JOURNAL OF BACTERIOLOGY, May 1993, p. 3043–3050
0021-9193/93/103043-08$02.00/0

Vol. 175, No. 10

Isolation of a Cytochrome-Deficient Mutant Strain of *Sporomusa sphaeroides* Not Capable of Oxidizing Methyl Groups

BEATE KAMLAGE AND MICHAEL BLAUT*

Institut für Mikrobiologie, Georg-August-Universität Göttingen, Grisebachstrasse 8, W-3400 Göttingen, Federal Republic of Germany

Received 17 December 1992/Accepted 4 March 1993

The homoacetogenic anaerobic bacterium *Sporomusa sphaeroides* was mutagenized with UV light. Taking advantage of the ampicillin enrichment technique and a newly developed test for the detection of heme in bacterial colonies, the cytochrome-deficient mutant strain *S. sphaeroides* BK824 was isolated. In contrast to the wild type, this mutant strain failed to grow on betaine, betaine plus methanol, H_2 plus CO_2, and methanol plus CO_2. Growth on betaine plus formate, betaine plus H_2, betaine plus pyruvate, methanol plus H_2 and CO_2, and acetoin was not impaired. All enzymes of the Wood pathway as well as hydrogenase and carbon monoxide dehydrogenase were detectable at comparable activities in both the wild type and the cytochrome-deficient mutant. Labeling experiments with [^{14}C]methanol demonstrated the inability of *S. sphaeroides* BK824 to oxidize methyl groups. The role of cytochromes in electron transport steps associated with the Wood pathway enzymes and their possible role in energy conservation during autotrophic growth in acetogens are discussed.

Sporomusa sphaeroides is an obligately anaerobic, gram-negative, endospore-forming bacterium which converts a number of substrates, such as betaine, methanol plus CO_2, H_2 plus CO_2, pyruvate, lactate, and acetoin mainly to acetate (24). Betaine is either dismutated according to equation 1 or, in the presence of an external electron donor such as formate or hydrogen, reductively split according to equation 2:

$$4 \text{ betaine} \rightarrow 3 \text{ acetate} + 3 \text{ trimethylamine} + N,N\text{-dimethylglycine} + CO_2 \quad (1)$$

$$\text{betaine} + 2e^- + 2H^+ \rightarrow \text{acetate} + \text{trimethylamine} \quad (2)$$

A reductive cleavage of betaine according to equation 2 was previously reported for *Clostridium sporogenes* and *Eubacterium acidaminophilum* (13, 26). The betaine reductase is assumed to function analogously to the glycine reductase system elucidated for *E. acidaminophilum* (5).

After the first report on the presence of cytochromes in *S. sphaeroides* and *Sporomusa ovata* (24), these electron carriers were also detected in other species of this genus (2, 4). A thorough characterization of the cytochromes of *S. sphaeroides* and *S. ovata* indicated the presence of two *b*- and two *c*-type cytochromes (19). Although these electron carriers are not unusual for homoacetogenic bacteria (10), their physiological function is not really understood. In *Clostridium thermoautotrophicum*, membrane-bound *b*-type cytochromes were found to interact with carbon monoxide dehydrogenase (14) or hydrogenase (15). Furthermore, *b*-type cytochromes were thought to be involved in the fumarate reductase reaction as performed by the homoacetogenic bacterium *Clostridium formicoaceticum* (6). In contrast to *S. sphaeroides*, *S. ovata* displays a clear dependence of cytochrome expression on the growth substrate: an 11-fold increase in the cytochrome content was observed after growth on betaine compared with that after growth on betaine plus formate (19), indicating a role of cytochromes during betaine disproportionation.

In order to find a clue to the role of these electron carriers, a cytochrome-deficient mutant strain of *S. sphaeroides* was isolated. Physiological investigations of this mutant demonstrated that the mutation affects the ability of the mutant to oxidize methyl groups.

MATERIALS AND METHODS

Bacterial strain. The experiments were performed with *S. sphaeroides* DSM 2875, which was obtained from the Deutsche Sammlung für Mikroorganismen (Braunschweig, Germany). For maintenance, the organism was grown at 37°C in the standard medium described below containing 50 mM betaine as the energy source.

Chemicals. Enzymes and cofactors, except tetrahydrofolate (H_4F) and NADH, were purchased from Boehringer (Mannheim, Germany). H_4F, dithioerythritol (DTE), heme, and δ-aminolevulinic acid were from Sigma (Deisenhofen, Germany), and NADH was obtained from Biomol (Hamburg, Germany). 3,3′,5,5′-Tetramethylbenzidine was purchased from Serva (Heidelberg, Germany). All other chemicals were of high purity and purchased from commercial sources. Gases were from Messer Griesheim (Kassel, Germany). All radiochemicals except $^{14}CH_3$-H_4F were purchased from New England Nuclear, Dreieich, Germany. $^{14}CH_3$-H_4F was from Amersham (Braunschweig, Germany).

Growth media and cultivation. *S. sphaeroides* was grown at 37°C under N_2 in the medium described previously (19) except that the phosphate concentration was increased to 3.48 g of K_2HPO_4 and 2.27 g of KH_2PO_4 per liter. Betaine, betaine plus formate, and acetoin served as the energy sources and were added to a 50 mM final concentration. The acetoin stock solution was filter sterilized. Solid medium was prepared in an anaerobic chamber (Megaplex, Grenchen,

* Corresponding author.

Switzerland) after addition of 1.5% agar (Oxoid, Basingstoke, England) to the medium and autoclaving.

