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Fermentation and Sulfur Reduction in the Mat-Building Cyanobacterium *Microcoleus chthonoplastes*

ROY MOEZELAAR,† SASKIA M. BIJVANK, AND LUCAS J. STAL*

Department of Microbiology, University of Amsterdam, Amsterdam, The Netherlands

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The mat-building cyanobacterium *Microcoleus chthonoplastes* carried out a mixed-acid fermentation when incubated under anoxic conditions in the dark. Endogenous storage carbohydrate was fermented to acetate, ethanol, formate, lactate, H_2, and CO_2. Cells with a low glycogen content (about 0.3 μmol of glucose per mg of protein) produced acetate and ethanol in equimolar amounts. In addition to glycogen, part of the osmoprotectant, glucosyl-glycerol, was degraded. The glucose component of glucosyl-glycerol was fermented, whereas glycerol was released into the medium. Cells with a high content of glycogen (about 2 μmol of glucose per mg of protein) did not utilize glucosyl-glycerol. These cells produced more acetate than ethanol. *M. chthonoplastes* was also capable of using elemental sulfur as the electron acceptor during fermentation, resulting in the production of sulfide. With sulfur present, acetate production increased whereas ethanol production decreased. Also, less formate was produced and the evolution of hydrogen ceased completely. In general, the carbon recoveries were satisfactory but the oxidation-reduction balances were too high. The latter could be explained by assuming the reduction of ferric iron, which is associated with the cells, mediated by the oxidation of formate. The switch from photoautotrophic to fermentative metabolism did not require de novo protein synthesis, and fermentation started immediately upon transfer to dark anoxic conditions. From the molar ratios of the fermentation products and from measurement of enzyme activities in cell extracts, we concluded that glucose derived from glycogen and glucosyl-glycerol is degraded via the Embden-Meyerhof-Parnas pathway.

Microbial mats are characterized by marked diel fluctuations of oxygen concentration, which are attributed largely to the cyanobacterial metabolism (19). In the light, these organisms grow photoautotrophically (24, 25) and their oxygenic plant-like photosynthesis results in oxygen supersaturation of the mat. In the dark, cyanobacteria switch to chemotrophic metabolism at the expense of endogenous glycogen, which was accumulated in the light (24, 25). Aerobically, glucose derived from glycogen is degraded via the oxidative pentose phosphate pathway and metabolic energy is generated by oxidative phosphorylation (24, 25). In addition, part of the glycogen may be used as a carbon source for biosynthetic purposes (32). However, in well-established mats, diffusion of oxygen into the mat is generally not sufficient to cover the demands and as a consequence, the mat becomes anoxic. Microbial mat cyanobacteria that have been tested for their capacity for anaerobic energy generation in the dark were all capable of fermentation. One of the first reports on fermentation in cyanobacteria dealt with *Oscillatoria limnetica*, which was isolated from the sulfide-rich hypolimnion of the hypersaline Solar Lake, Sinai, where it forms flocculant mats. This species carries out a homolactic fermentation at the expense of endogenous carbohydrates (17). Anaerobic respiration with elemental sulfur as the electron acceptor was also reported for *O. limnetica* (17). Another example concerns *Oscillatoria limosa*, isolated from a marine microbial mat. This organism ferments glycogen via the heterolactic pathway (9). In addition to glycogen, *O. limosa* degrades the osmoprotectant trehalose and ferments it via the homoacetic pathway (9). *Oscillatoria terebriformis*, isolated from a hot-spring mat, ferments exogenous glucose or fructose to lactate (20). Anoxia is vital for survival of this organism in dark periods, because the fast degradation of glycogen under aerobic conditions would result in exhaustion of the energy reserves within a few hours.

The filamentous *Microcoleus chthonoplastes* is a cosmopolitan cyanobacterium and is often the dominant organism in marine and hypersaline microbial mats (26). Since such mats become anoxic at night (19), it is obvious that the organism is capable of surviving under such conditions. This study was initiated to elucidate the mechanisms of anaerobic energy generation by this organism in the dark. It was found that *M. chthonoplastes* fermented endogenous storage carbohydrates according to a mixed-acid fermentation. Part of the osmoprotectant glucosyl-glycerol was also used as substrate for fermentation. The fermentation pathways were elucidated from the products excreted in the medium and by assaying cell extracts for enzyme activities.

MATERIALS AND METHODS

Organism and cultivation. The *M. chthonoplastes* strain used in this study was isolated from a marine microbial mat on the island of Mellum, Germany, by Stal and Krumbein (27) and obtained in pure culture by Visscher and Van Gemerden (33). The organism was grown in batch culture in 1-liter Kluyver flasks in ASN3 medium (21) with elevated concentrations of ferric ammonium citrate (final concentration, 46 μM). By increasing the concentration of ferric iron in the medium, clumping of the filaments was minimized and therefore cultures were rendered homogeneous (14). Aeration was 0.15 to 0.3 liter/min. The photon flux density was 30 μmol photons m^{-2} s^{-1} provided by one circular fluorescent tube (Philips 40W/33). The temperature was kept at 20°C.

Anaerobic dark incubations. For anaerobic dark incubations, actively growing cells (optical density at 750 nm [OD_{750}], 0.7 to 1) or cells from the stationary growth phase were used. An OD_{750} of 1 corresponds to 429 mg of protein $liter^{-1}$ or 16.5 mg of chlorophyll *a* $liter^{-1}$. Cells were harvested by centrifugation (2,000 × *g* for 5 min at 20°C), washed three times in buffer (25 mM Tris-HCl [pH 8]

* Corresponding author. Mailing address: Department of Microbiology, University of Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, The Netherlands. Phone: 31-20-5257090. Fax: 31-20-5257085. Electronic mail address: stal @sara.nl.

