Glycerol metabolism in the extremely halophilic bacterium

Salinibacter ruber

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Abstract

Growth of Salinibacter ruber, a red, extremely halophilic bacterium phylogenetically affiliated with the Flavobacterium/Cytophaga branch of the domain Bacteria, is stimulated by glycerol. In contrast to glucose consumption, which starts only after more easily degradable substrates present in yeast extract have been depleted, glycerol is consumed during the earliest growth phases. When U-14C-labeled glycerol was added to the culture, up to 25% of the radioactivity was incorporated by the cells. Glycerol kinase activity was detected only in cells grown in the presence of glycerol (up to 90 nmol mg protein$^{-1}$ min$^{-1}$). This enzyme functioned over salt concentrations from 0.6 to 2.8 M KCl. No significant activity of NAD-dependent glycerol dehydrogenase was found. It is suggested that Salinibacter may use glycerol as one of its principal substrates in its habitat, the saltern crystallizer ponds.

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1. Introduction

Salinibacter ruber is a red, rod-shaped, aerobic, extremely halophilic bacterium, first isolated from a saltern crystallizer pond in Spain [1]. Phylogenetically it is affiliated with the Flavobacterium/Cytophaga branch of the domain Bacteria. Studies with fluorescent oligonucleotide probes showed Salinibacter to be abundant in Spanish crystallizer ponds: between 5 and 25% of the total prokaryotic community belongs to this type [2]. S. ruber is an obligate halophile that grows optimally between 200 and 300 g l$^{-1}$ salts; no growth is obtained at NaCl concentrations below 150 g l$^{-1}$. The physiology of Salinibacter is surprisingly similar to that of the archaeal family Halobacteriaceae: it is an aerobic heterotroph that maintains high intracellular K$^+$ concentrations, possesses enzymes that are functional at high salt concentrations, and has proteins with a high content of acidic amino acids [3,4]. In contrast to the other aerobic halophilic representatives of the domain Bacteria, which accumulate organic osmotic solutes [5,6], no significant concentrations of such solutes were found within Salinibacter cells [4].

S. ruber was originally described as being unable to metabolize glycerol, simple sugars and related compounds. The species description [1] states that “Addition of sugars and related compounds (glucose, glycerol, sucrose, ribose, fructose, xylose, lactose, mannitol, galactose, sorbitol, maltose) at concentrations of 5 g l$^{-1}$ ... did not stimulate growth greatly.” Lack of glycerol utilization by Salinibacter was confirmed in a study in which microautoradiography combined with fluorescent in situ hybridization was used to obtain information about the organic substrates used by the microbial community in a saltern crystallizer pond on Mallorca [7]. However, glycerol has been suggested to be one of the most easily available and most rapidly turned over substrates in such environments [8,9].

A renewed examination in which sugars were added revealed a pronounced stimulatory effect of glucose, maltose, and starch on the growth of S. ruber [10]. The ability of Salinibacter to degrade certain sugars raises the question as to whether glycerol can be metabolized by this bacterium. Here we report the enzymatic pathway responsible for catabolism and incorporation of glycerol in S. ruber, and compare glycerol metabolism in Salinibacter

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with the corresponding pathways detected in the Halobacteriaceae.

2. Materials and methods

2.1. Bacterial and archaeal strains and culture conditions

*S. ruber* strain M31 (DSM 13855T) was grown in medium of the following composition (g l\(^{-1}\)): NaCl, 195; MgSO\(_4\)\(_7\)\(^{2}\)H\(_2\)O, 25; MgCl\(_2\)-6H\(_2\)O, 16.3; CaCl\(_2\)-2H\(_2\)O, 1.25; KCl, 5.0; NaHCO\(_3\), 0.25; NaBr, 0.625, and yeast extract, 1.0, pH 7.0 [1]. When indicated, this medium was supplemented with glycerol (0.1, 0.25, or 0.5 g l\(^{-1}\)). In selected experiments the yeast extract concentration was lowered to 0.1 g l\(^{-1}\). NH\(_4\)Cl and K\(_2\)HPO\(_4\) were then added to concentrations of 1 mM and 0.1 mM, respectively. *Halobacterium salinarum* R1 (ATCC 29341) was grown in medium composed of (g l\(^{-1}\)): NaCl, 250; MgCl\(_2\)-6H\(_2\)O, 5.0; KCl, 5.0; NH\(_4\)Cl, 5.0, and yeast extract, 5.0, pH 7.0 [3]. For enzymatic assays cells were grown in 0.5- or 1-l portions in 2-l Erlenmeyer flasks. Tests for growth stimulation by glycerol were conducted in 2-l Erlenmeyer flasks containing 500 ml of medium. All cultures were incubated on a rotary shaker (180 rpm) at 37°C.