When CO_2 is explicitly listed as a substrate component, *S. sphaeroides* was grown in bicarbonate-buffered medium (24) under N_2-CO_2 (80:20) or, when H_2 served as the electron donor, under H_2-CO_2 (80:20); the latter gas mixture was pressurized to 1.5 atm (151.9 kPa). Methanol and pyruvate were added from concentrated stock solutions to a final concentration of 50 mM. The pyruvate solution was filter sterilized. Autotrophic cultures were shaken at 30°C. For cultivation of the mutant strain on methanol plus H_2 and CO_2, the bicarbonate concentration was increased to 3 g/liter. Where indicated, heme and δ-aminolevulinic acid were added from stock solutions to the growth medium to give final concentrations of 4 μM and 1 mM, respectively. The stock solutions were prepared as follows. Hemin was dissolved in 10 mM KOH containing 50% methanol and filter sterilized; δ-aminolevulinic acid was neutralized, made anaerobic, and filter sterilized before addition to the medium.

The cultivation was performed under strictly anaerobic conditions. For liquid medium, rubber-stoppered bottles or tubes were used and additions were made with sterile syringes. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) in a Bausch & Lomb photometer with medium alone as a blank. Agar plates were inoculated in an anaerobic chamber, subsequently transferred to an anaerobic jar (Oxoid, Wesel, Germany) with N_2-H_2 (95:5) as the gas phase, and incubated at 37°C.

Isolation of cytochrome-deficient mutant strain *S. sphaeroides* BK824 after UV mutagenesis. All media used in these experiments were additionally supplied with 8 mg of vitamin B_{12} (Serva, Heidelberg, Germany) per liter. Ten milliliters of a betaine-grown culture of *S. sphaeroides* (OD_{600} of 0.3) was centrifuged at 4,000 × *g*, and the cells were suspended in 15 ml of sterile 50 mM potassium phosphate buffer (pH 7.0) containing 3% NaCl and 0.5 g of L-cysteine hydrochloride per liter. The cell suspension was transferred to an anaerobic chamber, and 10 ml was poured into a sterile glass petri dish. An aliquot of 0.1 ml was diluted and plated onto plates containing betaine plus formate in order to determine the number of viable cells. The cell suspension in the petri dish was irradiated with short-wavelength UV light (254 nm) for 20 s with a prewarmed UV lamp. Irradiation and all following steps were performed in dim light. A portion (0.1 ml) of the irradiated cell suspension was serially diluted and spread onto betaine-formate plates in order to estimate the rate of survival, which was 0.6%. A survival rate of 0.1 to 1% has been recommended for UV mutagenesis (3).

Five tubes, each containing 5 ml of betaine-formate medium, were inoculated with 0.5 ml of the irradiated cell suspension. After 40 h of incubation, the optical density was about 0.9. This incubation under conditions not requiring cytochromes served to dilute out the residual cytochromes in the mutants in order to prevent them from starting to grow during the following ampicillin enrichment step. The cultures were centrifuged at 4,000 × *g*, and the cells were washed once in 10 ml of the above-mentioned sterile buffer. Washed cells were suspended in 5 ml of buffer, 3 ml of which served as an inoculum for 10 ml of betaine medium for the mutant enrichment. The tubes were incubated until the optical density had doubled. Subsequently, 0.2 ml of sterile anaerobic ampicillin (15 mg/ml) was added to each tube. After 12 h of shaking, the cultures had cleared up, whereas an ampicillin-free control reached an optical density of 1.4. The cultures were centrifuged, and the pellets were washed twice in the aforementioned buffer to remove the ampicillin. The pellet was suspended in 3 ml of betaine-formate medium, and 0.2-ml aliquots were spread onto betaine-formate agar plates. After 7 days of incubation, colonies were picked. Each colony picked was transferred in parallel to a sterile nylon membrane (Biodyne A; 0.2 μm; Pall, Dreieich, Germany), which was placed on a betaine-formate agar plate and on a betaine-formate agar plate. When new colonies had appeared, the filters were tested for heme as described below. Four of 2,000 colonies picked stained heme negative. Since all of them originated from the same enrichment culture, they are assumed to be the same clone. Colonies staining negative for heme were picked and transferred to liquid betaine-formate medium. After growth had occurred, the stability of the mutation and purity of the clone were checked again by plating and staining for heme as described above.

Heme staining of colonies. Colonies grown on nylon transfer membranes as described above were lysed by incubating the filters in 1% sodium dodecyl sulfate (SDS)–0.2% NaOH for 5 min at room temperature with gentle shaking. The filters were subsequently rinsed with water for a few seconds and incubated in the dark for 1 to 2 h at room temperature in a mixture of seven parts 0.25 M sodium acetate (pH 5.0) and three parts of a freshly prepared solution of 6.3 mM 3,3′,5,5′-tetramethylbenzidine in methanol (31). The reaction was started by adding 0.1% (vol/vol) 30% H_2O_2. Heme-containing colonies stained turquoise blue within a few seconds, while heme-deficient colonies remained white. The reaction was stopped by rinsing the filters with water.

Redox difference spectroscopy and enzyme assays. Washed membrane fractions were prepared under aerobic conditions as described before (19). The redox difference spectra of washed membranes were recorded at room temperature by reducing a sample with a few grains of $Na_2S_2O_4$ and measuring it against an air-oxidized sample with a Perkin-Elmer/Hitachi spectrophotometer (model 556) in the double-beam mode (18). The spectral bandwidth was set to 1 nm, and the scanning speed was 30 nm/min.

Cell extracts for the determination of enzyme activities were prepared under strictly anaerobic conditions and assayed as described before (19). Hydrogenase activity was assayed under H_2 in 1.6-ml stoppered glass cuvettes filled with 0.8 ml of 50 mM anaerobic potassium phosphate buffer (pH 7.0) containing 5 mM DTE; 1 μl of 1 M benzyl viologen, 10 μl of 15 mM NAD, or 10 μl of 15 mM NADP was added as the electron acceptor. The reaction was started with 1 to 2 μl of crude extract, and the reaction was monitored at 578 and 365 nm, respectively. The extinction coefficients used for calculating the activity were $\varepsilon_{578} = 8.6\ mM^{-1}\ cm^{-1}$ for benzyl viologen and $\varepsilon_{365} = 3.4\ mM^{-1}\ cm^{-1}$ for NAD(P)H. When H_2 was replaced by N_2 as a control, no reaction occurred. CO dehydrogenase was assayed in the same way as hydrogenase except that H_2 was replaced by CO and only benzyl viologen served as the electron acceptor.