† Present address: Agrotechnological Research Institute, Wageningen, The Netherlands.

supplemented with 3% [wt/vol] NaCl), and resuspended in the same buffer (OD_{750}, 5 to 10). Aliquots of 10 ml were transferred to 30-ml serum bottles with screw caps and butyl rubber inlays. The bottles were wrapped in aluminum foil, and the headspace was flushed with argon for 10 min. Absence of oxygen was checked by gas chromatography. At regular time intervals, the contents of a bottle were analyzed for the amount of cell protein, storage polyglucose, glucosyl-glycerol, fermentation products, and sulfide. Carbon recoveries and oxidation-reduction (O/R) balances were calculated by the method of Gottschalk (7).

Elemental sulfur was prepared by the method of Roy and Trudinger (22) and autoclaved for 20 min at 110°C. It was added to the suspension at a final concentration of 0.3 g/liter.

Analysis. H_2 was determined gas chromatographically as described previously (15). Ethanol, acetate, formate, D- and L-lactate, and succinate were determined enzymatically with test combinations (Boehringer, Mannheim, Germany). The amount of CO_2 was assumed to equal the sum of the amounts of ethanol and acetate minus the amount of formate. Cell protein was determined by the method of Lowry et al. with bovine serum albumin as a standard (8). Storage polyglucose was hydrolyzed by incubating ethanol-extracted cells in 2 M HCl for 2 h. The hydrolysate was neutralized with NaOH and potassium phosphate buffer (pH 7; final concentration, 0.2 M), and glucose was determined enzymatically with a test combination (Boehringer). Glucosyl-glycerol was extracted from the cells by two consecutive extractions with 80% (vol/vol) ethanol at 40°C (28). The two fractions were pooled, and the ethanol was evaporated with a rotary evaporator. Glucosyl-glycerol was dissolved in water and hydrolyzed in 1 M HCl (100°C for 30 min). After the hydrolysate was neutralized with NaOH and potassium phosphate buffer (pH 7; final concentration, 125 mM), glucose and glycerol were determined enzymatically with test combinations (Boehringer). Sulfide was determined colorimetrically as described previously (29). Both whole-cell suspensions and supernatants were analyzed for sulfide.

Preparation of cell extracts. All steps were carried out at 0 to 5°C. Cells were harvested at an OD_{750} of 0.7 to 1 by centrifugation ($2{,}000 \times g$ for 5 min) and washed three times in 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–KOH buffer (pH 7.5) containing 5 mM $MgCl_2$ and decreasing NaCl concentrations (200, 100, and 0 mM, respectively). Subsequently, the cells were resuspended in 2 to 4 ml of 25 mM HEPES–KOH buffer (pH 7.5) to a final OD_{750} of 50 to 100. For the assay of alcohol dehydrogenase, dithiothreitol was added to the suspension (final concentration, 5 mM). Similarly, when 6-phosphofructokinase was to be assayed, the cell suspension was supplemented with its substrate, fructose 6-phosphate. Cells were disrupted by French press treatment at 1.4×10^8 Pa. Unbroken cells and debris were removed by centrifugation ($200{,}000 \times g$ for 30 min). To avoid NAD(P)H oxidase activity, only the upper half of the supernatant was used as cell extract in enzyme assays.

Assay of enzyme activity. Enzyme assays were performed at 25°C. In all cases, the observed reaction rate was constant for at least 2 min and linearly proportional to the amount of cell extract added. One unit of enzyme activity is defined as the amount of enzyme catalyzing the transformation of 1 μmol of substrate or the formation of 1 μmol of product in 1 min. Spectrophotometric assays were performed with a single-beam spectrophotometer (Ultrospec III; Pharmacia LKB) equipped with a thermostated rotating cuvette holder. The formation or disappearance of NAD(P)H was monitored at 340 nm ($\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$). The amount of protein was determined by the method of Bradford (2) with bovine serum albumin as the standard.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed in a reaction mixture containing 50 mM TAPS [*N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid]–KOH (pH 8.0), 5 mM $MgCl_2$, 0.4 mM NAD(P), and cell extract. The reaction was started by the addition of glucose-6-phosphate (5 mM).

6-Phosphogluconate dehydrogenase (EC 1.1.1.44) was assayed in a reaction mixture containing 50 mM TAPS–KOH (pH 9.0), 0.4 mM NAD(P), and cell extract. The reaction was started by the addition of 6-phosphogluconate (5 mM).

6-Phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.2.1.12 and EC 4.1.2.14, respectively) were assayed together by the method of Van Dijken and Quayle (31).

