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FACULTATIVE AND PARTIAL INTERSPECIES HYDROGEN TRANSFER - COMPETITION FOR REDUCING EQUIVALENTS

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SUMMARY

Sulfate reducing bacteria of the genus *Desulfovibrio* and homoacetogenic bacteria of the genus *Sporomusa* were sensitive to changes of hydrogen concentrations during the growth on an organic substrate. Increase of hydrogen concentrations competitively inhibited the organic substrate degradation and decrease of hydrogen concentration inhibited the respiration and the reduction of the external electron acceptor. Such hydrogen sensitive strains which seem to intermediarily produce and consume hydrogen ("hydrogen-cycling") were cultivated in the presence of a second hydrogen oxidizer. Both organisms competed for the hydrogen excreted by the first strain. The competence for H_2-oxidation of the strains depended not only on hydrogenase affinities but also on the free energy change of H_2-oxidation differing with the respective electron acceptors.

INTRODUCTION

Beside acetate, hydrogen is the most important intermediary product during anaerobic degradation of organic material. The competition for hydrogen plays a major role in endproduct formation in natural anaerobic environments. The interspecies hydrogen-transfer occurs in every investigated anaerobic environment as one of the general processes which allows finally the terminal oxidation of organic matter.

In interspecific hydrogen transferring cocultures, one species degrades an organic substrate and releases reducing equivalents in form of hydrogen (reduction of protons) which is oxidized by the second species. Generally the first organism profits from hydrogen removal by the second strain (Bryant et al. 1967, Bryant 1979). These bacteria are called obligate syntrophs and are depending on the presence of each other (symbiosis).

The present paper reports on facultative interspecies hydrogen transfer occurring between two bacteria which both can consume the hydrogen, produced during organic substrate degradation by one of them.

MATERIALS AND METHODS

Bacteria used

The following bacteria, which are all able to oxidize organic substrate as well as hydrogen under anaerobic conditions were used throughout this study:
Homoacetogens: *Sporomusa sphaeroides* (DSM 2875) , *Sporomusa acidovorans* (DSM 3132). Sulfate reducers: *Desulfovibrio vulgaris* G6 (isolated from the the defined syntrophic association with *Syntrophus bushwelli* (DSM 2612TB), *Desulfovibrio* strain JJ (DSM 3604), *Desulfobulbus* spec. (isolated on propionate from anaerobic digestor sludge). *Methanospirillum hungatei* (DSM 864), *Wolinella succinogenes* (DSM 1740), *Paracoccus denitrificans* (DSM strain N4).

Medium and growth conditions

The anaerobic bicarbonate-buffered medium was used as described for sulfate reducing bacteria (Widdel & Pfennig 1984), but sulfate was omitted and 1 g yeast extract per l was added. The stock

solutions of organic substrates and salts, serving as electron acceptors, were separately sterilized and added to the culture vessels when needed. Cells were cultivated in anaerobic Hungate tubes, or serum bottles when hydrogen was present in the gasphase.

Analytical methods

Alcohols and methane were determined gaschromatographically, fructose and organic acids by HPLC as previously described (Cord-Ruwisch et al. 1986) Sulfide was determined spectrophotometrically as colloidal CuS (Cord-Ruwisch 1984). Ammonium was measured potentiometrically by a special ammonium electrode, model 95-10, ORION Research Inc. Cambridge, MA 02139).

RESULTS

H_2-production and consumption during organic substrate degradation

In order to check which of the used strains was capable to produce and consume hydrogen ("hydrogen-cycling") during the growth on an organic substrate, two tests, which are normally applied to prove that hydrogen is an intermediary product in syntrophic associations, were carried out:

(i) The effect of the addition of hydrogen on the organic substrate degradation: Each strain was grown on an organic substrate in two parallel vessels. Hydrogen was added to the gasphase of one vessel after a part of the organic substrate had been degraded. The concentration of the organic substrate was monitored in both parallel essays. Both *Desulfovibrio* strains and both *Sporomusa* strains were immediately inhibited by H_2 to degrade the organic substrate. Instead they oxidized H_2. The inhibition was reversible: After replacing H_2 by N_2 the organic substrate was degraded. Lactate degradation by *Desulfobulbus* sp. was not effected by the presence of H_2. The addition of H_2 to cultures of *Par. denitrificans* and *Wol. succinogenes* slowed down or blocked (*Wol. succinogenes*) the degradation rate of formate after an adaptation time of about one generation.