2.2. Incorporation of \(^{14}\)C\]glycerol

To examine the fate of glycerol added to *Salinibacter* cultures, cells were inoculated as above in a 250-ml Erlenmeyer flask with 100 ml medium supplemented with 0.5 g l\(^{-1}\) glycerol and 25 μl of [U-\(^{14}\)C]glycerol (50 μCi ml\(^{-1}\); 149 mCi mmol\(^{-1}\); Amersham, UK). To assess the radioactivity incorporated in the cells during growth, 0.5-ml portions were centrifuged (3 min, 12000 \(\times\) g), the pellets were washed with 0.5 ml of growth medium without labeled glycerol, and then transferred to scintillation vials containing 5 ml Zinsser Analytic Quicksafe scintillation fluid. To additional 0.4-ml portions of the culture and to the culture supernatant from the 0.5-ml sample described above, 25 μl of 6 M HCl was added, and the tubes (1.5-ml Eppendorf) were incubated for 2 h at room temperature to remove any labeled CO\(_2\) or bicarbonate that may have been present. Subsequently the contents of the tubes were transferred to scintillation vials and counted with 5 ml of scintillation fluid as above.

2.3. Enzymatic assays

Exponential growth phase cells grown as described in Section 2.1 (OD\(_{600}\) approx. 0.25 for *S. ruber*, approx. 0.5 for *H. salinarum*) were harvested by centrifugation (15 min, 6000 \(\times\) g at 4°C). Cell pellets were resuspended in buffer (3 M KCl+100 mM KH\(_2\)PO\(_4\), adjusted to pH 7.4 with KOH for the glycerol kinase assays; 4 M NaCl+50 mM tetra-Na-pyrophosphate, adjusted to pH 9.5 with HCl for the glycerol dehydrogenase assays). Cells were broken by sonication (4×20 s at 140 W). Debris was removed by centrifugation (10 min, 12000 \(\times\) g, 4°C), and the supernatant, containing 5–15 mg protein ml\(^{-1}\), was used as the crude enzyme preparation.

Glycerol kinase (ATP:glycerol 3-phosphotransferase, EC 2.7.1.30) was assayed in the presence of glycerol and ATP, whereafter formation of α-glycerophosphate was quantified enzymatically in the presence of NAD and α-glycerophosphate dehydrogenase. The reaction mixture (5 ml) contained 1 ml cell extract, 3.7 ml 100 mM K-PO\(_4\) buffer, pH 7.4, 30 μmol ATP, and 100 μmol l-cysteine, with varying concentrations of KCl. The reaction was started by addition of 125 μmol glycerol. Negative controls lacked ATP and/or glycerol. At zero time and after 10, 20, and 30 min of incubation at 35°C, 1-ml samples were withdrawn and the reaction terminated by addition of 1 ml of 0.2 N H\(_2\)PO\(_4\). After centrifugation to remove precipitated proteins, the α-glycerophosphate content of 0.25-ml portions was assayed enzymatically by adding 0.25 ml 0.1 N NaOH for neutralization, followed by 0.1 ml 25 mM NAD, 1.5 ml 1 M hydrazine sulfate adjusted to pH 9.4 with NaOH, 0.15 ml 1% (w/v) nicotinamide-Na\(_2\)CO\(_3\) buffer, pH 9.4, and 20 μl of rabbit muscle α-glycerophosphate dehydrogenase (EC 1.1.1.8; Sigma, 400 U ml\(^{-1}\)). After 1 h incubation at 30°C the absorbance at 340 nm was measured.

Glycerol dehydrogenase (glycerol:NAD\(^+\) 2-oxidoreductase, EC 1.1.1.6) activity was quantified using the reduction of NAD in the presence of glycerol as outlined by Oren and Gurevich [11]. The reaction mixtures contained 0.2 ml cell extract, 1.6 ml 50 mM Na-pyrophosphate buffer, pH 9.5, containing NaCl concentrations varying from 0 to 4 M or KCl concentrations varying from 0 to 3 M, and 0.1 ml of 15 mg ml\(^{-1}\) NAD. The reaction was started by the addition of 0.1 ml of 5% (w/v) glycerol. Reduction of NAD was measured at 340 nm in a Hewlett-Packard model 8452A diode array spectrophotometer equipped with a temperature-controlled cuvette compartment. Alternatively, the reaction was followed at 600 nm by measuring reduction of 2,6-dichlorophenol-indophenol (0.1 ml of 1 mM added to the reaction mixture).

