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Evidence of interspecies hydrogen transfer from glycerol in saline environments

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Abstract Two halanaerobic bacteria – *Halanaerobium saccharolytica* subsp. *senegalense* and *Halanaerobium* sp. strain FR1H – produced acetate, H_2, and CO_2 from glycerol fermentation, but the glycerol consumption rate was low. In contrast, in the presence of the moderately halophilic hydrogenotrophic sulfate-reducing bacterium, *Desulfohalobium retbaense*, used as H_2 scavenger in the coculture, glycerol oxidation by both halanaerobes significantly increased. Cocultures of both halanaerobes with *D. retbaense* on glycerol led to acetate, hydrogen sulfide, and CO_2 production, whereas glycerol fermentation by the two strains led to the production of acetate, hydrogen, and CO_2. The increased glycerol oxidation by *H. saccharolytica* and strain FR1H in coculture with *D. retbaense* resulted from low H_2 partial pressure caused by the hydrogen-oxidizing activity of *D. retbaense*. These results provide the first evidence of interspecies hydrogen transfer in saline environments and indicate that this mechanism may play an important role in organic matter mineralization in hypersaline ecosystems.

Key words *Halanaerobium* spp. · *Desulfohalobium retbaense* · Interspecies hydrogen transfer · Saline environments · Sulfate reduction · Glycerol oxidation · Hydrogen oxidation

Introduction

In hypersaline lakes, glycerol is an osmolyte that can be synthesized in large quantities, mainly by *Dunaliella salina*. It is probably one of the major carbon sources used by natural communities of halophilic aerobic *Archaea* since it is subject to rapid turnover by these microorganisms (Oren 1993). It is known that some members of the family *Halanaerobiaceae* and particularly those belonging to the genus *Halanaerobium* isolated from saline environments ferment glycerol (Cayol et al. 1994; Cayol 1995). However, no attention has been paid so far to glycerol degradation by halanaerobes in saline to hypersaline ecosystems.

Under anaerobic conditions, glycerol is known to be (a) fermented by facultative anaerobes of the family *Enterobacteriaceae* (Bouvet et al. 1994) and anaerobes of the order *Clostridiales* (Nakas et al. 1983; Forsberg 1987; Heyndrickx et al. 1991; Biebl et al. 1992); (b) oxidized in the presence of sulfate by the anaerobic sulfate-reducing bacteria (SRBs) (Qatibi et al. 1991, 1998; Ouattara et al. 1992); or (c) oxidized via an interspecies hydrogen transfer between glycerol-oxidizing SRBs and hydrogenotrophic methanogens (Qatibi et al. 1991). The latter mechanism relies upon H_2 biological scavengers to render the oxidation of glycerol thermodynamically favorable (Qatibi et al. 1991).

Few anaerobic hydrogenotrophic microorganisms have been isolated from hypersaline ecosystems. They include some members of the genus *Desulfovibrio* and one species of the genus *Desulfohalobium* (Ollivier et al. 1991). The most halotolerant hydrogenotrophic methanogen, *Methanocalculus halotolerans*, cannot grow in the presence of more than 13% NaCl (Ollivier et al. 1998). In this respect, in saline environments, hydrogen is oxidized by SRBs rather than by methanogens (Ollivier et al. 1994; Oren 1999). Among SRBs, *Desulfohalobium retbaense* has been recognized as the most halophilic microorganism (Ollivier et al. 1991). It grew optimally between 15% and 20% NaCl and up to 24% NaCl. Despite its ability to oxidize hydrogen, its impact on the oxidation of organic reduced compounds such as glycerol in hypersaline ecosystems has never been studied. In addition, although the ecological importance of interspecies hydrogen transfer for organic matter mineralization is well known (Laube and Martin 1981; Wolin 1982), it has not yet been reported in hypersaline environments. Here we report for the first time on the existence of this transfer within halophilic communities of a hypersaline ecosystem.

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Materials and methods

Strains, media, and growth conditions

The fermentative halophile *Halanaerobium saccharolytica* subsp. *senegalense* strain H150 (DSM 7379), isolated from the same hypersaline African lake as *Desulfohalobium retbaense* strain HR100 (DSM 5692) (Ollivier et al. 1991), and *Halanaerobium* sp. strain FR1H, isolated from an oil field reservoir (Cayol 1995), were studied to determine their ability to ferment glycerol and to oxidize this compound in the presence of *D. retbaense*. Both *Halanaerobium* strains grew optimally at 10% NaCl.