6-Phosphofructokinase (EC 2.7.1.11) activity was detected only if fructose-6-phosphate was added to the cell suspension before cell disruption. The enzyme was assayed in a reaction mixture containing 50 mM TAPS–KOH (pH 8), 5 mM $MgCl_2$, 0.2 mM dithiothreitol, 2 mM fructose-6-phosphate, 0.9 U of aldolase per ml, 10 U of triose-phosphate isomerase per ml, 1.7 U of glycerol-3-phosphate dehydrogenase per ml, 0.15 mM NADH, and 2.5 mM ATP. The reaction was started by the addition of cell extract.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was assayed in the direction of glyceraldehyde-3-phosphate formation. The substrate for this reaction, 1,3-bisphosphoglycerate, was generated in situ from ATP and 3-phosphoglycerate by phosphoglycerate kinase. The reaction mixture contained 50 mM HEPES–KOH (pH 7.5), 5 mM $MgCl_2$, 5 mM 3-phosphoglycerate, 5 mM ATP, 2 U of phosphoglycerate kinase per ml, 0.2 mM dithiothreitol, and cell extract. After a preincubation of 3 min, the reaction was started by the addition of NAD(P)H (0.15 mM).

Pyruvate kinase (EC 2.7.1.40) was assayed in a reaction mixture containing 50 mM morpholinepropanesulfonic acid (MOPS)-HCl (pH 7.0), 5 mM $MgCl_2$, 5 mM ADP, 5.5 U of lactate dehydrogenase per ml, 0.15 mM NADH, and cell extract. The reaction was started by the addition of phosphoenolpyruvate (5 mM).

Pyruvate formate-lyase (EC 2.3.1.54) was assayed under an argon atmosphere in a cuvette closed with a Suba Seal rubber stopper. The reaction mixture contained 50 mM HEPES–KOH (pH 7.5), 5 mM $MgCl_2$, 5 mM pyruvate, 2 U of formate dehydrogenase per ml, and 0.4 mM NAD. The reaction was started by the addition of cell extract.

Lactate dehydrogenase (EC 1.1.1.27/28) was assayed in a reaction mixture containing 50 mM HEPES–KOH (pH 7.5), 5 mM $MgCl_2$, 0.15 mM NAD(P)H, and cell extract. The reaction was started by the addition of pyruvate (5 mM). The effect of fructose-1,6-bisphosphate at 10 mM was tested.

Hydrogenase (EC 1.18.3.1) was assayed as dithionite/methyl viologen-dependent H_2 production in a crimp-top vial (8 ml) with a butyl rubber septum under an argon atmosphere. The reaction mixture (final volume, 1 ml) contained 50 mM HEPES–KOH (pH 7.5), 5 mM $MgCl_2$, and 5 mM methyl viologen. After the headspace had been flushed with argon, sodium dithionite (20 mM) was added. The reaction was started by the addition of cell extract. At regular time intervals, H_2 in the gas phase was determined gas chromatographically as described previously (15).

Alcohol dehydrogenase (EC 1.1.1.1) activity was detected only if dithiothreitol was added to the cell suspension before cell disruption. The enzyme was assayed in a reaction mixture containing 50 mM HEPES–KOH (pH 7.5), 5 mM $MgCl_2$, 0.15 mM NAD(P)H, 5 mM dithiothreitol, and cell extract. The reaction was started by the addition of acetaldehyde (20 mM).

Acetate kinase (EC 2.7.2.1) was assayed discontinuously as the formation of acetyl phosphate from acetate and ATP. Acetyl phosphate was determined colorimetrically by using the principle of Lipmann and Tuttle (10). The assay mixture we used was derived from that of Fox and Roseman (5). It contained 92.3 mM HEPES–KOH (pH 7.5), 241 mM hydroxylamine–KOH (pH 7), 6.1 mM ATP, 12.3 mM $MgCl_2$, 0.62 mM dithiothreitol, and cell extract. The reaction was started by the addition of potassium acetate (77 mM). At regular time intervals, a 650-μl sample was withdrawn from the reaction mixture, acidified with 400 μl of 0.85 M HCl to stop the reaction, and colored with 200 μl of 5% (wt/vol) $FeCl_3$ in 0.1 M HCl. The A_{500} was measured. A calibration curve derived from addition to the assay mixture of known amounts of acetyl phosphate instead of cell extract was obtained.

RESULTS

Fermentation by cells from the exponential growth phase. The ability of *M. chthonoplastes* to survive anaerobically in the dark was examined by using cell suspensions in Tris-HCl buffer (pH 8.0) containing 3% NaCl. Cells from the exponential growth phase of batch cultures which were incubated in this way for 24 h did not lyse and were capable of growth on agar plates or in liquid medium, which indicated that they were still viable. During such incubations, the protein concentration remained constant or decreased only slightly (Fig. 1). However, incubations longer than 24 h resulted in progressive cell lysis. This was evident from the blue color of the supernatant, which was due to release of the light-harvesting pigment phycocyanin. Upon transfer to dark anoxic conditions, the polyglucose content of the cells (about 0.3 μmol of glucose per mg of protein) decreased according to first-order reaction kinetics (Fig. 1). Over the same period, formation of ethanol, acetate, formate, and hydrogen was observed. Characteristically, acetate and ethanol were formed in approximately equimolar amounts. The kinetics of production of these compounds correlated inversely with polyglucose degradation. From this, we concluded that glycogen was degraded and the glucose derived from it was fermented to acetate, ethanol, formate, CO_2, and H_2. Theoretically, fermentative degradation of 1 mol of glucose would result in the formation of 1 mol of acetate and 1 mol of ethanol, whereas the molar sum of formate and H_2 would equal 2 mol. However, from Fig. 1, it is evident that more acetate and ethanol are formed than can be explained from glycogen degradation. Therefore, the involvement of a second substrate was examined.

