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Hydrogenase Activity in Aged, Nonviable *Desulfovibrio vulgaris* Cultures and Its Significance in Anaerobic Biocorrosion†

C. CHATELUS,[1]* P. CARRIER,[1] P. SAIGNES,[1] M. F. LIBERT,[1] Y. BERLIER,[1] P. A. LESPINAT,[1] G. FAUQUE,[1,2] AND J. LEGALL[1,2]

Section d'Enzymologie et Biochimie Bactérienne, Association pour la Recherche en Bioénergie Solaire, CEN Cadarache, 13108 Saint Paul Lez Durance Cedex, France,[1] and Department of Biochemistry, School of Chemical Sciences, University of Georgia, Athens, Georgia 30602[2]

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Batch cultures of *Desulfovibrio vulgaris* stored at 32°C for 10 months have been found to retain 50% of the hydrogenase activity of a 1-day culture. The hydrogenase found in old cultures needs reducing conditions for its activation. Viable cell counts are negative after 6 months, showing that the hydrogenase activity does not depend on the presence of viable cells. These observations are of importance in the understanding of anaerobic biocorrosion of metals caused by depolarization phenomena.

To account for the phenomenon of anaerobic corrosion of iron, Von Wolzogen Kühr and Van Der Vlugt (16) proposed the cathodic depolarization theory, based on the metabolic activities of the sulfate-reducing bacteria which they had clearly established as the causative agents of this type of corrosion. Hydrogen plays a central role in the metabolism of sulfate-reducing bacteria (J. Le Gall and G. Fauque, *in* A. J. B. Zehnder, ed., *Biology of Anaerobic Microorganisms*, in press). These bacteria have hydrogenases with different cellular localizations: cytoplasmic, membrane-bound, and periplasmic. Recently, several hydrogenases have been detected in *Desulfovibrio vulgaris* (NCIB 8303) (4, 11): a periplasmic iron hydrogenase and three nickel-iron hydrogenases in the membrane fraction. The periplasmic (Fe) hydrogenase is highly sensitive to inhibition by CO (G. Fauque, Thèse d'Etat, Université de Technologie de Compiègne, Compiègne, France, 1985), whereas the membrane-bound (Ni-Fe) hydrogenases are relatively insensitive (4, 11). The periplasmic dehydrogenase catalyzes either hydrogen production or hydrogen consumption and can thus remove the protective layer of hydrogen attached to the metal surface (6). Growth on hydrogen and CO_2 plus acetate and sulfate is a widespread ability among *Desulfovibrio* and *Desulfotomaculum* species (3; F. Widdel, *in* A. J. B. Zehnder, ed., *Biology of Anaerobic Microorganisms*, in press). Furthermore, it has been shown that, in contrast to earlier findings (5), the latter genus also contains a high hydrogenase activity (10). *Desulfotomaculum orientis* can grow with hydrogen and CO_2 plus sulfate under autotrophic conditions or it can undergo homoacetogenic growth with hydrogen plus formate or methanol (8; Widdel, in press). Thus, in environments poor in organic compounds, sulfate-reducing bacteria are able to use the oxidation of cathodic hydrogen as a significant source of energy. In the natural environment, growth conditions are not always propitious. Significant variations in temperature, pH, and nutrient concentration can occur (7) and result in long periods during which bacteria are not able to grow or are no longer viable. During these adverse periods, some enzymatic activity may persist. Consequently, corrosion activity could occur independently of bacterial proliferation. We report here that hydrogenase activity of *D. vulgaris* Hildenborough grown on lactate-sulfate medium (15) is still present in batch cultures stored for several months at 32°C without shaking.