The growth and metabolism of the two halanaerobes were studied at various concentrations of glycerol, in the presence and in the absence of *D. retbaense*. All media were prepared anaerobically using the techniques of Hungate as previously described (Cayol et al. 1994). The medium contained (in $g\,l^{-1}$): 1.0 NH_4Cl; 0.3 K_2HPO_4; 0.3 KH_2PO_4; 2.0 $MgCl_2{\cdot}6H_2O$; 4.0 $CaCl_2{\cdot}2H_2O$; 1.0 $CH_3COONa{\cdot}3H_2O$; 1.0 bio-Trypticase; 1.0 yeast extract; 100 NaCl; 0.001 resazurin; and 1 ml of trace element solution (Imhoff-Stuckle and Pfennig 1983). In total, 20 mM of sulfate was added in culture medium when the halanaerobes were cocultivated with *D. retbaense*. Experiments were conducted in duplicate at 37°C.

Results and discussion

In hypersaline environments, several halophilic or halotolerant microorganisms accumulate various organic solutes including polyols, sugars and sugar derivatives, amino acids and derivatives, and quaternary amines such as glycine betaine to resist osmotic stress in the presence of high salt concentrations (Oren 1999). Among polyols, glycerol is known as the most predominant organic osmolyte, and natural communities of halophilic aerobic *Archaea* (*Halobacteriaceae*) are reported to be involved in the rapid turnover of this compound (Borowitzka 1981). Our results indicate that in saline to hypersaline ecosystems, where glycerol is abundantly produced mainly by *Dunaliella salina*, the ecological significance of halanaerobes in degrading this substrate also must be taken into account even if aerobic glycerol degradation is expected to prevail over anaerobic degradation (Oren 1993). We found that glycerol use by halanaerobes was greatly enhanced in the presence of hydrogenotrophic halophilic SRBs. Indeed, *Halanaerobium saccharolytica* subsp. *senegalense*, an isolate from a hypersaline lake in Africa, produced acetate, H_2, and CO_2 from glycerol, which was fermented at a low rate (Fig. 1a). In the presence of the sulfate reducer *D. retbaense*, an H_2 biological scavenger, glycerol utilization significantly increased and glycerol was completely oxidized after 7 days of incubation at 37°C (Fig. 1a). In contrast, less than 10% of the initial glycerol added to the culture medium was fermented during the same period of incubation with *H. saccharolytica* subsp. *senegalense* alone. During coculture of *H. saccharolytica* with *D. retbaense*, glycerol oxidation gave acetate, hydrogen sulfide, and CO_2 as end-products. Similar results were obtained when *Halanaerobium* sp. strain FR1H, an isolate from an oil reservoir, was cocultured with *D. retbaense* in the presence of glycerol (Fig. 1b). The importance of the hydrogenotrophic SRB in coculture with either *H. saccharolytica* or *Halanaerobium* sp. strain FR1H was exemplified by a much higher final optical density of both cocultures as compared to monocultures (Fig. 2a,b). Under the growth conditions tested, *H. saccharolytica* did not ferment more than 12 mM glycerol after 3 weeks of incubation at 37°C. One mole of glycerol degraded was converted to around 1 mol of acetate and 3 mol of H_2 (Table 1). In the presence of *D. retbaense*, higher amounts of glycerol were used, with acetate and H_2S being the major end-products. Hydrogen was not detected or detected as traces when the initial glycerol concentration was 23 mM (Table 1). Therefore, the presence of the hydrogenotrophic SRB not only