In the cyanobacterium *O. limosa*, the osmoprotectant trehalose is fermented under anoxic conditions in the dark (9). In *M. chthonoplastes*, glucosyl-glycerol, a compound consisting of a glucose unit and a glycerol unit, serves as an osmoprotectant (28). Measurements of the cellular glycosyl-glycerol content showed a decrease during dark anaerobic incubations, most notably after about 16 h of incubation (Fig. 2). The glycerol

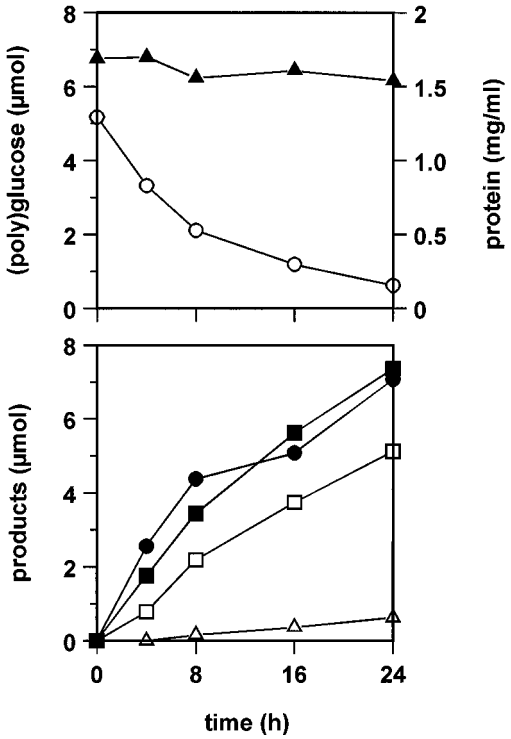

FIG. 1. Fermentation of endogenously stored polyglucose by *M. chthonoplastes* pregrown photoautotrophically in batch culture and harvested during the exponential growth phase. Symbols: ○, polyglucose; ▲, protein; ■, ethanol; ●, acetate; □, formate; △, hydrogen.

component was not used as a substrate but was released into the medium. Since glucose was found neither in the medium nor in the cells, we concluded that it was fermented. This conclusion was supported by a satisfactory C recovery (102%), which was obtained when glucosyl-glycerol was included (Table 1). However, the O/R balance (1.55) was too high, indicating an excess of oxidized products.

Effect of chloramphenicol on fermentation. Fermentation in *M. chthonoplastes* was not impaired when cells were washed and incubated in buffers containing the antibiotic chloramphenicol (0.1 mg/ml). This demonstrated that the switch from photoautotrophic to fermentative metabolism did not require de novo protein synthesis and indicated that all the enzymes involved in fermentation were already present.

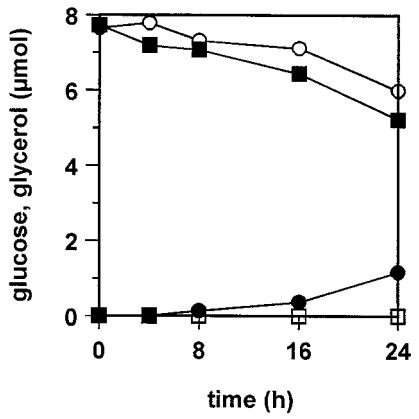

FIG. 2. Degradation of glucosyl-glycerol by *M. chthonoplastes* under dark anoxic conditions. For details, see the legend to Fig. 1. Symbols: ○, glycerol unit of glucosyl-glycerol; ■, glucose unit of glucosyl-glycerol; ●, glycerol released into the medium; □, glucose in the medium.

TABLE 1. Stoichiometry of glycogen degradation, glucosyl-glycerol degradation, and product formation in *M. chthonoplastes* during fermentation[a]

Product	Amt (µmol)
Ethanol	7.4
Acetate	7.1
Formate	5.1
H_2	0.6
CO_2	9.4

[a] Washed cells (10 ml, 1.5 mg/ml of protein) were incubated in a 30-ml serum bottle under an argon atmosphere for 24 h. The cells had been pregrown in batch culture and harvested in the exponential growth phase. The substrates were 4.6 µmol of glucose (from glycogen) and 2.5 µmol of glucose from glucosyl-glycerol. C recovery, 102%; O/R balance, 1.55.

Effect of elemental sulfur on fermentation. Addition of elemental sulfur to suspensions of *M. chthonoplastes* incubated anaerobically in the dark resulted in production of sulfide (Fig. 3). About 70% of the sulfide produced in whole-cell suspensions was found in the supernatant after centrifugation. The remaining part was associated with the cells. Since most of the iron present in cultures of *M. chthonoplastes* is also associated with the cells (26), it is likely that part of the sulfide produced during anaerobic incubations in the dark precipitates as FeS. That this indeed occurred was also evident from the observation that tiny black flocs were often present in the suspension.

