The Effect of Hydrogen on the Growth of *Desulfovibrio vulgaris* (Hildenborough) on Lactate

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(Received 28 April 1986; revised 2 July 1986)

Desulfovibrio vulgaris (Hildenborough) was grown on lactate with either a N₂/CO₂ or a H₂/CO₂ gas phase. H₂ increased the growth yield on lactate and had a sparing effect on lactate utilization, without altering the growth rate or hydrogenase level. Growth on acetate plus CO₂ with H₂ as sole energy source did not require an extensive adaptation period. Addition of lactate to cultures growing on acetate and H₂/CO₂ resulted in a switch from acetate to lactate utilization. In lactate-limited medium under H₂/CO₂ biphasic growth was observed. Lactate was oxidized first with production of acetate, followed by a second phase of growth on the acetate. Under this condition H₂ did not provide any supplementary energy during growth on lactate, as was evident from the ratio of lactate utilized to acetate produced.

INTRODUCTION

Sulphate-reducing bacteria (SRB) constitute an integral part of mixed microbial populations or consortia in sulphate-containing environments (Pfenning & Widdel, 1982; Hamilton, 1985). Their diverse metabolic capabilities (Peck & LeGall, 1982; Odom & Peck, 1984) allow them to carry out various interactions in different ecological niches such as marine and estuarine sediments, and biofilms. SRB are capable of growth on a wider range of substrates than had been previously appreciated (Pfenning *et al.*, 1981), and are thus very flexible ecologically. The relative importance of various intermediates of the mineralization process for the growth of SRB has recently been receiving increased attention (Jørgensen, 1982). Acetate in particular has been shown to be an important substrate for sulphate reduction, accounting for between 50% and 65% of the electron donors in marine environments (Sørensen *et al.*, 1981; Christensen, 1984). Many SRB capable of growth on acetate as sole source of carbon and energy with sulphate as terminal electron acceptor have been isolated by F. Widdel (Pfenning *et al.*, 1981).

Desulfovibrio vulgaris, on the other hand, is only able to grow on organic compounds such as lactate, oxidizing them incompletely to acetate. Badziong *et al.* (1978), however, isolated a *Desulfovibrio* species which utilized H₂ as the sole energy source and acetate and CO₂ as carbon sources. Approximately 70% of the cell carbon was derived from acetate and 30% from CO₂. It was believed at the time that this organism differed from the type strain *D. vulgaris* (Hildenborough) in this capacity to grow on H₂ in a minimal medium with acetate and CO₂. However, Brandis & Thauer (1981) were able to adapt *D. vulgaris* (Hildenborough) and other *Desulfovibrio* spp. to grow on H₂ as energy source.

H₂ is now believed to play a central role in the metabolism of *Desulfovibrio*, although the physiological function of H₂ and hydrogenase in the growth of SRB remains the subject of much discussion (Odom & Peck, 1984). Many *Desulfovibrio* spp. have the unique ability not only to oxidize H₂ (Brandis & Thauer, 1981; Nethe-Jaenchen & Thauer, 1984) but also to produce it,

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Abbreviation: SRB, sulphate-reducing bacteria.

even in the presence of sulphate (Tsuji & Yagi, 1980; Traore *et al.*, 1981). The possible physiological significance of this phenomenon has been discussed by several workers (Hatchikian *et al.*, 1976; Tsuji & Yagi, 1980; Odom & Peck, 1981, 1984; Lupton *et al.*, 1984). One hypothesis is the obligate H_2 -cycling mechanism linked to chemiosmotic energy metabolism (Odom & Peck, 1981). Electrons from lactate oxidation are transferred to protons, producing H_2 by the cytoplasmic hydrogenase. This H_2 then diffuses across the cell membrane where it is oxidized by the periplasmic hydrogenase, creating a proton gradient across the membrane and thus allowing energy transduction. Another model by Lupton *et al.* (1984) envisages the production of internal H_2 by cytoplasmic hydrogenase as regulating the redox state of electron carriers and so preventing the loss of energy by oxidizing H_2 in the periplasm.

In the investigation described here, the effect of H_2 on growth, lactate oxidation and hydrogenase levels in *D. vulgaris* was studied. The capability of H_2 /acetate-grown cultures to utilize lactate was also investigated.