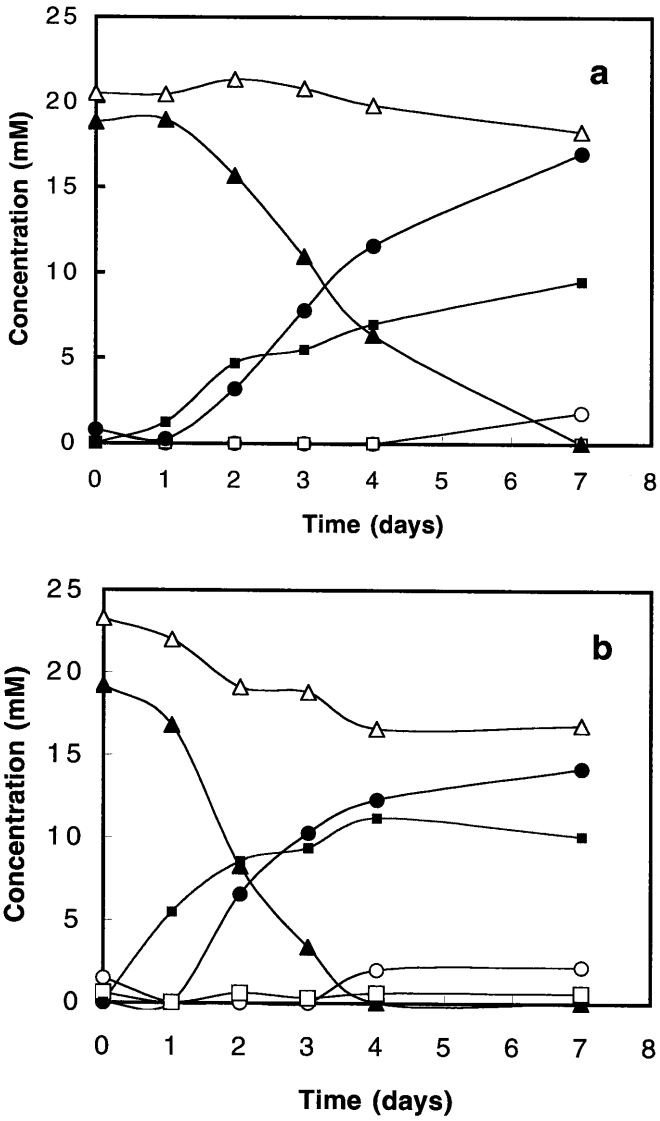

Fig. 1. Glycerol utilization by (**a**) *Halanaerobium saccharolytica* subsp. *senegalense* and (**b**) *Halanaerobium* sp. strain FR1H in the presence and in the absence of *Desulfohalobium retbaense* (Dr). Pure culture: *open triangles* glycerol, *open circle* acetate, *open squares* sulfide; coculture: *solid triangles* glycerol, *solid circles* acetate, *solid squares* sulfide

Table 1. End-products of glycerol catabolism by *Halanaerobium saccharolytica* subsp. *senegalense* (Hss) in the presence and in the absence of *Desulfohalobium retbaense* (Dr) after 23 days of incubation at 37°C

	Initial glycerol (mM)	Glycerol consumed (mM)	Acetate produced (mM)	Final pH	Final H_2S (mM)	Final H_2 (mM)
Hss	11.81	11.81	10.32	6.86	0.17	31.7
	17.54	9.62	9.42	6.84	0.33	25.1
	24.48	12.39	10.63	6.84	0.39	26.75
Hss+Dr	10.47	10.47	9.83	6.94	6.26	0
	13.6	13.6	12.47	6.91	8.2	0
	22.98	22.98	21.25	6.88	13.14	1