Enumeration of viable cells was made by the most-probable-number method (9) in lactate-sulfate medium. Hydrogenase activity was measured on 10 ml of cell suspension withdrawn from the batch cultures. Bacterial cells or cell debris (or both) was harvested by centrifugation at 31,500 × *g* for 30 min, and the pellet was suspended in 20 mM potassium phosphate buffer at pH 7.6. Hydrogenase activity was measured by two methods: hydrogen production and deuterium-proton (D_2-H^+) exchange reaction. The hydrogen evolution assay from dithionite-reduced methyl viologen (14) was done in 15-ml gastight serum flasks with 3 ml of an anaerobic Tris hydrochloride buffer (0.1 M, pH 7.6) containing 1 mg of bovine serum albumin, 1 mM methyl viologen, and 15 mM sodium dithionite. The cells were introduced into the reaction vessel, and then H_2 production was monitored on a sample of the flask atmosphere injected into a Varian Aerograph A90 P3 gas chromatograph. The second method made use of the deuterium-proton exchange reaction in a vessel connected by a membrane inlet to the ion source of a mass spectrometer (VG8-80 model) equipped with an Apple II data acquisition system (1). The culture medium (pH 7.2) in the vessel was sparged to equilibrium with an appropriate gas mixture (80% argon–20% deuterium). The experiment was done in the absence of the gas phase and of artificial electron donors or mediators. The reaction was then initiated by injecting a sample of cell preparation into the vessel. The reaction consisted of the disappearance of D_2 and of the evolution of HD and H_2. Diverse reagents or inhibitors could also be introduced in gaseous or dissolved forms. The specific activity is expressed in the first case as micromoles of H_2 and in the second case as micromoles of H_2 plus HD produced per minute per milligram of protein. The protein determination was made by a modification of the Lowry method (13).

The hydrogenase activity of *D. vulgaris* Hildenborough was measured in H_2 evolution at the beginning of the stationary growth phase, and then at intervals during a 10-month period at 32°C. During the first 3 months, the specific activity of hydrogenase decreased to half of its initial value; then it became relatively stable until the end of the experiment. At that time, enumeration of viable cells indi-

* Corresponding author.
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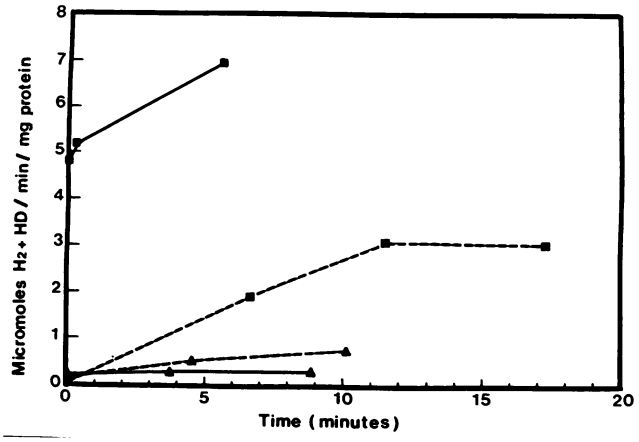

FIG. 1. Kinetics of D_2-H^+ exchange reaction of *D. vulgaris* cells. ——, After 1 day of growth; -----, after 10 months of batch culture. Symbols: ■, activity in the pellet; ▲, activity in the supernatant. At 45 s, a sodium dithionite solution (0.4 mM final concentration) was added to both 1-day-old and 10-month-old cultures.

cated that none was able to proliferate. In another set of experiments, no viable cells could be detected after an incubation time of 6 months at the same temperature. Figure 1 shows the time course of hydrogenase activity in the H_2 evolution assay of *D. vulgaris* cells at the beginning of the stationary growth phase and after 10 months of incubation.

Two types of control experiments confirmed the enzymatic origin of the hydrogen production activity. First, the activity was completely lost after the cultures were boiled. Second, the high sensitivity of periplasmic hydrogenase from *D. vulgaris* to CO inhibition was used. When CO was injected into the reaction vessel (4% in the gas phase), hydrogenase activity was completely suppressed after a few minutes in aged cultures and greatly reduced in younger cells (Fig. 1). Although the hydrogenase activity decreased during the storage period, the activity pattern and the CO inhibition were similar at the earliest stage of growth and after 10 months, suggesting that the enzyme was able to maintain its function independently of bacterial proliferation.

Figure 2 shows the D_2-H^+ exchange activity of *D. vulgaris* Hildenborough after 1 day of growth and after 10 months. With young cultures (1 day), the activity was present almost entirely in the cells, although the supernatant obtained after centrifugation at 31,500 × *g* contained some activity. This low activity was probably caused by some lysis occurring at the end of the growth phase. With 10-month-old cultures, the activity was detected only after addition of dithionite; in contrast, dithionite had but a small activation effect when added to young cultures (Fig. 2).

The total activity in the sample also decreased with time, and at the end of the experiment it was nearly half of that found at the beginning (Table 1). A good correlation was thus found between the two experimental methods. The total and specific activities of cell pellets declined with age. In contrast, both activities increased with time in the supernatant, showing that some hydrogenase was able to maintain its function independently of the cell structure.