METHODS

Organism and media. *D. vulgaris* (Hildenborough) NCIB 8303 was obtained from the National Collections of Industrial and Marine Bacteria Ltd, Aberdeen, UK. The bacteria were maintained in a medium containing ($g\ l^{-1}$): Na_2SO_4 , 3.0; KH_2PO_4 , 0.2; NH_4Cl , 0.25; $NaCl$, 1.0; $MgCl_2 \cdot 6H_2O$, 0.4; KCl , 0.5; $CaCl_2 \cdot 2H_2O$, 0.15; resazurin, 0.001; sodium lactate (70% w/w), 5 ml. The medium was boiled, cooled under O_2 -free N_2 and 50 ml volumes were dispensed into 100 ml Wheaton bottles (Supelco).

The bottles were stoppered with black butyl rubber stoppers and clamped with aluminium caps (Bellco). A solution of $Na_2S \cdot 9H_2O$ (1%, w/v) was injected into each bottle to give a final concentration of $0.01\ g\ l^{-1}$. The culture bottles were pressurized to 0.5 bar with N_2 and autoclaved for 20 min at $121\ ^\circ C$. After autoclaving, $1\ ml\ l^{-1}$ sterile anoxic trace element solution (Widdel, 1980) was added and the pH adjusted to 6.8 with $0.3\ ml\ 8.4\%$ (w/v) $NaHCO_3$. The medium was inoculated with a 2% (v/v) inoculum and incubated at $35\ ^\circ C$. The culture was transferred into fresh medium every second day.

The growth medium contained the following ($g\ l^{-1}$): $(NH_4)_2SO_4$, 6.6; $NaCl$, 1.8; KH_2PO_4 , 0.9; $MgCl_2 \cdot 6H_2O$, 0.36; $CaCl_2 \cdot 2H_2O$, 0.26; resazurin, 0.001; sodium lactate (70%, w/w), 5 ml or sodium acetate, 2.5; trace mineral solution (Brandis & Thauer, 1981), 12.5 ml. The trace mineral solution was autoclaved separately and the pH of the medium adjusted to 6.8 with sterile 10% (w/v) Na_2CO_3 .

pH-stat batch culture. The apparatus is shown in Fig. 1. Each vessel was autoclaved aerobically and then connected up with butyl rubber tubing (Esco Rubbers). The gas mixture (I) (80:20, v/v, N_2/CO_2 or H_2/CO_2) was passed over copper turnings which were heated at $350\ ^\circ C$ to remove traces of O_2 and through a 1 l bottle (II) containing sterile distilled water and resazurin ($0.001\ g\ l^{-1}$). It was then bubbled through a 5 l bottle (III) containing sterile medium before entering the culture vessel (IV), which contained 400 ml growth medium. After equilibration with N_2/CO_2 or H_2/CO_2 (flow rate $250\ ml\ min^{-1}$), $10\ ml\ 5\%$ (w/v) $Na_2S \cdot 9H_2O$ was added to the 1 l bottle (II) and within 20 min the medium was reduced as evidenced by the disappearance of the blue colouration of the resazurin. The pH in the culture vessel was maintained at 6.8 by the automatic addition of 10% (v/v) H_2SO_4 using a pH controller (LH Engineering). A butyl rubber tubing connection was made between the bottle containing acid and the culture vessel (IV) so as to compensate for the negative pressure that would be formed due to the automatic removal of acid from the bottle. The samples for the measurement of growth, substrate utilization and product formation were withdrawn from the culture vessel using the sampling port (V).

Growth was followed by measuring optical density at 578 nm in a Pye-Unicam SP305 spectrophotometer after diluting the sample 1:1 with 1 M-maleinate/NaOH buffer pH 6.5 and adding a few granules of sodium dithionite. For dry weight measurements 20 ml culture was withdrawn and immediately filtered through a $0.45\ \mu m$ membrane filter (Millipore) and washed twice with $20\ ml\ 1\%$ (w/v) NaCl. The filters were dried at $90\ ^\circ C$ overnight in an oven. Appropriate corrections were made for the blank prepared by filtering uninoculated growth medium and washing with NaCl.

Hydrogenase. The enzyme was assayed as H_2 -consuming activity by following spectrophotometrically the reduction of methyl viologen at 578 nm (Pankhania *et al.*, 1986a), and as H_2 -producing activity by an electrochemical method using a H_2 electrode (Hansatech) (Gow *et al.*, 1986). Hydrogenase activity was sensitive to inactivation by O_2 if the cells were grown under H_2/CO_2 , whereas under N_2/CO_2 the enzyme was extremely stable (I. P. Pankhania, unpublished results). For this reason the cells grown under a H_2/CO_2 gas phase were collected anaerobically (Pankhania *et al.*, 1986b). Cultures were centrifuged at $10000\ g$ for 20 min and the pellets were resuspended in 50 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl and 1 mM-dithiothreitol. Cells were broken by passage through a French pressure cell, centrifuged at $10000\ g$ for 20 min, and the supernatants were examined for the hydrogenase activities in the absence or presence of CO as described by Gow *et al.* (1986).