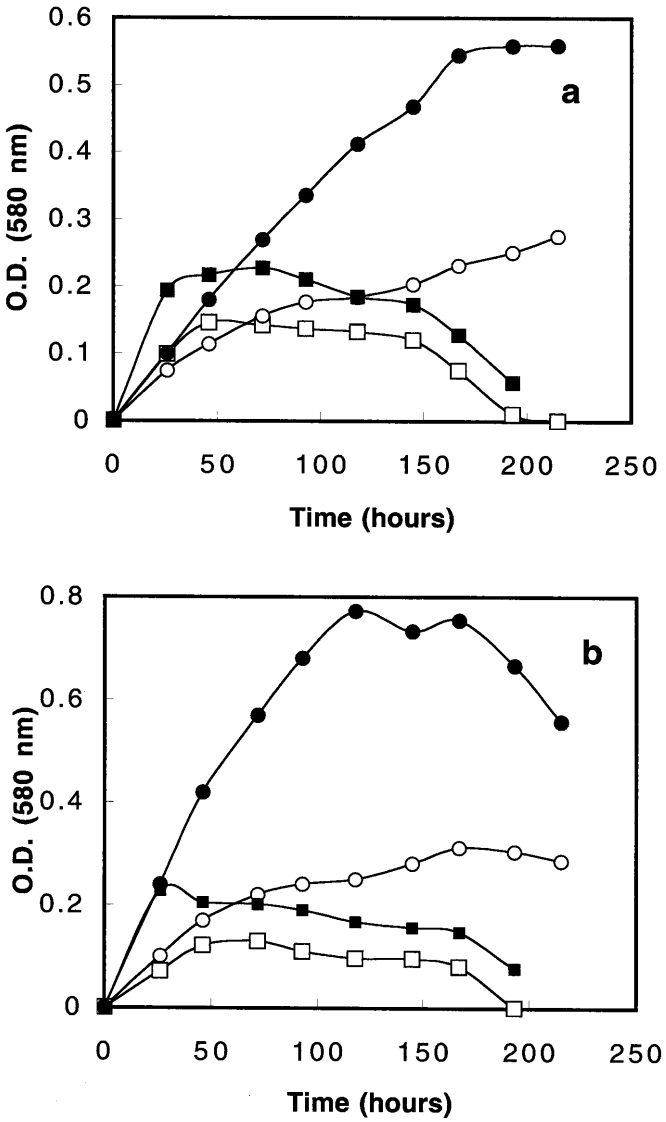

Fig. 2. Effect of *Desulfohalobium retbaense* on growth of (**a**) *Halanaerobium saccharolytica* subsp. *senegalense* and (**b**) *Halanaerobium* sp. strain FR1H when cultivated on 20 mM glycerol. *Open squares* pure culture, *open circles* pure culture with glycerol, *solid squares* coculture, *solid circles* coculture with glycerol

increased the rate of glycerol oxidation, but also the amount of glycerol consumed.

Studies on organic matter degradation by mixed defined populations of anaerobes including hydrogenotrophs have been mostly performed under mesophilic or thermophilic nonsaline conditions (Laube and Martin 1981; Cord-Ruwish and Ollivier 1986; Ollivier et al. 1986; Smiti et al. 1986), aiming in particular at improving the overall performance of anaerobic digestion of biological polymers. Here, we provide evidence for the first time of the existence of a hydrogen interspecies transfer mechanism that may occur within hypersaline environments. Hydrogen interspecies transfer probably plays an important ecological role in such ecosystems by facilitating organic matter mineralization. This improvement is probably not limited to glycerol but may also apply to carbohydrates, proteins, amino acids, or other reduced compounds (e.g., ethanol) and therefore needs further investigation. Similar to other experiments conducted with mixed defined cultures (Laube and Martin 1981; Cord-Ruwish and Ollivier 1986; Ollivier et al. 1986; Smiti et al. 1986), we hypothesize that glycerol oxidation under anaerobic conditions is highly dependent on hydrogenotrophic partners, such as *D. retbaense*, which decrease H_2 partial pressure during the catabolism of glycerol and renders its degradation thermodynamically more favorable. Taking into account the NaCl range for growth of *D. retbaense* and *H. saccharolytica* subsp. *senegalense*, originating from the same hypersaline lake in Africa, their coculture might be efficient in oxidizing glycerol in situ in the salinity range 5%–25% NaCl. In addition, similar types of cocultures could be expected to occur in saline environments.

In contrast to other environments where methanogenic or sulfate-reducing H_2-oxidizing bacteria are potential H_2-scavenging partners with heterotrophic or syntrophic bacteria, SRBs are probably the only microorganisms able to perform interspecies hydrogen transfer during organic matter degradation in saline environments containing more than 15% NaCl. At high NaCl concentrations, the methanogenic activity toward hydrogen is low or absent and is limited to the use of noncompetitive substrates, such as methylamines originating from the breakdown of osmoregulatory amines (Ollivier et al. 1994).

Similar results on glycerol degradation were obtained when *Halanaerobium* sp. strain FR1H, isolated from an oil field, was cocultured with *D. retbaense*. Since hydrogenotrophic SRBs were found in saline oil fields (Magot et al. 2000), it can be hypothesized that they play a similar ecological role as an H_2 scavenger partner in the mineralization of organic matter in saline subsurface ecosystems.

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