Sugar 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 a small number of sugars (glucose, maltose, starch at 1 g l\(^{-1}\)). Glucose consumption starts after other substrates have been depleted. Glucose metabolism proceeds via a constitutive, salt-inhibited hexokinase and a constitutive salt-dependent nicotinamide adenine dinucleotide phosphate (NADP)-linked glucose-6-phosphate dehydrogenase. Glucose dehydrogenase and fructose-1,6-bisphosphate aldolase activity could not be detected. It is therefore suggested that *Salinibacter* metabolizes glucose by the classic Entner–Doudoroff pathway and not by the Embden–Meyerhof glycolytic pathway or by the modified Entner–Doudoroff pathway present in halophilic Archaea of the family Halobacteriaceae, in which the phosphorylation step is postponed. However, activity of 2-keto-3-deoxy-6-phosphogluconate aldolase could not be detected in extracts of *Salinibacter* cells, whether or not grown in the presence of glucose.

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1. Introduction

*Salinibacter ruber* is a red, rod-shaped, aerobic, extremely halophilic bacterium, first isolated from saltern crystallizer ponds 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 Archaea of the 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 [3–6]. In contrast to the other aerobic halophilic representatives of the domain Bacteria, which accumulate organic osmotic solutes [3,7], no significant concentrations of such solutes were found within *Salinibacter* cells [4].

*S. ruber* was originally described as being unable to metabolize sugars. The species description [1] states that ‘Simple sugars ... did not support growth as sole carbon and energy sources. 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’. However, a renewed examination, in which sugars were added at lower concentrations, as presented below, revealed a pronounced stimulatory effect of glucose, maltose, and starch.

The ability of *Salinibacter* to degrade certain sugars raises the question of the biochemical pathway(s) used in their catabolism. In the halophilic Archaea glucose is metabolized using a modification of the Entner–Doudoroff pathway, in which the phosphorylation step is postponed. Glucose is oxidized via gluconate to 2-keto-3-deoxygluco-
nate, followed by phosphorylation to 2-keto-3-deoxy-6-phosphogluconate (KDPG), which is then split into pyruvate and glyceraldehyde-3-phosphate [8,9]. However, those halophilic Archaea that degrade fructose do so by means of the glycolytic Embden–Meyerhof pathway, with fructose-1,6-bisphosphate (FBP) aldolase as the key enzyme [9–13]. We here report data on the enzymatic pathway responsible for catalysis of glucose in *S. ruber*, comparing sugar metabolism in *Salinibacter* with the corresponding pathways detected in the halophilic archaeon *Halofex rax* *volcanii*.

2. Materials and methods

2.1. Bacterial and archaeal strains and culture conditions

*S. ruber* strain M31T (DSM 13855) was grown in medium of the following composition (g l−1): NaCl, 195; MgSO4·7H2O, 25; MgCl2·6H2O, 16.3; CaCl2·2H2O, 1.25; KCl, 5.0; NaHCO3, 0.25; NaBr, 0.625; and yeast extract, 1.0, pH 7.0. Media were supplemented with glucose or fructose (1 g l−1), was used as crude enzyme preparation. For enzymatic assays cells were grown in 1 l portions in 2 l Erlenmeyer flasks on a rotary shaker (180 rpm) at 35°C. Tests for growth stimulation by sugars were conducted in 250 ml Erlenmeyer flasks containing 100 ml of medium.

2.2. Enzyme assays

Exponential growth phase cells were harvested by centrifugation (15 min, 6000×g) at 4°C. Cell pellets were suspended in 3 M KCl+50 mM Tris–HCl buffer, pH 8.0. Cells were then broken by sonication (4× 20 s at 140 W). Debris was removed by centrifugation (10 min, 12000×g, 4°C), and the supernatant, containing between 5 and 15 mg protein ml−1, was used as crude enzyme preparation. All enzymatic activities were tested at salt concentrations varying from near-zero to 3–3.5 NaCl or KCl.