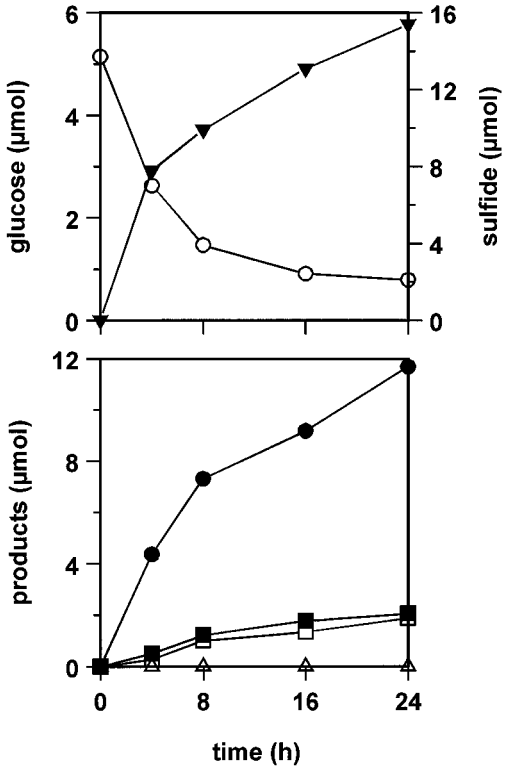

FIG. 3. Fermentation of endogenously stored polyglucose by *M. chthonoplastes* in the presence of elemental sulfur. The cells had been pregrown photoautotrophically in batch culture and harvested during the exponential growth phase. Symbols: ○, polyglucose; ▼, sulfide; ■, ethanol; ●, acetate; □, formate; △, hydrogen. The cell protein concentration was 1.4 mg/ml.

TABLE 2. Stoichiometry of glycogen degradation, glucosyl-glycerol degradation, and product formation in *M. chthonoplastes* during fermentation in the presence of elemental sulfur[a]

Product	Amt (μmol)
Ethanol	2.1
Acetate	11.7
Formate	1.9
H_2	0
CO_2	11.9
Sulfide	15.5

[a] Washed cells (11 ml, 1.4 mg/ml of protein) were incubated in a 30-ml serum bottle under an argon atmosphere for 24 h. The cells had been pregrown in batch culture and harvested in the exponential growth phase. The substrates were 4.4 μmol of glucose (from glycogen) and 2.4 μmol of glucose from glucosyl-glycerol. C recovery, 101%; O/R balance, 1.30.

TABLE 3. Stoichiometry of glycogen degradation and product formation in *M. chthonoplastes* during fermentation[a]

Product	Amt (μmol)
Ethanol	17.6
Acetate	22.9
Formate	25.9
H_2	1.4
Lactate	0.8
CO_2	9.3

[a] Washed cells (10 ml, 3.5 mg/ml of protein) were incubated in a 30-ml serum bottle under an argon atmosphere for 24 h. The cells had been pregrown in batch culture and harvested in the stationary growth phase. The substrate was 20.0 μmol of glucose (from glycogen). C recovery, 99%; O/R balance, 1.22.

The reduction of elemental sulfur to sulfide was accompanied by three marked changes in the fermentation pattern but did not influence the rate of glycogen degradation. These three changes are as follows. (i) A shift in the ratio of ethanol and acetate was observed; production of acetate was increased, whereas production of ethanol decreased. (ii) H_2 was not produced anymore. (iii) Less formate was produced. Also in these cells, which had a low polyglucose content, part of the glucosyl-glycerol was degraded. The glycerol component was released into the medium, whereas the glucose component was used as a substrate during fermentation (carbon recovery, 101%). The O/R balance (1.30) was lower than during comparable incubations without elemental sulfur but was still significantly too high (Table 2). Calculation of the O/R balance included both the free sulfide in the suspension and the cell-associated sulfide.

Fermentation by cells from the stationary growth phase. Cells of *M. chthonoplastes* harvested from the stationary phase of batch cultures had a significantly higher polyglucose content (about 2 μmol of glucose per mg of protein). As a result, survival under dark anoxic conditions could be extended to 3 to 4 days. These cells used only glycogen as the substrate and did not degrade glucosyl-glycerol. The fermentation pattern obtained with these cells differed considerably from that displayed by cells with a low polyglucose content (Fig. 4). Cells with a high polyglucose content produced significantly more acetate than ethanol. In addition, more formate was produced per mole of glucose degraded and some L-lactate was produced as well. Succinate and D-lactate were not formed. Since degradation of glucose via the mixed-acid fermentation pathway without external electron acceptors cannot result in acetate/ethanol ratios higher than 1, it was concluded that part of the acetate was produced via another pathway. The fermentation pattern in Table 3 was interpreted as follows: (i) 17.6 μmol of both acetate and ethanol were derived from 17.6 μmol of glucose via the mixed-acid fermentation pathway; (ii) 0.8 μmol of lactate was derived from 0.4 μmol of glucose; and (iii) the remaining 5.3 μmol of acetate was derived from the 2 μmol of glucose left, i.e., via the homoacetic pathway. Assuming simultaneous operation of the mixed-acid pathway and the homoacetic pathway, we calculated a C recovery of 99% and an O/R balance of 1.22 (Table 3).

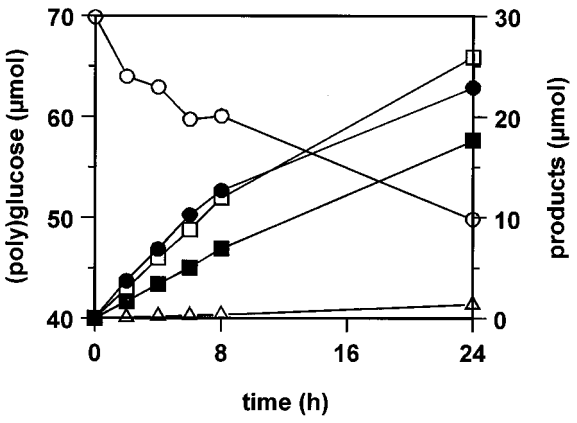

FIG. 4. Fermentation of endogenously stored polyglucose by *M. chthonoplastes* pregrown photoautotrophically in batch culture and harvested during the stationary growth phase. Symbols: ○, polyglucose; ■, ethanol; ●, acetate; □, formate; △, hydrogen. The cell protein concentration was 3.5 mg/ml.