Fig. 1. Diagram of the pH-stat batch culture apparatus. I, Gas cylinder; II, 1 l bottle containing 750 ml distilled water and $0.001 \text{ g resazurin l}^{-1}$; III, 5 l medium reservoir bottle; IV, 500 ml fermenter with a water jacket; V, sampling port.

Measurement of sulphate, lactate and acetate. Sulphate was assayed turbidimetrically (Taras *et al.*, 1971). Lactate and acetate were measured by gas chromatography (Pye-Unicam PU4500) on a GP 15% SP-1220/1% H_3PO_4 on 100–120 Chromosorb WAW (Supelco) column ($1.82 \text{ m} \times 4 \text{ mm i.d.}$) with a flame ionization detector. The carrier gas was N_2 at a flow rate of 35 ml min^{-1} . The flow rates of H_2 and air were 45 ml min^{-1} and 450 ml min^{-1} respectively. The temperatures of the detector and injector were 170°C and that of the column was 115°C . Culture (5 ml) was centrifuged at 13000 g in a Micro Centaur (MSE) centrifuge for 10 min and the supernatant was used for the analyses. For acetate, 500 μl supernatant was acidified with 50 μl concentrated HCl and a 2 μl sample was injected in the chromatograph. Lactate was first methylated as described in the Supplement to Supelco Bulletin 748F and a 2 μl sample was used for analysis. Appropriate standards were run to calculate the concentrations of acetate and lactate in the samples.

RESULTS

Effect of H_2 on growth on lactate

Growth of SRB is generally poor in closed bottles because of the toxic effects of the H_2S produced (Postgate, 1984; Klemps *et al.*, 1985). Additionally, even if the medium is well chelated the accumulation of sulphide can cause precipitation of iron, which makes it difficult to measure growth by following the increase in optical density. A further problem in our experiments was that during growth of *D. vulgaris* under N_2/CO_2 the pH shifted from 6.8 to 7.2, whereas under H_2/CO_2 the increase was up to pH 7.8, which resulted in lysis of the cells (data not shown). Therefore, the batch culture with a pH controller was set up in which at the constant pH of 6.8, sulphide was removed from the culture as H_2S in the gas stream (Fig. 1).

Growth of *D. vulgaris* on lactate under N_2/CO_2 or H_2/CO_2 was followed by measuring increase in optical density, lactate utilization, acetate production and hydrogenase activity (data not shown). Growth stopped upon exhaustion of lactate. The periplasmic hydrogenase activity increased with time and followed growth. No increase in the hydrogenase activity was observed when the enzyme was assayed in the presence of Triton X-100. Odom & Peck (1981) have reported an increase in the enzyme activity in the presence of Triton X-100 which has been thought to be due to the presence of a second cytoplasmic hydrogenase.

Fig. 2

Fig. 3

Fig. 2. Growth (O) of *D. vulgaris* on lactate under N_2/CO_2 followed by H_2/CO_2 (the change in gas phase is indicated by the dashed line). Lactate (Δ) and acetate ($\blacktriangle$) were measured by gas chromatography as described in Methods.

Fig. 3. Growth (O) of *D. vulgaris* on acetate under H_2/CO_2 . The arrow indicates the time (9 h) when 25 mM-lactate was added to the growth vessel. Lactate (Δ), acetate ($\blacktriangle$) and sulphate ($\bullet$) were measured as described in Methods.

Table 1. Growth parameters of *D. vulgaris* on lactate under N_2/CO_2 or H_2/CO_2 gas phases

The results are means of triplicate determinations.

Gas phase	Y (g l ⁻¹)	t_d (h)	Lactate/ acetate	$Y_{lactate}$ (g dry wt cells mol ⁻¹)	Hydrogenase* [U (g dry wt) ⁻¹]	
					H_2 production	H_2 consumption
N_2/CO_2	0.255	3.7	1.17	5.95	10.3	7.18
H_2/CO_2	0.43	3.8	1.70	8.75	9.56	5.45

* U = mmol H_2 produced or consumed min⁻¹.