2.4. Miscellaneous analytical methods

Glycerol was assayed colorimetrically, based on periodate oxidation to formaldehyde, followed by colorimetric determination of formaldehyde with 3-methyl-2-benzothiazolone hydrazone (MBTH) [8]. To 1-ml portions of sample or standard glycerol solutions in glass test tubes, 0.1 ml of 25 mM Na-meta-periodate in 1 N H\(_2\)SO\(_4\) was added. After a 10-min incubation at room temperature, 0.1 ml 0.25 M Na-meta-arsenite was added. After a second 10-min incubation, 0.2 ml 2 N HCl was added, incubated for 1 min, then 0.2 ml of 2.76% (w/v) MBTH (Sigma, dissolved by
gentle heating in 0.1 N HCl) was added. The tubes were heated for 3 min in a boiling water bath, cooled, and 0.2 ml of 5% (w/v) FeCl₃·6H₂O was added. After 30 min 1 ml acetone was added and the absorbance measured at 670 nm. All incubations were performed in the dark.

The protein content of cell extracts was assayed by the Lowry procedure, using bovine serum albumin as a standard.

3. Results and discussion

3.1. Growth experiments

When media with 0.1 or 1 g l⁻¹ yeast extract were supplemented with glycerol, final cell densities of S. ruber increased. Upon addition of 0.25 and 0.5 g l⁻¹ glycerol to media containing 1 g l⁻¹ yeast extract, the OD₆₀₀ reached the stationary growth phase increased from around 0.3 to 0.4 and 0.5, respectively (Fig. 1). Addition of 0.5 g l⁻¹ glycerol to medium containing 0.1 g l⁻¹ yeast extract led to an increase in OD₆₀₀ from 0.035 to about 0.2 (data not shown). Glycerol was consumed in the process, with the greatest decrease in glycerol concentration coinciding with the exponential part of the growth curve (Fig. 1). In medium containing 0.1 g l⁻¹ yeast extract only about half of the 0.5 g l⁻¹ glycerol was consumed, in spite of the fact that ammonia and phosphate had been added to the medium (data not shown). This suggests that glycerol alone is not sufficient to support growth, and that other organic substrates may be required. Consumption of glycerol was not accompanied by acidification of the medium. In contrast, acids such as acetate, pyruvate, and D-lactate are formed during breakdown of glycerol by halophilic Archaea of the genera Haloferax and Halocarcula, even when glycerol was present in micromolar concentrations [12]. Metabolism of glucose by Salinibacter also did not lead to medium acidification [10], this in contrast to the massive acid production observed when certain members of the Halobacteriaceae grow in the presence of glucose or other sugars [5,13].

Comparison of glycerol and glucose consumption by Salinibacter shows that different kinetics are involved. Glycerol was consumed throughout the exponential growth phase (Fig. 1), while glucose consumption only started when other, more easily degradable substrates were exhausted [10]. Sugars are not among the preferred growth substrates for S. ruber, and the range of sugars used is small [10]. No diauxic growth pattern such as found in glucose/yeast extract media was observed in glycerol/yeast extract media, as shown in the right panels of Fig. 1.

3.2. The fate of glycerol metabolized by Salinibacter

To determine the fate of the glycerol consumed by Salinibacter, we followed the distribution of the label from
[U-14C]glycerol added to a growing culture (Fig. 2). After 100 h of incubation, when all glycerol had been consumed (compare Fig. 1), about 12% of the label had been incorporated by the cells. The total radioactivity remaining in the culture had decreased by about 40%, a decrease that may be attributed to oxidation of part of the glycerol to CO2. The nature of the remaining dissolved radiolabeled fraction was not ascertained. Part of this labeled material was slowly incorporated by the cells upon prolonged incubation, and after 190 h of incubation the cellular fraction contained about 25% of the initial radioactivity.

3.3. Enzymatic assays

Two pathways of glycerol metabolism are found in the prokaryotic world. One starts with the phosphorylation of glycerol by glycerol kinase, followed by dehydrogenation of glycerol 3-phosphate. In the second pathway, glycerol is first oxidized to dihydroxyacetone by glycerol dehydrogenase, whereafter phosphorylation occurs. In both cases the final product is dihydroxyacetone phosphate. Bacteria that use glycerol as carbon and energy sources may possess either or both pathways [14].