Metabolic pathways during fermentation. The pathway involved in degradation of glucose derived from glycogen or glucosyl-glycerol was examined by assaying enzymes in cell extracts of *M. chthonoplastes*. The results are given in Table 4. Aerobically, glycogen is degraded via the oxidative pentose phosphate pathway. For comparison, the specific activities of the key enzymes of this pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are also given in Table 4.

On the basis of the ratios of the amount of glucose degraded to the amount of acetate and ethanol produced, involvement of either the Embden-Meyerhof-Parnas pathway or the Entner-Doudoroff pathway was expected. The key enzyme of the former pathway, 6-phosphofructokinase, has been detected in several cyanobacteria (15, 16, 23, 25). Initially, we failed to demonstrate its presence in cell extracts of *M. chthonoplastes*. However, a low but significant specific activity of 6-phosphofructokinase was uncovered when its substrate, fructose-6-phosphate, was added to cell suspensions before passage through the French press. Omission of fructose-6-phosphate from the buffer led to a complete loss of activity, which was not restored by its presence in the assay mixture. A stimulator of 6-phosphofructokinase in many eukaryotes is fructose-2,6-bisphosphate, which is produced from fructose-6-phosphate by phosphorylation (4). However, addition of fructose-2,6-bisphosphate to the assay mixture did not restore activity either. Apparently, 6-phosphofructokinase in *M. chthonoplastes* is unstable without its substrate. The key enzymes of the Entner-Doudoroff pathway, 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase, were not detected.

The appearance of formate as a fermentation product suggested the involvement of the enzyme pyruvate formate-lyase in pyruvate degradation. However, the presence of this enzyme could not be demonstrated in cell extracts. Attempts to detect

TABLE 4. Specific activities of enzymes in cell extracts of *M. chthonoplastes*

Enzyme	Cofactor	Sp act (U/mg of protein)
Glucose-6-phosphate dehydrogenase	NADP	0.118
	NAD	0
6-Phosphogluconate dehydrogenase	NADP	0.085
	NAD	0
6-Phosphofructokinase		0.008
Glyceraldehyde-3-phosphate dehydrogenase	NAD	0.162
	NADP	0.012
Pyruvate kinase		0.037
Lactate dehydrogenase	NAD	0.041
	NADP	0
Hydrogenase		0.052
Alcohol dehydrogenase	NAD	0.010
	NADP	0
Acetate kinase		0.076

the enzyme after anaerobic preincubation of cells in the dark also met with failure. The enzymes catalyzing the formation of the end products lactate (lactate dehydrogenase), acetate (acetate kinase), ethanol (alcohol dehydrogenase), and H_2 (hydrogenase) were all found in cell extracts. Alcohol dehydrogenase was strictly NAD dependent. The activity of this enzyme was detected only when dithiothreitol was added to the cell suspension before cell disrupture. Also, the lactate dehydrogenase was strictly NAD dependent. As has been found for some *Lactobacillus* and *Streptococcus* spp. (6), the activity was stimulated by 50% when fructose-1,6-bisphosphate (10 mM) was added to the assay mixture.

Although the specific activities of 6-phosphofructokinase and alcohol dehydrogenase were relatively low, they were still sufficiently high to account for the rates of glucose degradation and product formation in cell suspensions. From Fig. 1 to 4, it can be deduced that glucose degradation occurred at an initial rate of about 0.6 to 0.8 nmol min^{-1} mg of cell $protein^{-1}$. As the cytoplasmic protein constituted approximately 50% of the cell protein, this corresponded to a rate of 1.2 to 1.6 nmol min^{-1} mg of $protein^{-1}$ in cell extracts.

DISCUSSION

In its natural environment, the mat-building cyanobacterium *M. chthonoplastes* is confronted with anoxia at night. The data presented in this paper show that under such conditions, the organism is capable of generating metabolic energy by fermentation of endogenous substrates. This capacity is constitutive, and therefore fermentation commences immediately when the organism is transferred to anoxic dark conditions. Particularly in environments in which anoxic conditions are established within minutes, such metabolic reactivity is of paramount importance for the survival of the organism. Also, in other cyanobacteria, the potential to ferment is constitutive (9, 13, 15, 17, 30).

In cyanobacteria, glycogen serves as the carbon and energy source in the dark. However, in cells of *M. chthonoplastes* that have a low glycogen content, the osmoprotectant glucosylglycerol is also degraded during dark anaerobic incubations. The glucose component is used as an additional substrate for fermentation, whereas the glycerol residue is released into the medium. Degradation of the osmoprotectant has also been observed in *O. limosa*, which accumulates the disaccharide trehalose (9). It is conceivable that inorganic ions such as K^+ and Na^+ may temporarily serve to maintain osmotic equilibrium (18).