An increase of the specific activity was noted in the supernatant fraction after 1 month of incubation. This phenomenon may result from the fact that the proteolytic enzymes present are more sensitive to denaturation than is the hydrogenase itself. A low sensitivity of *D. vulgaris* periplasmic hydrogenase to some proteolytic enzymes has

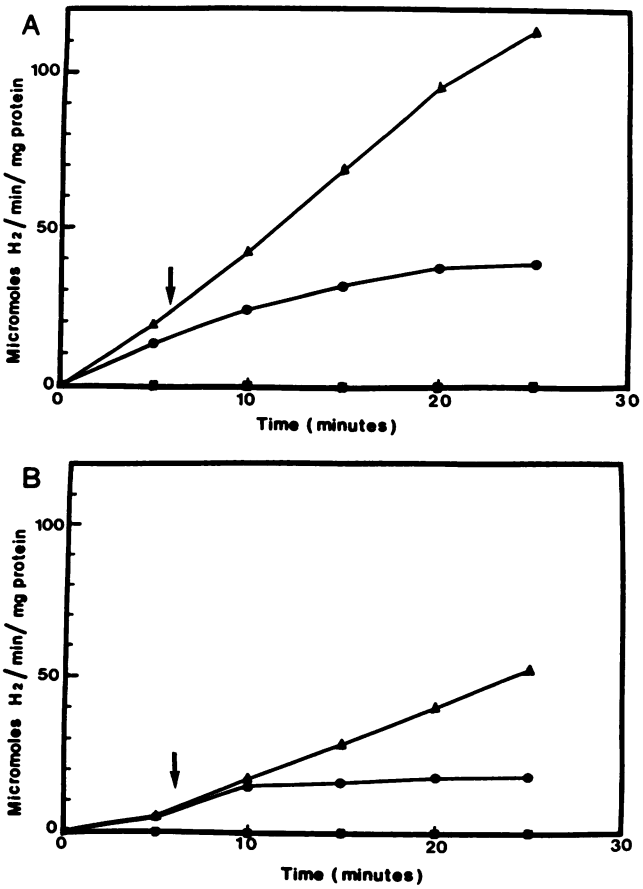

FIG. 2. Hydrogenase activity of *D. vulgaris* cells as measured with the H_2 evolution assay. (A) At the beginning of the stationary phase of growth; (B) after 10 months. Symbols: ▲, intact cells; ●, effect of 4% CO added after 5 min (↓); ■, boiled cells.

already been noted by Gow et al. (4). Another possible explanation for the long-term stability of hydrogenase is that the enzyme does not turn over and is actually in a resting state, as suggested by the necessity of adding dithionite to

TABLE 1. Total and specific hydrogenase activities measured by the D_2-H^+ exchange reaction

Time	Fraction[a]	Hydrogenase activity[b]	
		Total activity	Sp act
1 day	P	11.52	6.93
	S	2.22	0.30
	P + S	13.74	
1 month	P	5.72	5.82
	S	5.17	0.63
	P + S	10.89	
10 months	P	3.17	3.07
	S	4.50	0.75
	P + S	7.67	

[a] Volume of the pellet (P) = 0.25 ml; volume of the supernatant (S) = 10 ml.

[b] Total hydrogenase activity is expressed as micromoles of H_2 and HD produced per minute in the sample. Specific hydrogenase activity is expressed as micromoles of H_2 and HD produced per minute per milligram of protein.

restore its full activity in old cultures. The stability of several purified hydrogenases from *Desulfovibrio* species upon storage is well known (2, 12).

In our experimental conditions, the concentrations of CO used were sufficient to inhibit the Fe periplasmic enzyme but to only partially inhibit the Ni-Fe membrane-bound hydrogenases (4, 11). Since the inhibition of hydrogenase activity is partial in young cultures but complete in old ones, it is clear that the residual activity of old cultures results mainly from the periplasmic hydrogenase.

These results are of particular importance in the understanding of metal biocorrosion by anaerobic bacteria since hydrogenase is still active for months independent of viable cells. Thus, bacteria that are still attached to metallic surfaces and that cannot be detected by the usual methods, such as viable counts or production of H_2S, may retain their depolarizing capabilities, which could be expressed immediately if reducing conditions were created in the environment. Thus, the opinion of Postgate (15) indicating that enumeration of sulfate-reducing bacteria is of little value because it does not distinguish between active and dormant cells should be modified to include the possibility that nonviable cells retaining detrimental enzymatic activities may be present in the environment.

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