*S. ruber* cells grown in the presence of glycerol possess glycerol kinase activity. Rates between 23 and 90 nmol mg protein\(^{-1}\) min\(^{-1}\) were detected in extracts of cells grown for 2–3 days in the presence of 1 g l\(^{-1}\) glycerol. Salt concentration did not significantly affect the activity of the enzyme; rates were similar between 0.6 M KCl (the lowest concentration tested) and 2.8 M (data not shown). No glycerol kinase activity was found when glycerol was omitted from the medium, and activity started to appear within 2–3 h following exposure to glycerol (Fig. 3).

The induction pattern found for glycerol kinase suggests that this enzyme may be responsible for degradation of the glycerol supplied in the medium. Glycerol kinase has also been found in all genera of the Halobacteriaceae tested [11,15,16]. In *Haloferax mediterranei* and in *Halocarcula vallismortis* the activity increased four- and two-fold, respectively, when glycerol was included in the medium, as compared to glucose- or pyruvate-grown cells. Glycerol kinases of halophilic Archaea were most active at 0.4 M salt (either NaCl or KCl), but their activity in 3 M salt was still about 70% of the optimum [15]. Our earlier studies showed great variation in the behavior of the intracellular enzymes of *S. ruber* with respect to their salt requirement and tolerance. Some enzymes, such as the NAD-dependent isocitrate dehydrogenase and the NADP-dependent glucose-6-phosphate dehydrogenase, showed a markedly halophilic character [3,10], as expected for an organism that accumulates KCl intracellularly at levels comparable to those found in the Halobacteriaceae [4]. Also the fatty acid synthetase complex is stimulated by salt, and requires 0.5–1.5 M KCl for optimal activity (A. Oren, unpublished results). The malate dehydrogenase and hexokinase, on the other hand, do show a halophilic behavior as their optimal activity is found in the absence of salt [3,10]. The organism probably contains two NAD-dependent glutamate dehydrogenases, one salt-sensitive and one salt-requiring [3,17].

No significant activity of NAD-dependent glycerol dehydrogenase was detected in extracts of cells of *S. ruber*, whether or not grown in the presence of 1 g l\(^{-1}\) glycerol (data not shown). Rates of around 9 nmol mg protein\(^{-1}\) min\(^{-1}\) were detected in *H. salinarum* [11]; see also [18]. No glycerol dehydrogenase could be detected in representatives of the genera *Halorubrum*, *Haloferax* and *Halocarcula*, whether or not grown in the presence of glycerol [11,15].

3.4. Conclusions

The results of growth experiments and enzymatic assays show that glycerol is used as a substrate for growth for *S. ruber*, in contrast to what was published earlier [2]. Glycerol may well be one of the most abundantly available substrates in the habitat of *Salinibacter*: hypersaline salt lakes in which the unicellular green alga *Dunaliella* is the sole or main primary producer. *Dunaliella* produces massive amounts of glycerol as an osmotic stabilizer, and this glycerol is probably one of the main carbon and energy sources for the heterotrophic microbial community in such environments [19]. Some glycerol leaks through the cell membrane of *Dunaliella* even under optimal conditions, and release of glycerol into the surrounding medium is increased at elevated temperatures [20]. Glycerol is taken up and turned over rapidly by the microbial communities in saltern crystallizer ponds. Turnover times between 2.6 and 7.2 h have been reported for glycerol in the saltern crystallizer ponds in Eilat, Israel [8]. The finding that *Salinibacter* is able to use glycerol as a growth substrate raises interesting questions about the possible competition between *Salinibacter* and the halophilic Archaea of the...
family Halobacteriaceae, with which it shares its habitat. It is intriguing to note that microautoradiography combined with fluorescence in situ hybridization applied to a saltern crystallizer pond sample from Mallorca did not provide evidence for glycerol uptake, either by *Salinibacter* or by the members of the Halobacteriaceae present [7].

The present study shows that *S. ruber* is capable of glycerol uptake, even when other potential substrates are present in the medium. The reason for the failure to detect glycerol uptake in situ by *Salinibacter* cells in the Spanish saltern crystallizer ponds [7] remains unclear. Our radioactive labeling experiments (Fig. 2) suggest that part of the glycerol metabolized by *Salinibacter* may be converted to soluble products other than CO$_2$. The nature of these intermediates will be studied further in our laboratory.

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References


