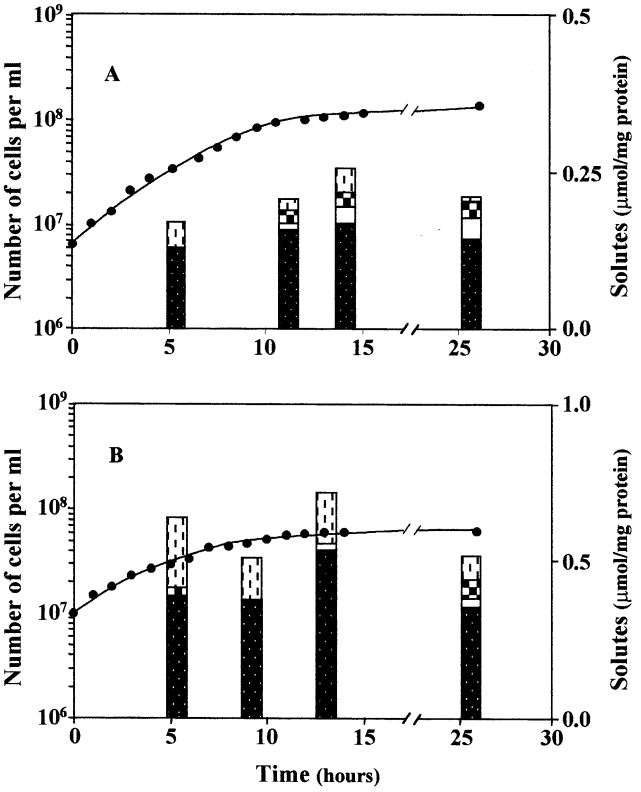

Fig. 5. Correlation between growth phase and accumulation of organic solutes by *A. fulgidus* VC-16S. The culture was grown in medium containing 1.8% (A) and 4.5% NaCl (B), at 83°C. The intracellular concentrations of the solutes were determined in different phases of growth. DGP (black dotted), DIP″ (white), DIP (checkered) and α-glutamate (stippled).

not tolerated by the parental organism, variant VC-16S accumulated huge amounts of compatible solutes (Table 1).

3.5. *Effect of growth phase on the accumulation of solutes in A. fulgidus VC-16S*

The profile of solute accumulation was not drastically affected by the growth phase (Fig. 5). The maximum accumulation of organic solutes occurred at the beginning of the stationary phase and the total pool decreased during the late stationary phase of growth. DGP was the major solute during all growth phases decreasing towards the stationary phase, while DIP and DIP″ tended to increase relative to DGP during the late exponential and stationary phases of growth.

4. Discussion

Most hyperthermophilic organisms isolated from marine hot springs are slightly halophilic, the vast majority of these organisms being unable to grow in media containing more than about 5.0% NaCl. The species of the genus *Archaeoglobus*, for example, do not grow in media con-

taining NaCl above 4.5% [6,7]. We have selected a variant of the type strain of *A. fulgidus* VC-16 that is clearly better adapted to high salinity than the parental strain, being able to grow in medium containing 6.0% NaCl making it one of the most halophilic hyperthermophilic archaea known. This variant also has a higher growth rate than the parental strain in media with low salt concentrations (up to 4.5% NaCl) and it could be argued that we selected for and isolated a fast-growing variant. However, the fact remains that variant VC-16S also grows at higher salinities than the parental strain and is, therefore, more halotolerant.

Hyperthermophilic organisms, like most other microorganisms, accumulate compatible solutes for osmotic adjustment. The physiological conditions leading to the accumulation of compatible solutes in thermophiles and hyperthermophiles have been studied, in some detail only in a few organisms, namely in *Pyrococcus furiosus*, *Thermococcus litoralis*, *Rhodothermus marinus*, and *Thermotoga neapolitana* [13,15–17]. From these studies we notice a general trend that mannosylglycerate generally accumulates in response to salt stress, while DIP and its derivatives respond primarily to heat stress [18]. Mannosylglycerate has not been detected in *A. fulgidus* strain VC-16 or its variant. The genes involved in the synthesis of mannosylglycerate in *Pyrococcus horikoshii* and *R. marinus* have been characterised, but homologues of these genes are absent from the complete genome sequence of *A. fulgidus* [19–21]. DGP, which has not been found in other organisms, and DIP constitute the major organic solutes of *A. fulgidus*. It is relevant to report here that mannosylglycerate has been detected in the type strain of *A. profundus* and *A. veneficus*. These organisms also accumulate glutamate and DIP, but surprisingly DGP was not detected in *A. profundus* (T.Q. Faria, L.G. Gonçalves, R. Huber and H. Santos, unpublished results). It appears, therefore, that the type strain and the variant of *A. fulgidus*, unlike other strains of this genus, cannot synthesise mannosylglycerate because the genes leading to the synthesis of mannosylglycerate must have been lost during the evolution of this strain. It is interesting that *A. fulgidus* VC-16, unable to synthesise mannosylglycerate, was able to develop an alternative solute, DGP, to cope with osmotic stress.

This study shows that DGP is the major compatible solute during osmoadaptation in strain VC-16 and its variant. On the other hand, and corroborating previous results for other hyperthermophiles [15,17], the intracellular levels of DIP increase at growth temperatures above the optimum. Therefore, these results further support the view that compatible solutes in hyperthermophiles have specialised roles; some, such as mannosylglycerate and DGP, have a primary role in osmoadaptation, while others, namely DIP, are preferentially involved in thermoadaptation. For now, this hypothesis remains speculative because definite experimental evidence obtained with, for example, suitable knockout mutants is missing. These experiments would show if mannosylglycerate and DGP could replace DIP in thermal protection of the organisms at supra-optimal growth temperatures.

The differential profile of accumulation of the two isomers of di-*myo*-inositol phosphate in *A. fulgidus* VC-16S in response to osmotic and heat stress seems rather interesting. DIP was absent during growth at high NaCl concentrations, while the pool of DIP″ increased in response to the salt stress. This observation indicates that the synthesis of the two DIP isomers is subject to distinct regulatory mechanisms.

Surprisingly, the variant appears to have a discontinuous behaviour for osmotic adaptation; at moderate salinities (up to 4.5% NaCl) the total organic solute pool in *A. fulgidus* VC-16S was considerably lower than in the parental strain, but at high salinities, not tolerated by the parental strain, there was an enormous accumulation of compatible solutes, clearly reflecting that osmolytes are essential for osmotic adjustment at these extreme salinities.

Therefore, it seems that at moderate salinities this variant relies less on the accumulation of osmolytes for osmoadaptation. The hypothesis that the accumulation of inorganic solutes, such as KCl, contributed to the osmotic equilibrium of the cytoplasm was not supported by quantification of these ions in total cell extracts (not shown). It is conceivable that the nature of the cell wall and/or membrane has been altered in the salt-adapted variant to endow the cell with a higher resistance to gradients of osmotic pressure or that macromolecules may replace small osmolytes in protecting cell components against dehydration.

Greater knowledge of the molecular aspects of osmoadaptation in *A. fulgidus* is clearly necessary, not only because the major compatible solute, DGP, has only been found in the genus *Archaeoglobus* and appears to replace the ubiquitous mannosylglycerate as a compatible solute, but also because of the practical interest of DGP as a protein stabiliser. Work is in progress to elucidate the biosynthesis of DGP and regulation.

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