Basically, *M. chthonoplastes* degrades glucose derived from glycogen and glucosyl-glycerol according to a mixed-acid fermentation (Fig. 5A). This type of fermentation, which is characterized by the production of substantial amounts of formate, has been studied especially in members of the family *Enterobacteriaceae* (7). Among cyanobacteria, the mixed-acid fermentation is not usual. Only in one other species, *Cyanothece* strain PCC7822, has this type of fermentation been reported to occur (30). As in the family *Enterobacteriaceae*, formation of formate by *M. chthonoplastes* most probably results from cleavage of pyruvate, catalyzed by the enzyme pyruvate formate-lyase, which also yields acetyl coenzyme A (acetyl-CoA). In its active state, this enzyme is extremely sensitive to oxygen, and our failure to detect it in cell extracts may have been due to oxygen inactivation during cell disruption in the French press. Most probably, release of H_2 by *M. chthonoplastes* results from cleavage of formate. Hydrogenase, which is a component of formate hydrogen-lyase, is present in *M. chthonoplastes*. The acetyl-CoA formed by pyruvate formate-lyase may be converted to either ethanol or acetate. The enzymes catalyzing the formation of these products, alcohol dehydrogenase and acetate kinase, respectively, were found in cell extracts of *M. chthonoplastes*. This fermentation requires the formation of equimolar amounts of ethanol and acetate, as is the case in cells with a low glycogen content (Table 1). In addition, 2 mol of formate must be formed. Assuming that part of the formate can be cleaved into H_2 and CO_2, the molar sum of formate and H_2 should equal 2 mol per mol of glucose degraded. Obviously, this was not the case in experiments with *M. chthonoplastes* (Table 1). Possible causes of the deviation from the theoretical ratio are discussed below.

Cells with a high glycogen content exhibited a different fermentation pattern. In addition to the fermentation products excreted by cells with a low glycogen content, some L-lactate was produced. Characteristically, these cells formed approximately 1.1 to 1.2 mol of acetate and about 0.8 to 0.9 mol of ethanol per mol of glucose degraded. Since degradation of glucose via the mixed-acid pathway without external electron acceptors cannot result in such ratios and since no other products such as succinate were found, we concluded that part of the glucose was degraded via the homoacetic fermentation pathway (11). Although the homoacetic fermentation is unusual in this type of organism, it has been reported to occur in two other cyanobacteria (9, 13).

From the fermentation patterns obtained with cell suspensions and the presence of the enzyme 6-phosphofructokinase in cell extracts, we concluded that *M. chthonoplastes* employs the Embden-Meyerhof-Parnas pathway for the degradation of glucose to pyruvate during fermentation (Fig. 5A). The occurrence and significance of this pathway in cyanobacteria have been the subject of controversy for a long time (25). A reasonable level of activity of the key enzyme 6-phosphofructokinase has been found in several species (15, 16, 23, 25), whereas virtually no activity has been detected in others (25). Our data indicate that the enzyme of *M. chthonoplastes* is unstable without its substrate, fructose-6-phosphate; therefore, failure to detect the enzyme in other cyanobacteria may have been due