Acetogenesis from CH_3-H_4F by crude extracts was assayed by the method of Drake et al. (7). Crude extracts were prepared under strictly anaerobic conditions in 50 mM potassium phosphate buffer, pH 6.5, containing 5 mM $MgCl_2$, 10 mM DTE, and 1 mg of resazurin per liter. The test was performed in 4.7-ml rubber-stoppered tubes under H_2-CO (75:25) containing 250 μl of crude extract, 2 mM ATP, and 0.2 mM coenzyme A (CoA). The tubes were incubated at 30°C for 1 min, and the reaction was started by the addition of $^{14}CH_3$-H_4F to a final concentration of 2 mM, with a specific radioactivity of 136.9 kBq/μmol. Samples (25

μl) were withdrawn at 1-min intervals and injected into 250 μl of the aforementioned buffer without DTE but with 10 mM sodium acetate as a carrier. After addition of 125 μl of 2.2 M $HClO_4$ and then of 300 μl of 1 M KOH to bring the pH to about 3, the samples were incubated at 0°C for 15 min and centrifuged. The supernatants were loaded onto 300 μl of cation exchanger (Dowex 50 WX8, 200-400 mesh, H^+ form, equilibrated with water). Acetate was eluted by washing the columns three times with 0.5 ml of water. $^{14}CH_3$-H_4F bound tightly to the cation exchanger at pH 3 and was eluted by washing the columns three times with 0.5 ml of 1 M KOH. The acetate fraction was alkalinized by addition of 0.25 ml of 1 M KOH. Radioactivity was determined by scintillation counting as described below.

Labeling experiments. Growth experiments with [^{14}C]methanol were performed in 16-ml rubber-stoppered Hungate tubes containing 5 ml of medium under H_2-CO_2 (80:20) pressurized to 2 atm (203 kPa) or under N_2-CO_2 (80:20) (101 kPa). Since phosphate disturbs the quantification of $^{14}CO_2$ (see below), the potassium phosphate concentration was decreased to 4 mM. [^{14}C]methanol (specific radioactivity, 160.6 Bq/μmol) was added to a final concentration of 46 mM. Immediately after inoculation, a sample (1 ml) was removed and mixed with 0.2 ml of stop mix (0.2 M NaOH, 0.1 M sodium acetate, 0.4 M Na_2CO_3). Cells were removed by centrifugation, and the supernatant was stored at −20°C. The cultures were shaken at 30°C in a water bath. When growth was complete, 0.8 ml of stop mix was added by syringe, resulting in a pH of about 10. The tubes were shaken vigorously in order to absorb the CO_2 from the gas phase as carbonate. Cells were pelleted by centrifugation, and the supernatants from the beginning and the end of the growth experiments were tested for [^{14}C]methanol, [^{14}C]acetate, and $^{14}CO_2$.

For the determination of the total radioactivity added (100%), 0.3 ml of each sample was mixed with 5 ml of scintillation fluid (Opti-Fluor; Canberra Packard, Frankfurt/Main, Germany), and the radioactivity was determined in a scintillation counter (Tri-Carb; Canberra Packard). Another 0.3-ml aliquot of each sample was mixed with 0.6 ml of 0.15 M $Ba(OH)_2$ solution to precipitate $^{14}CO_2$ as $Ba^{14}CO_3$. After centrifugation, the supernatants were carefully removed, and the pellets were resuspended in 1 ml of 0.1 M $BaCl_2$. Centrifugation and removal of the supernatants were repeated, and the $Ba^{14}CO_3$ pellets were resuspended in 0.2 ml of 0.1 M $BaCl_2$. Radioactivity was determined by scintillation counting. To verify that the material precipitated with Ba^{2+} was $BaCO_3$, it was resuspended in water and placed in a rubber-stoppered tube. Subsequently, 2 M HCl was added by syringe until the precipitate was dissolved (about 0.5 ml). The released CO_2 was carried by a stream of air via rubber tubing to a vial containing 4 ml of Carbo-Sorb (Packard, Frankfurt, Germany). By this method, 98% of the radioactivity was recovered in the Carbo-Sorb fraction by scintillation counting, demonstrating that the material precipitated with Ba^{2+} was actually $^{14}CO_3^{2-}$.

The supernatants from the first centrifugation step were loaded onto Dowex anion-exchange columns equilibrated with 2 M NaOH and then washed with water until the pH of the eluent was <9 (Dowex 1×8, 200-400 mesh; bed volume, 0.3 ml; Serva). The columns were washed three times with 0.5 ml of distilled water. The radioactive compound which eluted in these fractions was assumed to be [^{14}C]methanol because the corresponding standard eluted in the same fraction. The radioactivity was quantified by scintillation counting. [^{14}C]acetate bound tightly to Dowex type 1 and was eluted by washing three times with 0.3 ml of 0.6 M KI. The pooled KI fractions were mixed with 1 ml of distilled water, and the radioactivity was determined by scintillation counting. The efficiency of this separation method was determined by adding known amounts of $NaH^{14}CO_3$, [^{14}C]methanol, and [^{14}C]acetate (alone and mixed) to the culture medium and processing as described above. The recovery of radioactivity was in the range of 94 to 98%.