Hexokinase (EC 2.7.1.1) was determined according to the reduction of nicotinamide adenine dinucleotide phosphate (NADP) in the presence of glucose, ATP, and glycose-6-phosphate (G-6-P) dehydrogenase as outlined by Sharma et al. [14]. The assay mixture (final volume 2 ml) contained: glucose, 5.5 μmol; ATP, 1 μmol; MgCl2, 5 μmol; Tris–HCl, 50 μmol, pH 8.5; NADP, 2.5 μmol; G-6-P dehydrogenase (from yeast; Sigma), 0.1 mg, 0.66 U; KCl or NaCl as indicated, and cell extract (0.6–1.2 mg protein). The reaction was started by the addition of glucose or by addition of ATP (with similar results), and NADP reduction was followed at 340 nm in a Hewlett-Packard model 8452 diode array spectrophotometer, provided with a heated (35°C) cuvette holder. Control experiments showed that the yeast G-6-P dehydrogenase was not significantly inhibited by KCl at concentrations up to 2.5 M, while NaCl caused significant inhibition. Still, sufficient activity remained at NaCl concentrations up to 2.8 M, as checked by addition of G-6-P to the experimental systems, ensuring that the G-6-P dehydrogenase activity in the reaction mixture was not the limiting step in the assay.

Glucose dehydrogenase (EC 1.1.1.47) and G-6-P dehydrogenase (EC 1.1.1.49) were assayed spectrophotometrically by measuring reduction of nicotinamide adenine dinucleotide (NAD) or NADP. The reaction mixtures contained 0.56 μmol glucose or 3.5 μmol G-6-P (Sigma), 2.5 μmol NAD or NADP, 50 μmol Tris–HCl (pH 8.0), and NaCl or KCl as indicated, and cell extract (between 0.6 and 1.5 mg protein) in a final volume of 2 ml. The reactions were started by the addition of the substrates (glucose and G-6-P, respectively).

FBP aldolase (EC 4.1.2.13) was assayed colorimetrically as outlined by Sibley and Lehninger [15]. The reaction mixtures contained in a final volume of 2.5 ml: 12.5 μmol FBP, 125 μmol Tris–HCl, pH 8.6, NaCl and KCl as indicated, 140 μmol hydrazine-sulfate adjusted to pH 8.6 with NaOH, and cell extract (between 0.6 and 1.5 mg protein). The mixtures were incubated in a water bath at 35°C. At different times (zero time, 15 min and 30 min) the reactions were terminated by addition of 2 ml 10% trichloroacetic acid. After centrifugation, 1 ml of supernatant was added to 1 ml 0.75 M NaOH. Following 10 min incubation at room temperature, 1 ml 2,4-dinitrophenylhydrazine solution (0.1% in 2 M HCl) was added, and the mixtures were further incubated at 38°C for 10 min. Finally, 7 ml 0.75 M NaOH was added, and after 10 min the absorbance at 540 nm was measured. Glyceraldehyde-3-phosphate (Sigma) was used as standard for calibration.

KDPG aldolase (EC 4.1.2.14) was determined by measuring the formation of KDPG from glyceraldehyde-3-phosphate and pyruvate [9]. The assay mixture (1 ml) contained 100 μmol Tris–HCl, pH 8.5, KCl as indicated, 100 μmol pyruvate, 1 or 2 μmol glyceraldehyde-3-phosphate (Sigma), and cell extract (between 1 and 1.5 mg protein). At time zero and after 10, 20, and 30 min incubation at 35°C, 0.2 ml samples were transferred to 2.5 ml of 10% trichloroacetic acid + 0.025 N periodic acid + 0.125 N H2SO4. The precipitated protein was removed by centrifugation, and the amount of KDPG in the supernatant was quantified by the thiobarbituric acid assay [16].

2.3. Miscellaneous analytical methods

Glucose was assayed colorimetrically using the glucose oxidase–dianisidine–peroxidase method (Sigma diagnostic kit, St. Louis, MO, USA, cat. no. 510-DA). The total sugar content of media was measured using the colorimetric reaction with anthrone [17]. Glucose or fructose were
used as standards. The protein content of cell extracts was assayed by the Lowry procedure, using bovine serum albumin as a standard.