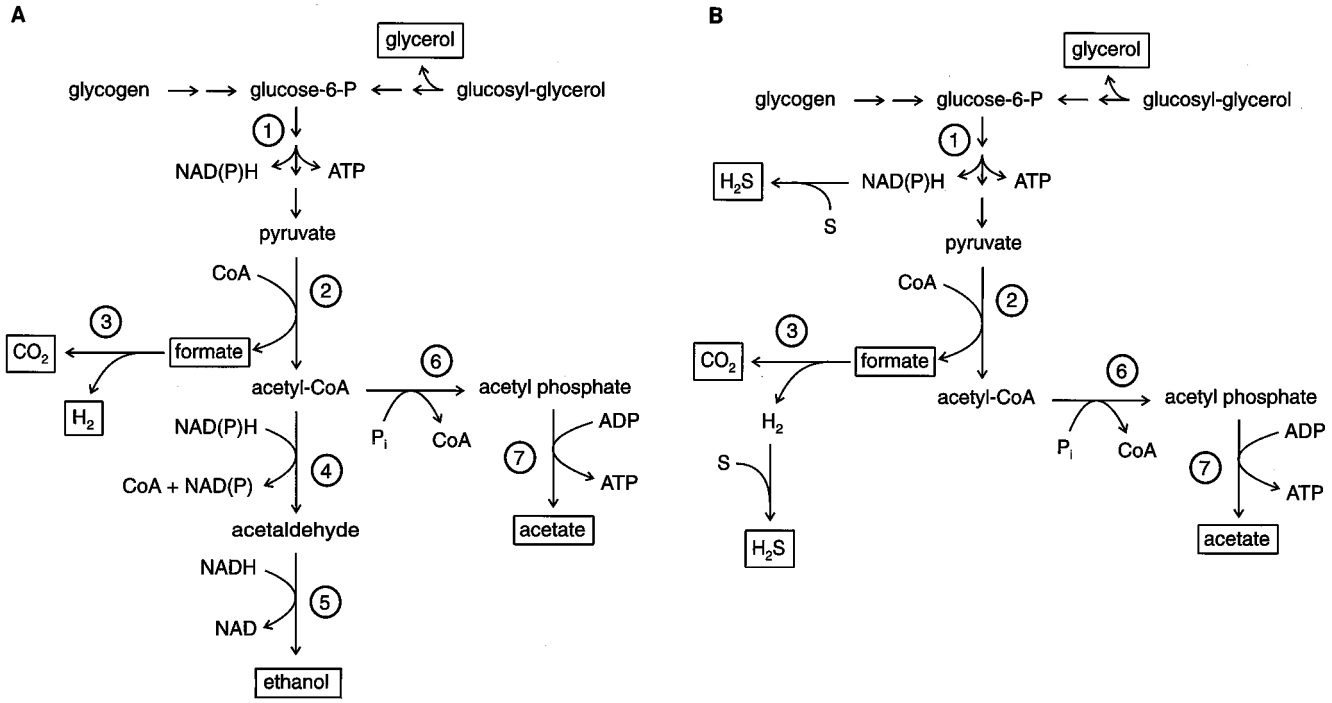

FIG. 5. Proposed pathways of anaerobic energy generation in the dark by *M. chthonoplastes* pregrown photoautotrophically in batch culture and harvested during the exponential growth phase. (A) Fermentation; (B) in the presence of elemental sulfur. Numbers refer to the enzymes involved: 1, enzymes of the Embden-Meyerhof-Parnas pathway; 2, pyruvate formate-lyase; 3, formate hydrogen-lyase; 4, CoA-linked aldehyde dehydrogenase; 5, alcohol dehydrogenase; 6, phosphotransacetylase; 7, acetate kinase.

to the same phenomenon. The presence of the complete glycolytic pathway and its operation during fermentation was demonstrated in the cyanobacterium *Microcystis* strain PCC 7806 (15). Also, in other cyanobacteria capable of fermentation, the involvement of the glycolytic pathway can be deduced from the fermentation pattern (9, 13, 17, 30). Thus, the glycolytic pathway serves an important catabolic function in cyanobacteria for which fermentative metabolism is essential.

M. chthonoplastes is able to use elemental sulfur as the electron acceptor during fermentation. The reduction of elemental sulfur to sulfide can be attributed to two different processes. (i) The first is NAD(P)H-dependent reduction of sulfur. The formation of ethanol serves to regenerate NAD(P), whereas the formation of acetate allows the synthesis of ATP (Fig. 5A). However, when sulfur is present, the formation of ethanol as an electron sink becomes superfluous and consequently more acetate is produced (Fig. 5B). This NAD(P)H-dependent sulfur reduction therefore results in a higher yield of ATP per mol of glucose degraded. (ii) The second process is formate- and/or H_2-dependent reduction of sulfur. The amount of sulfide recovered is larger than can be accounted for by the shift in the ratio of ethanol to acetate. Since less formate and no H_2 are formed in the presence of elemental sulfur, it can be concluded that these compounds can donate electrons to elemental sulfur as well (Fig. 5B). Theoretically, transfer of electrons to sulfur may involve a membrane-associated electron transport chain as in *Wolinella succinogenes* (12). This anaerobic sulfur respiration was reported to occur in *O. limnetica* (17). Sulfur reduction was also found in another cyanobacterium, *O. limosa*, but this did not result in a shift of fermentation products as in *M. chthonoplastes*, except that H_2 evolution ceased (9).

A common feature of the fermentation results obtained with *M. chthonoplastes* is the high O/R balances, indicating an excess of oxidized products. In contrast, the carbon recoveries are satisfactory. From Table 1, it can be concluded that the production of NAD(P)H in the Embden-Meyerhof-Parnas pathway is balanced by the NAD(P)H-consuming formation of ethanol, since 1 mol of ethanol and 1 mol of acetate are formed per mol of glucose degraded. However, when pyruvate is cleaved into formate and acetyl-CoA and formate can be cleaved into H_2 and CO_2, the molar sum of ethanol and acetate should equal the molar sum of formate and hydrogen. From Table 1, it is clear that this is not the case. Thus, the low electron recoveries are caused by low recoveries of formate and hydrogen. On the other hand, the amount of CO_2 produced, calculated as the molar sum of acetate and ethanol minus the molar amount of formate produced, makes an even carbon balance. Therefore, we concluded that either formate or H_2 is oxidized by an external electron acceptor that is associated with the cells, since the incubation buffer contains only Tris and NaCl. We assume that part of the formate formed during fermentation is oxidized to CO_2 by ferric iron (3) associated with the cells (26):

$$HCOO^- + 2Fe^{3+} \rightarrow CO_2 + H^+ + 2Fe^{2+}$$

That iron is associated with the cells is supported by two observations. *M. chthonoplastes* was grown in a medium with elevated ferric ammonium citrate levels, since this resulted in homogeneous cultures (14). However, iron was undetectable in the growth medium. The appearance of tiny black flocs during dark anaerobic incubations of *M. chthonoplastes* in the presence of elemental sulfur suggests the formation of iron sulfide. This is in agreement with the observation that about 30% of the sulfide formed is associated with the cells. The high O/R

balances found after incubations with elemental sulfur may also be explained by oxidation of sulfide by ferric iron (3):

$$S^{2-} + 2Fe^{3+} \rightarrow S^0 + 2Fe^{2+}$$

From the amount of ferric iron added to the medium, it is calculated that the two reactions mentioned above can account for approximately 30 to 50% of the formate or sulfide lacking in Tables 1 to 3.

Microbial mats are composed of physiologically different groups of microorganisms. In addition to cyanobacteria, which are the primary producers of such communities, anoxygenic phototrophic purple sulfur bacteria, colorless sulfur bacteria, and sulfate-reducing bacteria are the main groups. These groups all depend on the presence of cyanobacteria, which supply them with organic matter. Although organic matter also becomes available to the microbial community by processes like photorespiration (1) or cell lysis, fermentation by cyanobacteria may prove the most important. It is evident that a considerable part of the CO_2 fixed daily will be excreted at night during fermentation. The fermentation products of *M. chthonoplastes*, acetate, ethanol, formate, lactate, and H_2, all support the growth of sulfate-reducing bacteria.

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