Identification of Dowex-binding radioactivity as acetate. Dowex-1-binding radioactivity was identified as [^{14}C]acetate by its enzymatic conversion to [^{14}C]acetylchloramphenicol via [^{14}C]acetylphosphate and [^{14}C]acetyl-CoA by the combined action of acetate kinase, phosphotransacetylase, and chloramphenicol acetyltransferase. An ATP-generating system was established by addition of phosphoenolpyruvate and pyruvate kinase. [^{14}C]acetylchloramphenicol was specifically separated from [^{14}C]acetate and [^{14}C]acetyl-CoA by extraction with ethyl acetate (29). A 1-ml portion of the culture supernatant and a [^{14}C]acetate standard solution were separated over Dowex as described above. The acetate in the KI-eluted fractions was converted to acetylchloramphenicol as described before (29). The reaction mixture contained, in a total volume of 0.5 ml, 100 mM Tris-HCl (pH 7.8), 6 mM $MgCl_2$, 3 mM ATP, 20 mM phosphoenolpyruvate, 2.6 mM CoA, 4.5 mM chloramphenicol, 10 U of acetate kinase, 20 U of pyruvate kinase, 10 U of phosphotransacetylase, 30 U of chloramphenicol acetyltransferase, and 2.4 to 3 mM [^{14}C]acetate (about 12,000 dpm). A negative control received H_2O instead of chloramphenicol acetyltransferase. After 11 h of incubation at 25°C, acetylchloramphenicol was extracted with 0.7 ml of ethyl acetate by vortexing for 1 min followed by centrifugation for phase separation. The extraction was repeated four times, resulting in recovery of 70 to 80% of the total radioactivity in the pooled ethyl acetate extracts. In the absence of chloramphenicol acetyltransferase, only 0.8% of the radioactivity was detectable in the ethyl acetate fraction. The pooled ethyl acetate fractions were dried in an air stream, taken up in 40 μl of ethyl acetate, and spotted onto a silica gel thin-layer sheet (Merck, Darmstadt, Germany). The chromatogram was developed in ascending fashion with water-saturated ethyl acetate, dried, and subjected to autoradiography. As a result, all spots appearing could be identified by their R_f values (28) as 1-acetoxy chloramphenicol, 3-acetoxy chloramphenicol, or 1,3-diacetoxy chloramphenicol, demonstrating that the fractions eluted from Dowex contained [^{14}C]acetate.

SDS-PAGE. Extracts for SDS gel electrophoresis were prepared as described before (19) except that aerobic conditions were employed and 0.04 M Tris-HCl buffer (pH 7.1) was used instead of potassium phosphate buffer. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described before (20) with 12% (wt/vol) polyacrylamide gels (7 by 15 by 0.1 cm) which were run at 25 mA for about 1 h. Standard proteins of low molecular weight were from Pharmacia (Freiburg, Germany). Proteins were stained with Coomassie brilliant blue (32). Proteins to be stained for heme after separation by SDS-PAGE were prepared and stained as described before (31) except that the SDS concentration in the sample preparation buffer was increased to 8%.

Determination of acetate and protein. Acetate content was determined enzymatically by coupling acetyl-CoA formation by acetyl-CoA synthetase via myokinase and pyruvate kinase with the NADH-dependent reduction of pyruvate to lactate (6). Protein was determined by the method of Lowry et al. (22). Bovine serum albumin served as a standard.

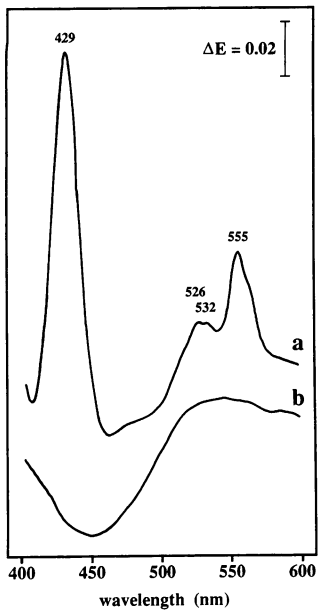

FIG. 1. $Na_2S_2O_4$ reduced minus O_2 oxidized difference spectra of the washed membrane fractions from two strains of *S. sphaeroides* after growth on betaine plus formate. Wild-type (a) and BK824 (b), each contained 10.8 mg of protein per ml in 50 mM potassium phosphate buffer (pH 7.0). The peaks at 555 nm (α-band), 532 and 526 nm (β-band), and 429 nm (γ-band) in spectrum a demonstrate the presence of cytochromes.

RESULTS AND DISCUSSION

Isolation of a cytochrome-deficient mutant of *S. sphaeroides*. In order to obtain a clue about the role of cytochromes in the metabolism of *S. sphaeroides*, the organism was mutagenized with UV light, and cytochrome-deficient mutants were isolated. It was previously found that the cytochrome content of *S. ovata* is 11 times higher during growth on betaine than during growth on betaine plus formate, suggesting a role of these electron carriers in the disproportionation of betaine (19). Mutant enrichment was based on the assumption that cytochromes are needed for the conversion of betaine alone but not for the conversion of betaine in the presence of formate. Therefore, *S. sphaeroides* cells mutagenized with UV light were first grown on betaine plus formate and subsequently on betaine in the presence of ampicillin to select for mutants not capable of growth on betaine alone. Mutants were detected by testing for the presence of heme after plating the mutagenized cells on agar plates with betaine plus formate as the substrate. For this purpose, a new test was developed which allowed the detection of heme-free mutant colonies on nylon filters by applying a heme stain. (Details are given in Materials and Methods.) This procedure led to isolation of the *S. sphaeroides* mutant BK824, which was used for all further experiments.

Characteristics of the mutant strain. Membranes isolated from the mutant strain were tested for the presence of cytochromes by redox difference spectroscopy. The spectrum in Fig. 1 shows that the mutant membranes were devoid of cytochromes. This is in agreement with the negative heme stain behavior of the mutant. SDS gels of crude extracts and membrane proteins from the wild type and the mutant showed the same protein pattern, but only the membrane proteins of the wild type displayed bands when stained for the presence of heme (not shown).