When the gas phase was changed from N_2/CO_2 to H_2/CO_2 a short lag of about 1 h was observed before there were further increases in cell mass (Fig. 2) and hydrogenase activities (data not shown). The growth parameters under N_2/CO_2 and H_2/CO_2 gas phases are summarized in Table 1. H_2 had a pronounced effect on growth of *D. vulgaris* on lactate. In the presence of H_2 , there were increases in growth yield and in the ratio of lactate consumed to acetate produced. However, there was no significant effect on the growth rate or the hydrogenase activity, suggesting that the periplasmic hydrogenase may be a constitutive enzyme. Hydrogenase activity was therefore measured in crude extracts of *D. vulgaris* grown with lactate under N_2/CO_2 or H_2/CO_2 , and with acetate under H_2/CO_2 . The activities were respectively, 5.7, 5.2 and 3.4 $\mu\text{mol } H_2$ produced min⁻¹ (mg protein)⁻¹. When the enzyme was measured in the presence of CO, however, cells grown on lactate under N_2/CO_2 retained nearly 18% of the original activity (1.0 $\mu\text{mol } H_2$ min⁻¹ mg⁻¹). This indicates that under such growth conditions there may be more membrane-bound hydrogenase (Gow *et al.*, 1986). Comparable figures for lactate and acetate under H_2/CO_2 were respectively, 3.8% (0.2 $\mu\text{mol } H_2$ min⁻¹ mg⁻¹) and 5.9% (0.2 $\mu\text{mol } H_2$ min⁻¹ mg⁻¹).

Fig. 4

Fig. 5

Fig. 4. Growth (○) of *D. vulgaris* in lactate-limited medium under N_2/CO_2 . After 54 h the gas was changed to H_2/CO_2 (indicated by the dashed line). Acetate in the growth vessel at zero time was carried over in the inoculum. Lactate (△) and acetate (▲) were measured as described in Methods.

Fig. 5. Growth (○) of *D. vulgaris* in lactate-limited medium under H_2/CO_2 . Lactate (△), acetate (▲) and sulphate (●) were measured as described in Methods.

Preferential utilization of lactate

It was not known whether cultures adapted to grow on H_2/CO_2 and acetate would utilize lactate. The data presented above show that *D. vulgaris* did not use acetate as a carbon source when growing on lactate with either N_2/CO_2 or H_2/CO_2 as gas phase. However, the addition of lactate to cultures growing with H_2 as energy source resulted in a switch from acetate to lactate utilization, which was evident from the further production of acetate (Fig. 3).

Growth with H_2 as the sole energy source

D. vulgaris (Hildenborough) was originally thought to differ from *D. vulgaris* strains Marburg and Madison in that it could not be grown with H_2 as sole energy source (Badziong *et al.*, 1978). It was later demonstrated that the ability to grow on H_2 was common to other species (Brandis & Thauer, 1981); *D. vulgaris* (Hildenborough), however, required an elaborate adaptation period prior to growth on H_2 . In our studies no such adaptation period was required (Pankhania *et al.*, 1986b) although a lag period of about 18 h was observed when a lactate-grown culture was subcultured into acetate medium under H_2/CO_2 . After four subcultures growth and acetate utilization were measured (data not shown). The growth rate was similar to that observed with lactate.

Growth of *D. vulgaris* in lactate-limited medium

Growth of *D. vulgaris* in medium containing 9 mM-lactate was followed under a gas phase of N_2/CO_2 . The growth rate was low and growth stopped when lactate was completely utilized (Fig. 4), the acetate produced from lactate not being utilized further. However, when the gas phase was changed to H_2/CO_2 , growth was much faster, after a lag phase of approximately 2 h. When the above experiment was carried out entirely under H_2/CO_2 , a biphasic growth pattern was observed, lactate first being oxidized to acetate, followed immediately by growth on acetate after exhaustion of the lactate (Fig. 5). A surprising observation was that the ratio of lactate consumed to acetate formed was nearly 1 rather than 2 as found with excess lactate (Table 1). These data suggest that H_2 may not serve as an energy source in the presence of limiting lactate.