Table 1: Ability of the strains tested to serve as hydrogen producing syntroph

Strain	immediate inhibition of organic substrate degradation by H_2	with Methanospirillum as hydrogen sink	
		methane produced	growth
Sporomusa acidovorans	+	+	+
Sporomusa sphaeroides	+	+	+
Desulfovibrio vulgaris	+	+	+
Desulfovibrio strain JJ	+	+	+
Desulfobulbus spec.	-	-	-
Wolinella succinogenes	+	n.d.	n.d.
Paracoccus denitrificans	-	-	-

n.d. = not determined (Methanospirillum uses formate)

(ii) The capability to produce hydrogen as a facultative syntroph: The used strains were inoculated into a medium, in which the final electron-acceptor was replaced by a second hydrogenophilic organism of which the electron-acceptor was present. The growth of such cocultures, compared to control essays without hydrogen removing organism, and methane formation indicated whether an interspecies H_2 transfer occurred or not (Tab.1).

The results of those two tests went together: Strains capable to produce hydrogen in coculture were inhibited by H_2 to degrade an organic substrate (Table 1): These strains (*Du. vulgaris; Du. strain JJ; Sp. acidovorans; Sp. sphaeroides*) were susceptible to produce and consume H_2 as intermediary product during organic substrate degradation and have been called "hydrogen cycling" organisms (Peck & Odom 1984).

Coculture of an "hydrogen-cycling" organism with a second hydrogenophile:

Pure cultures of the homoacetogens *Sp. acidovorans* and *Sp. sphaeroides* degraded organic substrates only to acetate, using CO_2 as external electron acceptor. In coculture with *Wol. succinogenes* on nitrate as electron-acceptor or with *Desulfovibrio* species and Sulfate as electron acceptor a total transfer of reducing-equivalents was observed. Only sulfate or nitrate were reduced but not CO_2 (Table 2). The CO_2 reducing *Sporomusa* species could not outcompete sulfate-reducing or nitrate-reducing H_2-oxidizers for the hydrogen produced during organic substrate degradation. However, if methanol was the organic substrate, it was not degraded completely (not more than 15mM) in these cocultures and the growth as determined by OD was not significant. After transferred into new medium such cocultures failed to continue methanol degradation. In the coculture of *Sp. acidovorans* with *Msp. hungatei* (Cord-Ruwisch and Ollivier 1985) and of *Sp. acidovorans* with *Du. vulgaris* on sulfur as electron acceptor, both strains reduced the electron acceptor CO_2, resulting in the formation of acetate as well as of methane. The reducing equivalents produced during substrate oxidation were partially transferred as H_2 but also reoxidized, possibly also in the form of H_2.

Table 2: Coculture of homoacetogens of the genus *Sporomusa* with different hydrogenophilic partners in the presence of their respective electron acceptors and of an organic substrate only degradable by the homoacetogen.

organic substrate	Strain I	Strain II	electron-accept. II	electron acceptor reduced per mole substrate oxidized	Transfer of H_2 [%]	inhibition of growth of strain I
methanol	Sporomusa	Methanosprill.	CO_2	acetate, methane	20 - 30	-
"	acidovorans	Desulfovibrio	sulfate	sulfide	100	+
"		Wolinella	nitrate	ammonium	100	+
fructose		Desulfovibrio	sulfate	sulfide	100	-
methanol	Sporomusa	Desulfovibrio	sulfur	acetate, sulfide	30 - 50	-
"	sphaeroides	Wolinella	fumarate	succinate	100	+
lactate		Wolinella	fumarate	succinate	100	-

***) acetate was produced less than 1mole/mole lactate and was regarded as resulting from lactate conversion to H_2, CO_2 and acetate.**
The relations of both electron acceptors reduced were not constant during the growth and varied even in parallel assays. The values given are mean values obtained from two or three measurements.

Desulfovibrio vulgaris did not transfer significant amounts of H_2 when cocultured in the presence of sulfate with other H_2-consuming bacteria (Table 3). Small amounts of NH_4^+

produced in coculture with Wol. succinogenes on Nitrate may be due to the slow sulfide oxidation by Wol. succinogenes. However, with elemental sulfur as electron-acceptor for *Du. vulgaris* and with *Wol. succinogenes* as hydrogenophilic partner a complete H_2-transfer established: no sulfide was produced (Table 3). The coculture of *Du. vulgaris* with sulfur as electron acceptor and with the homoacetogen *Sp. acidovorans* or with *Msp. hungatei* as hydrogenophilic partner represented another example of a coculture performing partial interspecies hydrogen-transfer (Table 3). The electron acceptors of both strains were reduced.