3. Results

3.1. Growth experiments

When media with 0.1 g l⁻¹ or 1 g l⁻¹ yeast extract were supplemented with 1 g l⁻¹ glucose, mannose, or starch, final cell densities of *S. ruber* were increased. The addition of these carbohydrates increased the OD₆₀₀ upon reaching the stationary growth phase from around 0.035 (0.1 g l⁻¹ yeast extract) or 0.33 (1 g l⁻¹ yeast extract) to 0.15–0.18 and 0.5–1.0, respectively. No growth stimulation was found upon addition of fructose, galactose, arabinose, ribose, xylose, sucrose, lactose, cellobiose, or raffinose. Sugar metabolism was not accompanied by acidification of the medium.

Glucose is not the preferred growth substrate of *S. ruber*. When grown in medium containing 1 g l⁻¹ glucose and 1 g l⁻¹ yeast extract, glucose consumption started after other available carbon and energy sources had been exhausted and the culture had reached a turbidity equal to that obtained in the stationary phase when glucose was replaced by fructose (Fig. 1A) or in the absence of added sugar (data not shown). A semilogarithmic plot of the culture density in the presence of glucose as a function of time clearly shows a diauxic behavior: during the first phase the culture grew with a doubling time of about 0.65 days, slowing down to a doubling time of 3.4 days during the glucose consumption phase (Fig. 1B). When the glucose concentration was increased to 3 or 5 g l⁻¹, the final OD₆₀₀ increased to about 1.5.

3.2. Enzyme studies

Hexokinase was detected in cell extracts of *S. ruber*, both in cells grown in the presence of glucose and in its absence. Activities were in the order of 4 nmol mg protein⁻¹ min⁻¹. The activity was markedly inhibited by salt, and no activity was recorded in the presence of 2.1 M KCl (Fig. 2) or 2.8 M NaCl (data not shown). A similar salt sensitivity was reported for the hexokinases of *Haloferax mediterranei* and *Haloarcula vallismortis* [18].

*S. ruber* has a constitutive NADP-linked G-6-P dehydrogenase activity. Activities around 10 nmol mg protein⁻¹ min⁻¹ were detected in extracts of cells, whether or not glucose had been included in the growth medium. The enzyme is markedly salt-dependent: activity was optimal above 1.5–2 M NaCl or KCl, and no activity was found below 0.8 M salt (Fig. 3). No NAD- or NADP-dependent glucose dehydrogenase activity was detected in the cell extracts; activities of up to 6.5 nmol mg protein⁻¹ min⁻¹ of NAD-dependent glucose dehydrogenase.
and 13C-labeled glucose was transformed to gluconate by Halobacterium rose dehydrogenase and for 2-keto-3-deoxygluconate ketoreductase. This resembles many of the halophilic Archaea of the Halobacteriaceae. The range of sugars used is small, and sugar consumption starts only after the supply of other substrates has been exhausted. In this respect Salinibacter ruber does not grow on sugars, in spite of the fact that the genes for sugar catabolism have been identified in the genome of Halobacterium NRC-1 [19], and the fact that the Halobacterium glucose dehydrogenase has been isolated and characterized [20]. However, species of Haloferax, Haloarcula, Halorubrum, and a few additional genera do grow on sugars. Carbohydrate utilization within the group was first documented in Halorubrum saccharovorum [8,21]. Breakdown of glucose follows a modified Entner–Doudoroff pathway in which the phosphorylation step is postponed: glucose is first oxidized to gluconate, and the phosphorylation step acts on 2-keto-3-deoxygluconate to form KDPG [8]. Genes coding for glucose dehydrogenase and for 2-keto-3-deoxygluconate kinase have been identified in the Halobacterium genome, and 13C-labeled glucose was transformed to gluconate by Halobacterium salinarum [22]. However, the gene for KDPG aldolase remains to be assigned in the Halobacterium NRC-1 genome [19].