DISCUSSION

One of the aims of this work was to look for evidence for or against the H_2 -cycling mechanism as proposed by Odom & Peck (1981). A major problem for this hypothesis is that the standard redox potentials for the two half reactions $\text{lactate} \rightarrow \text{pyruvate} + 2H^+ + 2e^-$ ($E'_0 = -197 \text{ mV}$) and $2H^+ + 2e^- \rightarrow H_2$ ($E'_0 = -420 \text{ mV}$) are such that H_2 production from the oxidation of lactate is endergonic. Because of the unfavourable position of the equilibrium of the reaction therefore, the oxidation of lactate can only proceed at very low H_2 concentrations and/or high lactate:pyruvate ratios. Even with a lactate:pyruvate ratio of 100:1, this would require a partial pressure of H_2 less than 10^{-5} atm . According to the H_2 -cycling hypothesis therefore, oxidation and growth on lactate should be inhibited by H_2 partial pressures in excess of 10^{-5} atm . Data obtained in this study, however, suggest that H_2 did not inhibit lactate oxidation. Instead it had a sparing effect on lactate utilization and increased the growth yield on lactate. Lupton *et al.* (1984) also observed no inhibition of growth or substrate consumption when *D. vulgaris* (Madison) was cultured on lactate in the presence of H_2 . We have additionally shown that *D. vulgaris* grown on lactate in a sulphate- (10 mM) limited chemostat does not suffer wash-out at low dilution rate under a H_2/CO_2 gas phase (I. P. Pankhania, unpublished results). Similar results using sulphate-limited chemostats were obtained by Nethe-Jaenchen & Thauer (1984) with *D. vulgaris* (Marburg) and by Cypionka & Pfennig (1986) with *Desulfotomaculum orientis*. Other evidence against the H_2 -cycling hypothesis comes from the lack of inhibition of synthesis or activity of lactate dehydrogenase by H_2 (Hamilton, 1982) and the production of H_2 during growth on lactate plus sulphate (Tsuji & Yagi, 1980; Traore *et al.*, 1981). Lupton *et al.* (1984) have proposed a trace H_2 transformation model for the physiological function of H_2 metabolism during growth of *D. vulgaris* on organic electron donors. In this model, hydrogenases function in regulating electron flow and preventing energy loss by producing and consuming H_2 respectively. An alternative explanation would be that the energetically unfavourable oxidation of lactate is driven by energy-dependent reversed electron transport in line with the model suggested by Thauer & Morris (1984).

Another requirement for the mechanism of H_2 -cycling is the presence of two hydrogenases, one in the periplasm, functioning as a H_2 -consuming hydrogenase, and another in the cytoplasm, functioning as a H_2 -producing hydrogenase (Odom & Peck, 1984). Our results suggest that two hydrogenases are present in *D. vulgaris*. The second, membrane-bound hydrogenase can be detected in the presence of CO. The membrane-bound enzyme differs from periplasmic hydrogenase in a number of properties: (a) R_F values are 0.12 and 0.45 for membrane-bound and periplasmic hydrogenase respectively, (b) membrane-bound hydrogenase does not cross react with antisera raised against periplasmic hydrogenase but it is digested by pancreatin, and (c) the membrane-bound enzyme is not inhibited by CO (Gow *et al.*, 1986). Evidence for two hydrogenases, one a CO-sensitive enzyme and the other CO-stimulated, has also been reported in *D. vulgaris* (Madison) by Lupton *et al.* (1984). Although we have found evidence for a second hydrogenase, we still do not know the physiological role of this enzyme. The highest activity of the membrane-bound enzyme was found in the cells grown on lactate under N_2/CO_2 .

In lactate-limited growth conditions under N_2/CO_2 , lactate was oxidized to acetate and no further oxidation of acetate occurred. It is well established that *D. vulgaris* lacks a complete tricarboxylic acid cycle (Lewis & Miller, 1977). However, under a H_2/CO_2 gas phase, biphasic growth occurred. Firstly lactate was oxidized, followed by acetate utilization for the anabolic pathways (Badziong *et al.*, 1978), using H_2 as sole energy source. In lactate-limited conditions H_2 did not provide any supplementary energy, as was evident from the lactate/acetate ratio. Similar results were obtained by Khosrovi *et al.* (1971); as yet we are unable to explain the above observation physiologically. Another important point which must be mentioned here is that Khosrovi & Miller (1975) reported specific growth yields of 9.2 and 25.8 g (mol lactate) $^{-1}$ for *D. vulgaris* under Ar/CO_2 (95:5, v/v) and H_2/CO_2 (95:5, v/v) respectively. However, the yields reported in the present paper are much lower and H_2 increased the yield by only 47%. The apparent 180% increase in yield reported by Khosrovi & Miller (1975) might be explained on the basis that the culture was in fact capable of biphasic growth under H_2/CO_2 , growing on lactate first then switching to a second phase of growth on acetate.

In this study we have shown that if *D. vulgaris* is given a choice between lactate and acetate it will preferentially utilize lactate irrespective of the presence or absence of H_2 . Also the capacity of *D. vulgaris* to grow biphasically on lactate and acetate may have a significant ecological implication.

We wish to thank the Science and Engineering Research Council and British Petroleum plc for their financial support of this project.

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