Table 3: Coculture of Desulfovibrio vulgaris on lactate (20mM) in the presence of sulfate or sulfur as external electron acceptor with different hydrogenophilic partners.

electron acceptor I	strain II	electron acceptor II	% of H_2 transferred
sulfate	Msp. hungatei	CO_2	0
sulfate	Sp. sphaeroides	CO_2	0
sulfate	Wolinella	nitrate	0-20
sulfur	Msp. hungatei	CO_2	50-80
sulfur	Sp. acidovorans	CO_2	60-90
sulfur	Wolinella	nitrate	100

In the absence of an external electron acceptor *Desulfovibrio* strain JJ degrades fructose according to the following equation (Cord-Ruwisch & Ollivier 1986)

$$2\,C_6H_{12}O_6 \longrightarrow 2\,^-OOC\text{-}CH_2\text{-}CH_2\text{-}COO^- + CH_3\text{-}COO^- + CH_3\text{-}CH_2OH + 5H^+ + H_2O$$

The formation of succinate and the ability of strain JJ to use fumarate as electron-acceptor during the growth on H_2 indicated that the reoxidation of the reducing equivalents formed during fructose oxidation may be coupled to electron transport phosphorylation (fumarate ---> succinate).

The production and consumption of H_2 seems to be envolved even during the fermentation of fructose in the absence of external electron acceptors by Dv. strain JJ, as indicated by the fact that the addition of H_2 to a fructose-fermenting culture of Dv. strain JJ inhibited fructose degradation.

Therefore the system reducing internal electron acceptors such as fumarate can be regarded as competing for H_2 with the systems using external hydrogen acceptors such as sulfate sulfur or hydrogen consuming partners.

The amount of H_2 oxidized by an accepting system was correlated to the free energy change of the oxidation of H_2 with the respective electron acceptor (Table 4,5). In the presence of sulfate or nitrate all reducing equivalents were finally oxidized by these electron acceptors, whereas in the presence of CO_2 or sulfur a part of the

Table 4: Effect of different external hydrogen acceptors on the reduction of internal electron acceptors (succinate formation) by Desulfovibrio strain JJ during the growth on fructose. [mole / mole fructose degraded].

external H_2-sink	succinate produced	H_2 trapped by external sink
without	1.7	0.0
sulfur	1.0	1.0
sulfate	0.0	4.0
Sporomusa (CO_2) sphaeroides	1.0	1.6
Meth. spir. (CO_2) hungatei	0.3	2.8
Wolinella (NO_3) succinog.	0.1	4.0

reducing equivalents were reoxidized by internal electron acceptors resulting in succinate formation.

Competition of two "hydrogen cycling" organisms for an organic substrate

Growing on lactate in excessive concentration the homoacetogen Sp. sphaeroides immediately stopped its respiration (dissimilatory CO_2-reduction to acetate) when the lactate and H_2-oxidizing Dv. vulgaris was added to the coculture.

DISCUSSION

To artificial increase of H_2-concentration homoacetogens of the genus *Sporomusa* and sulfidogens of the genus *Desulfovibrio* reacted with hydrogen consumption and inhibition of organic substrate degradation (table 1, fig.1) and to the decrease of hydrogen concentration they reacted with H_2-production, as if to have an interest in keeping a particular H_2 partial pressure (maintenance of external hydrogen pool). Cocultures of these "H_2 cycling" bacteria with other hydrogenophilic bacteria resulted predictably in competition for hydrogen.

Thermodynamical explanation of competition for H_2 of low concentrations

Because of the extremely low concentrations of hydrogen in anoxic biotops the competition is won by organisms (e.g. sulfate reducers) able to hold the H_2 partial pressure below the level which allows H_2 oxidation by concurrent organisms (e.g. methanogens). Therefore the threshold levels of hydrogen oxidation of two competing bacteria has been seen as the deciding factor (Lovley 1985) , depending on the hydrogenase-affinities (Kristiansson et al. 1982).

Enzymes can catalyse only reactions which are thermodynamically favorable. And hydrogenases can not catalyse H_2 oxidation when the subtrate concentration (H_2) is to low to yield energy. This energetic threshold concentration of H_2 (the value below that the H_2 oxidation becomes endergonic) depends on the electron acceptor of the reaction and can be calculated from the free energy changes of the respective reactions (Table 5).