TABLE 1. Growth behavior of the cytochrome-deficient mutant strain *S. sphaeroides* BK824 and the wild-type strain[a]

Substrate(s)	Final OD_{600}	
	Wild type	BK824
Betaine + formate[b]	1.3–1.6	1.3–1.6
Betaine	1.3–1.6	0.07–0.13
Acetoin	0.4–0.5	0.4–0.5
Betaine + methanol	1.3–1.5	0.12
Methanol + CO_2	1.5	0.12
Betaine + H_2 + CO_2	1.1	1.4
H_2 + CO_2	0.6	0
Betaine + pyruvate	1.5	1.5
Methanol + H_2 + CO_2	ND[c]	0.8

[a] Both strains were grown first on betaine plus formate and then transferred to fresh medium containing the substrate(s) listed. Growth was monitored photometrically at 600 nm, and the final optical density is given. Uninoculated medium served as a blank. Without substrate, the final OD_{600} was 0.
[b] Formate in the absence of betaine is a very poor growth substrate (24).
[c] ND, not determined.

From these results, it was assumed that the mutant strain was not capable of synthesizing heme. In contrast to this idea, addition of heme or δ-aminolevulinic acid to the growth medium did not result in reversion of the observed phenotypical defects of the mutant. However, this finding does not exclude a defect in heme synthesis, since it has to be kept in mind that *Escherichia coli* or *Salmonella typhimurium* mutants defective in heme synthesis are unable to take up heme from the medium unless secondary mutations are introduced that allow heme to enter the cells (17, 23).

It was now of interest to find out which catabolic functions were affected by this mutation. Therefore, as a first approach, the ability of the mutant strain to grow on a number of substrates was compared with that of the wild type. Table 1 shows that growth on betaine was impaired in the mutant but not in the wild type, whereas betaine utilization in the presence of formate was equally good in the wild-type and the mutant strain. No or only little growth of the mutant was observed on H_2 plus CO_2, betaine plus methanol, or methanol plus CO_2. In contrast, growth of the mutant on acetoin, betaine plus H_2 and CO_2, betaine plus pyruvate, or methanol plus H_2 and CO_2 could not be distinguished from that of the wild type. These results suggest that the mutation does not affect betaine reduction itself but methyl group oxidation. This idea was substantiated by the finding that H_2, formate, and pyruvate could serve as electron donors for the reductive betaine cleavage to trimethylamine and acetate (Fig. 2), whereas methanol or betaine could not.

From the recent demonstration of the Wood pathway enzymes in *S. sphaeroides* (19), it is reasonable to assume that oxidation of methanol and betaine involves the formation of methyl-H_4F and its subsequent oxidation to CO_2 (Fig. 2) (21, 27). That the mutation affects a reaction associated with this pathway is supported by the inability of the mutant to grow on H_2 plus CO_2. The absolute dependence on methanol for growth on H_2 plus CO_2 points to the inability of the mutant to form the methyl moiety of acetate from CO_2; therefore, when an external methyl group donor such as methanol was supplied, acetate was formed. Acetogenesis from betaine or H_2 plus CO_2 involves reactions of the Wood pathway, although in opposite directions (Fig. 2). From the ability of the mutant to grow on formate plus betaine or H_2

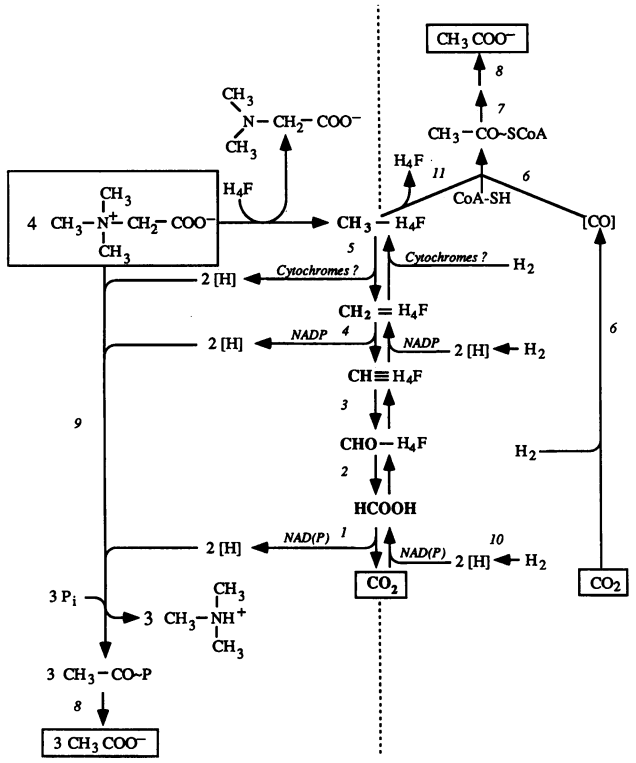

FIG. 2. Scheme depicting the central role of the Wood pathway for the dismutation of betaine (left) and acetogenesis from H_2 plus CO_2 (right) as performed by *S. sphaeroides*. Boldface letters indicate the C_1 moieties of the Wood pathway intermediates. *1*, Formate dehydrogenase; *2*, formyl-H_4F synthetase (ATP dependent); *3*, methenyl-H_4F cyclohydrolase; *4*, methylene-H_4F dehydrogenase; *5*, methylene-H_4F reductase; *6*, CO dehydrogenase; *7*, phosphotransacetylase; *8*, acetate kinase; *9*, betaine reductase; *10*, hydrogenase; *11*, methyltransferase and corrinoid-FeS protein. CoA-SH, coenzyme A; CH_3–Co~SCoA, acetyl coenzyme A.

plus betaine, it must be concluded that the oxidation of neither formate nor H_2 is affected by the mutation.