A functional (modified) Embden–Meyerhof pathway is also present in some members of the Halobacteriaceae. Fructose breakdown in Har. vallismortis proceeds via an initial phosphorylation step to yield fructose-1-phosphate, catalyzed by an ATP-dependent fructose-1-phosphotransferase (ketohexokinase). Fructose-1-phosphate is then converted by 1-phosphofructokinase to FBP [10,11]. In Har. vallismortis and in Hfx. mediterranei fructose originating from catabolism of sucrose and mannitol is metabolized in a modified Embden–Meyerhof pathway, initiated by ketohexokinase [11]. Existence of activities related to the Embden–Meyerhof pathway in Hbt. salinarum was also shown in nuclear magnetic resonance (NMR) studies with 13C-labeled glucose: G-6-P, fructose-6-phosphate and FBP were detected in addition to labeled gluconate [22]. A recent study of sugar metabolism in Halococcus saccharolyticus, in which 13C-labeled substrates and NMR techniques were used to identify the intermediates and products formed, confirmed the use of separate dissimilatory pathways for glucose and fructose. Glucose is metabolized via the modified Entner–Doudoroff pathway, while fructose utilization almost entirely follows the Embden–Meyerhof pathway. Glucose-grown cells showed increased activities of gluconate dehydratase and 2-keto-3-deoxygluconate kinase, while fructose-grown cells contained higher activities of ketohexokinase and fructose-1-phosphate kinase, the key enzymes of the modified Embden–Meyerhof pathway [9].

Our studies with S. ruber suggest that this organism uses the enzymes of the classic Entner–Doudoroff pathway, with G-6-P dehydrogenase as a key enzyme, rather than the archaeal-type modification based on glucose dehydrogenase, or the Embden–Meyerhof pathway with FDP aldolase as the key enzyme. The picture is still incomplete in view of our inability to demonstrate KDPG aldolase activity in S. ruber extracts. The nature of the inducible step, responsible for the diauxic growth behavior, is also not clear as yet. Both hexokinase and G-6-P dehydrogenase activities were present constitutively. A study of the mechanism by which glucose is transported inside the cells may possibly yield insight into the induction mechanism.

To our knowledge no enzymological studies of glucose dissimilation have yet been performed in other halophilic representatives of the domain Bacteria. However, a partial analysis of the genome of Halomonas elongata DSM 3043T has enabled the tentative identification of genes encoding hexokinase, G-6-P isomerase and FBP aldolase, enzymes of the Embden–Meyerhof pathway. In addition, genes for glucose dehydrogenase and gluconolactonase were found. Genes for phosphofructokinase and for the Entner–Doudoroff pathway enzymes 6-phosphogluconate dehydratase and KDPG aldolase have not been identified in the genome fragments sequenced thus far (Laszlo Csonka, Purdue University, personal communication).

Fig. 3. Dependence of the S. ruber NADP-dependent G-6-P dehydrogenase activity on salt. The NaCl-based systems all contained in addition 150 mM KCl, introduced with the enzyme preparation. The plot shows representative results from two independent experiments.
Our earlier studies showed a variable 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, showed a markedly halophilic character, being stimulated about three-fold by 1.2 M KCl [6]. The fatty acid synthetase complex also needs salt (0.5–1.5 M KCl) for optimal activity (A. Oren, unpublished results). The malate dehydrogenase, on the other hand, does not behave as a halophilic enzyme as its optimal activity is found in the absence of salt [6]. The organism probably contains two NAD-dependent glutamate dehydrogenases, one salt-sensitive and one salt-requiring [6]; [Maria-Jose Bonete, University of Alicante, personal communication]. The finding of a salt-dependent G-6-P dehydrogenase and a salt-sensitive hexokinase adds to disparate behavior of the enzymes of *Salinibacter*, an organism that shares many properties both with the halophilic Archaea that contain extremely high intracellular KCl concentrations and with the halophilic representatives of the domain Bacteria, to which it phylogenetically belongs.

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**References**