Table 5: Minimal partial pressure of hydrogen, which thermodynamically allows H2 oxidation, by the respective electron acceptor. Calculated from the equation: $\Delta G' = \Delta G^{0\prime} + 1.36 \log [C][D] / [A][B]$

electron acceptor	CO_2 to Acet.	Sulfur	CO_2 to CH_4	SO_4^{2-}	fumarate	NO_3^-
$\Delta G^{0\prime}$ [kJ/mole H_2]	-26.2	-28.0	-33.8	-38.0	-86.2	-149.9
pH_2 for $\Delta G' = 0$ [atm.]	$10^{-4.4}$	$10^{-4.7}$	$10^{-5.2}$	$10^{-6.3}$	$10^{-14.7}$	10^{-}

The results of these competitions were correlated to the free energy change of the H_2 oxidation with the respective electron acceptor (Tables 2,3,4,5). *Desulfovibrio* species were less successfull in competition for H_2 when elemental sulfur replaced sulfate as electron acceptor (Table 3). This indicated, that with Sulfur as electron acceptor

Desulfovibrio species were limited to oxidize hydrogen, by thermodynamical reasons rather than by hydrogenase affinity (presuming that hydrogenase was the same in both cases).

Ecological aspects of the results

(i) Indirect competition for organic substrate. The competition for an organic substrate by two organisms with different substrate affinities only takes place under substrate limiting conditions. If the organic substrate is present in excess a coexistence of both organisms is to be expected until the substrate concentration gets limiting for one. However, if the organisms are intermediarily producing and consuming hydrogen the competition for the organic substrate is actually a competition for low concentrations of hydrogen. Even in the presence of organic substrate in excess this "indirect competition" can take place.

(ii) Parasitism by hydrogen removal. The production of hydrogen from organic substrates and the oxidation of hydrogen are two completely different metabolic ways of energy conservation: substrate level phosphorylation and electron transport phosphorylation. In pure culture, "hydrogen cycling" bacteria use both of this energy saving mechanisms. If the hydrogen is completely removed by a hydrogen oxidizing partner (here: concurrent), substrate level phosphorylation remains the only mechanism to save energy by the first strain ("H_2-cycler"). The known mechanism of substrate level phosphorylation during H_2-production from organic substrates such as ethanol or lactate is the acetate-kinase reaction. Hydrogen formation from methanol, however, is not known to be a possible energy saving reaction. Probably for that reason homoacetogenic bacteria failed to grow, when 100 % of hydrogen produced during methanol oxidation was removed by the hydrogeophilic concurrent (Table 2). This kind of parasitism finally is a disadvantage for both bacteria. To my knowledge this is the first case, where the addition of a hydrogen consuming bacterium, providing a more favorable electron acceptor, to a hydrogen producing organism (amelioration of thermodynamical conditions) drastically decreases the substrate degradation potential of a bacterial culture.

(iii) Possible ecological advantage of "hydrogen cycling". The theory of "hydrogen cycling" (Odom and Peck 1981), opposed by Lupton et al. (1984), originally assumes that all reducing equivalents are reoxidized via H_2. In the present study, which could not confirm this mechanism, the term "hydrogen cycling" means only, that the pool of reducing equivalents is continuously in a dynamic chemical equilibrium, catalised by hydrogenase(s), with an external hydrogen pool. However for ecological considerations the turnover of hydrogen in pure culture is of minor interest.

The maintenance of an external H_2-pool during the degradation of organic substrate may seem a complex and useless mechanism taking part in the reoxidation of reducing equivalents. What is the sence of "hydrogen cycling"? In fact, in pure culture this may represent nothing but a loss of energy and useless maintenance of hydrogenase activity.

In anaerobic environments however, the availability of fermentable organic substrate or electron acceptors such as sulfate, creates changes of hydrogen concentration, which thermodynamically favor hydrogen production or hydrogen consumption (Table 4). The mechanism of "H_2-cycling", or more precisely the maintenance of a defined external H_2 pool, serves as H_2-antenna, allowing the bacteria to react rapidly to changing hydrogen concentrations: Without major modifications of enzymatical equippement, they can switch from only H_2-production (100% H_2-transfer) to only H_2-consumption and to any intermediary position (partial reoxidation of the H_2 excreted). In a similar way "H_2-cycling" enables SRB to switch from sulfate respiration to hydrogen production as a facultative syntroph, without adaptation time (in contrast to diauxy), when sulfate becomes depleted.

In conclusion the interspecies hydrogen transfer can mean more than symbiotic association of two species, also: competition, parasitism and indirect competition for an organic substrate.

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