To further substantiate these conclusions, wild-type and mutant cells were grown on [^{14}C]methanol under N_2-CO_2 (80:20) or under H_2-CO_2 (80:20), and the fate of the label was determined. It is evident from experiments 1 to 4 in Table 2 that 73 to 99% of the label was converted to $^{14}CO_2$ and [^{14}C]acetate, with a [^{14}C]acetate/$^{14}CO_2$ ratio of 1.3 to 1.4. It can be assumed that the majority of the methanol was converted directly into the methyl moiety of acetate by means of a specific methyl group transfer (30). Since methanol has to be oxidized to CO_2 in order to gain reducing power for the generation of the carboxyl group of acetate, a small portion of the label was probably also incorporated into the carboxyl group of acetate. With H_2 as the electron donor (Table 2, experiments 5 to 8), much less methanol was oxidized to CO_2. Only 55 to 71% of the methanol was converted, 46 to 52% being incorporated into acetate and 4 to 9% being oxidized to CO_2.

The situation encountered in growing cultures of mutant strain BK824 was completely different. A negligible proportion (<1%) of the label was recovered in the $^{14}CO_2$ fraction under both N_2-CO_2 (Table 2, experiments 9 to 12) and H_2-CO_2 (Table 2, experiments 13 to 15). This shows that the mutant cells were not capable of oxidizing methanol to CO_2 and is in agreement with the very little growth on methanol plus CO_2 (Table 1) and with the observation that the majority of the added methanol was not converted, the rest being converted almost exclusively to acetate (Table 2, experiments 9 to 12).

That some [^{14}C]acetate could be formed may be explained as follows. Since methanol was obviously not oxidized, it has to be concluded that it is converted into the methyl group of acetate and that the reducing equivalents for the reduction of CO_2 to the carboxyl group of acetate were derived either from some storage material or from the yeast extract and casamino acids present in the growth medium. Under H_2 plus CO_2, reducing equivalents for the reduction of CO_2 to the carboxyl group of acetate can be generated from H_2 (see Fig. 2) (Table 2, experiments 13 and 14). This agrees with the finding that *S. sphaeroides* BK824 is not able to grow on either methanol or H_2 plus CO_2 but does grow on the combination of these substrates (Table 1). Interestingly, in experiments performed with resting cells, free CO could substitute for H_2 plus CO_2 when methanol was present (data not shown). In conclusion, these experiments show that methanol can serve as a methyl group donor in acetate synthesis but not as an electron donor in the mutant.

The ability of the cytochrome-deficient mutant to form acetate from H_2 plus CO_2 and methanol implies that cytochromes are not required for the reduction of CO_2 to the carboxyl carbon of acetate, as catalyzed by CO dehydrogenase. This is in contrast to experiments performed with vesicles of *Clostridium thermoautotrophicum*, which suggest the involvement of *b*-type cytochromes in the reduction of CO_2 to CO and vice versa (14).

Enzyme activities in the mutant. From these results, only the following reactions could theoretically be affected by the mutation: formyl-H_4F synthetase, methenyl-H_4F cyclohydrolase, methylene-H_4F dehydrogenase, methylene-H_4F reductase, methyltransferase, or an electron transfer reaction connected to these reactions.

Therefore, the activity of the enzymes of the acetyl-CoA pathway and of enzymes associated with this pathway were tested in crude extracts of both the wild type and the mutant strain. It is evident from Table 3 that there was no marked difference in the activity of the Wood pathway enzymes between the wild-type and the mutant strains of *S. sphaeroides*. The differences encountered are normal variations in the measurements; they cannot explain the inability of the mutant to grow on certain substrates. Acetogenesis from CH_3-H_4F and CO by crude extracts occurred only at low specific activities (Table 3). However, since low in vitro rates of acetate formation are a general phenomenon observed in crude extracts (8), the observed activities are considered significant.

From these data, formyl-H_4F synthetase, methenyl-H_4F cyclohydrolase, methylene-H_4F dehydrogenase (Fig. 2), and the synthesis of acetate from CH_3-H_4F and CO are considered to be cytochrome-independent activities, since these activities with physiological substrates were detectable in comparable amounts in both the wild type and the cytochrome-deficient mutant.

Furthermore, our results allow conclusions about the hydrogen metabolism of *S. sphaeroides*. Acetogenesis from H_2 plus CO_2 necessitates an electron transfer from H_2 to NAD(P) as well as to methylene-H_4F to generate the methyl moiety of acetate. In addition, CO_2 has to be reduced to the carboxyl group of acetate (Fig. 2). However, information on the enzymes involved in these electron transfer reactions is very limited. In case of H_2-dependent NAD(P) reduction,

TABLE 2. Conversion of [^{14}C]methanol by growing cultures of wild-type and mutant *S. sphaeroides*[a]

Strain and substrate(s)	Expt no.	% of total radioactivity recovered as:			% of $^{14}CH_3OH$ converted[b]	Recovery[c] (%)	Ratio, [^{14}C]acetate/$^{14}CO_2$
		Unconverted $^{14}CH_3OH$	[^{14}C]acetate	$^{14}CO_2$			
Wild type							
$^{14}CH_3OH + CO_2$	1	0.5	53.1	40.2	99.5	93.8	1.32
	2	0.4	50.9	39.2	99.6	90.5	1.30
	3	25.2	39.8	28.0	74.8	93.0	1.42
	4	23.7	36.7	26.8	76.3	87.2	1.37
$^{14}CH_3OH + H_2 + CO_2$	5	29.9	46.4	7.4	70.1	83.7	6.27
	6	25.0	51.7	9.0	75.0	85.7	5.74
	7	42.9	47.1	4.1	57.1	94.1	11.5
	8	42.5	46.3	4.1	57.5	92.9	11.3
Mutant BK824							
$^{14}CH_3OH + CO_2$	9	82.5	8.3	0	17.5	90.8	∞
	10	85.4	8.6	0	14.6	94.0	∞
	11	75.7	15.2	0.1	24.3	91.0	152
	12	77.1	15.6	0.1	22.9	92.8	156
$^{14}CH_3OH + H_2 + CO_2$	13	5.4	88.4	0.7	94.6	94.5	126
	14	0.9	93.0	0.7	99.1	94.6	133
	15	0.7	93.8	0.7	99.3	95.2	134
	16	0.7	90.4	0.6	99.3	91.7	151

[a] Immediately after inoculation, radioactivity was found exclusively in the methanol fraction (data not shown). At the end of the experiments, the radioactivity in an aliquot of alkalized cell culture supernatant was arbitrarily set to 100%, and the radioactivity recovered in the various fractions is given as the percentage of that value.
[b] Calculated from $^{14}CH_3OH$ added (100%) minus $^{14}CH_3OH$ unconverted.
[c] Sum of [^{14}C]acetate, $^{14}CO_2$, and $^{14}CH_3OH$ unconverted.

the information available suggests the involvement of a ferredoxin-reactive hydrogenase, ferredoxin, and ferredoxin:NAD(P) oxidoreductase in acetogenic bacteria (21). Since extracts of both the wild-type and mutant strains of *S. sphaeroides* catalyze the H_2-dependent reduction of NAD(P) (Table 3), it has to be concluded that this electron transfer is not affected by the mutation and therefore does not involve cytochromes. This is in contrast to *C. thermoautotrophicum*, in which *b*-type cytochromes have been shown to act as primary electron acceptors of a periplasmic hydrogenase (15). The reduction of CO_2 by H_2 to CO is likewise not affected by the mutation, as clearly shown by the growth experiments with [^{14}C]methanol (Table 2).

Nothing is known about the physiological electron donor

TABLE 3. Comparison of various enzyme activities in cell extracts of *S. sphaeroides* wild type and BK824 after growth on betaine plus formate

Enzyme activity	Electron acceptor or substrate	Sp act (μmol min^{-1} [mg of protein]$^{-1}$)	
		Wild type	BK 824
CO dehydrogenase	Benzyl viologen	1.0	2.0
Hydrogenase	Benzyl viologen	15.6	15.0
	NAD	0.09	0.12
	NADP	0.21	0.96
Formate dehydrogenase	Benzyl viologen	0.39	0.61
	NAD	0.02	0.02
	NADP	0.11	0.10
Formyl-H_4F synthetase		0.44	0.15
Methenyl-H_4F cyclohydrolase		0.10	0.13
Methylene-H_4F dehydrogenase	NAD	0	0
	NADP	1.47	1.22
Methylene-H_4F reductase	Benzyl viologen	0.76	0.70
	NAD	0	0
	NADP	0	0
Acetogenesis	CH_3-H_4F + CO	0.0016	0.001

in the methylene-H_4F reductase reaction during acetogenesis from H_2 plus CO_2 in *S. sphaeroides* and in other acetogenic bacteria, the only exception being *Peptostreptococcus productus*, in which NADH was shown to be the physiological electron donor (33). The methylene-H_4F reductase has been proposed to be involved in energy generation in acetogens (8). The membrane location of this enzyme in *C. thermoautotrophicum* and in *P. productus* is in favor of this suggestion (14, 33). Since the extracts of *S. sphaeroides* were prepared by French pressing, it is not surprising that the majority of the methylene-H_4F reductase was recovered from the soluble fraction (data not shown); this is also observed in *C. thermoautotrophicum* and in *P. productus*. It is most noteworthy that the benzyl viologen-dependent methylene-H_4F reductase activity was present in both the wild type and the cytochrome-deficient mutant of *S. sphaeroides*. Therefore, the enzyme itself cannot be affected by the cytochrome deficiency. However, it is conceivable that cytochromes serve as primary physiological electron donors in this reaction. This hypothesis is in very good agreement with the observed features of the cytochrome-deficient mutant strain. Future experiments will have to show whether this is really the case.

It is not presently understood how acetogenic bacteria gain net ATP during acetogenesis from H_2 plus CO_2 (8). However, there seems to be a general agreement that net ATP synthesis needs the generation of a transmembrane gradient of protons or sodium ions (8, 15, 25). The reaction of the acetogenic pathway coupled to the extrusion of Na^+ has not yet been identified, but experiments performed with *Acetobacterium woodii* and *P. productus* indicate that either methylene-H_4F reduction or the formation of the methyl group of acetyl-CoA from methyl-H_4F has to be involved (9, 11). The methyl group transfer from methyltetrahydromethanopterin to coenzyme M was recently shown in *Methanosarcina* strain Göl to be involved in the generation of a transmembrane sodium ion potential (1). It is conceivable that an analogous reaction exists in acetogenic bacteria that depend on Na^+ for growth and acetogenesis from H_2 plus CO_2.

Na^+ dependence has been demonstrated for *A. woodii*, *P. productus*, and *Acetogenium kivui* (9, 11, 34), and it seems possible that these organisms generate a primary Na^+ gradient which is taken advantage of for ATP synthesis via an Na^+-dependent ATP synthase. In agreement with this notion, Heise et al. reported the presence of an Na^+-translocating ATPase in *A. woodii* (12). Autotrophic growth of a second group of acetogenic bacteria (e.g., *C. thermoaceticum* and *Sporomusa* species) seems to be independent of Na^+ (34; unpublished results) and to require cytochromes for autotrophic growth (16; this study). Cytochromes were suggested to participate in electron transport from various electron donors to methylene-H_4F in *C. thermoautotrophicum* (16). Our study clearly shows that cytochromes are absolutely required in *S. sphaeroides* for autotrophic growth and methyl group oxidation. The cytochrome-deficient mutant described here will give us new insights into the role of these electron carriers in this group of acetogenic bacteria.

ACKNOWLEDGMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

We are indebted to G. Gottschalk for generous support. We thank Lynne Rogers-Blaut for proofreading.

REFERENCES

1. **Becher, B., V. Müller, and G. Gottschalk.** 1992. N^5-Methyltetrahydromethanopterin:coenzyme M methyltransferase of *Methanosarcina* strain Göl is an Na^+-translocating membrane protein. J. Bacteriol. **174:**7656–7660.
2. **Breznak, J. A., J. M. Switzer, and H.-J. Seitz.** 1988. *Sporomusa termitida* sp. nov., an H_2/CO_2-utilizing acetogen isolated from termites. Arch. Microbiol. **150:**282–288.
3. **Carlton, B. C., and B. J. Brown.** 1981. Gene mutation, p. 222–242. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general microbiology. American Society for Microbiology, Washington, D.C.
4. **Dehning, I., M. Stieb, and B. Schink.** 1989. *Sporomusa malonica* sp. nov., a homoacetogenic bacterium growing by decarboxylation of malonate or succinate. Arch. Microbiol. **151:**421–426.
5. **Dietrichs, D., M. Meyer, M. Rieth, and J. R. Andreesen.** 1991. Interaction of selenoprotein P_A and the thioredoxin system, components of the NADPH-dependent reduction of glycine in *Eubacterium acidaminophilum* and *Clostridium litoralis*. J. Bacteriol. **173:**5983–5991.
6. **Dorn, M., J. R. Andreesen, and G. Gottschalk.** 1978. Fermentation of fumarate and L-malate by *Clostridium formicoaceticum*. J. Bacteriol. **133:**26–32.
7. **Drake, H. L., S.-I. Hu, and H. G. Wood.** 1981. Purification of five components from *Clostridium thermoaceticum* which catalyze synthesis of acetate from pyruvate and methyltetrahydrofolate. Properties of phosphotransacetylase. J. Biol. Chem. **256:**11137–11144.
8. **Fuchs, G.** 1986. CO_2 fixation in acetogenic bacteria: variations on a theme. FEMS Microbiol. Rev. **39:**181–213.
9. **Geerligs, G., P. Schönheit, and G. Diekert.** 1989. Sodium dependent acetate formation from CO_2 in *Peptostreptococcus productus* (strain Marburg). FEMS Microbiol. Lett. **57:**253–258.
10. **Gottwald, M., J. R. Andreesen, J. LeGall, and L. G. Ljungdahl.** 1975. Presence of cytochrome and menaquinone in *Clostridium formicoaceticum* and *Clostridium thermoaceticum*. J. Bacteriol. **122:**325–328.
11. **Heise, R., V. Müller, and G. Gottschalk.** 1989. Sodium dependence of acetate formation by the acetogenic bacterium *Acetobacterium woodii*. J. Bacteriol. **171:**5473–5478.
12. **Heise, R., V. Müller, and G. Gottschalk.** 1992. Presence of a sodium-translocating ATPase in membrane vesicles of the homoacetogenic bacterium *Acetobacterium woodii*. Eur. J. Biochem. **206:**553–557.
13. **Hormann, K., and J. R. Andreesen.** 1989. Reductive cleavage of sarcosine and betaine by *Eubacterium acidaminophilum* via enzyme systems different from glycine reductase. Arch. Microbiol. **153:**50–59.
14. **Hugenholtz, J., D. M. Ivey, and L. G. Ljungdahl.** 1987. Carbon monoxide-driven electron transport in *Clostridium thermoautotrophicum* membranes. J. Bacteriol. **169:**5845–5847.
15. **Hugenholtz, J., and L. G. Ljungdahl.** 1989. Electron transport and electrochemical proton gradient in membrane vesicles of *Clostridium thermoautotrophicum*. J. Bacteriol. **171:**2873–2875.
16. **Hugenholtz, J., and L. G. Ljungdahl.** 1990. Metabolism and energy generation in homoacetogenic clostridia. FEMS Microbiol. Rev. **87:**383–390.
17. **Janzer, J. J., H. Stan-Lotter, and K. E. Sanderson.** 1981. Isolation and characterization of hemin-permeable, envelope-defective mutants of *Salmonella typhimurium*. Can. J. Microbiol. **27:**226–237.
18. **Kamlage, B., and M. Blaut.** 1992. Characterization of cytochromes from *Methanosarcina* strain Göl and their involvement in electron transport during growth on methanol. J. Bacteriol. **174:**3921–3927.
19. **Kamlage, B., A. Boelter, and M. Blaut.** 1993. Spectroscopic and potentiometric characterization of cytochromes in two *Sporomusa* species and their expression during growth on selected substrates. Arch. Microbiol. **159:**189–196.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.

21. **Ljungdahl, L. G.** 1986. The autotrophic pathway of acetate synthesis in acetogenic bacteria. Annu. Rev. Microbiol. **40:**415–450.
22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
23. **McConville, M. L., and H. P. Charles.** 1979. Isolation of haemin-requiring mutants of *Escherichia coli* K 12. J. Gen. Microbiol. **113:**155–164.
24. **Möller, B., R. Ossmer, B. H. Howard, G. Gottschalk, and H. Hippe.** 1984. *Sporomusa*, a new genus of gram-negative anaerobic bacteria including *Sporomusa sphaeroides* spec. nov. and *Sporomusa ovata* spec. nov. Arch. Microbiol. **139:**388–396.
25. **Müller, V., M. Blaut, R. Heise, C. Winner, and G. Gottschalk.** 1990. Sodium bioenergetics in methanogens and acetogens. FEMS Microbiol. Rev. **87:**373–376.
26. **Naumann, E., H. Hippe, and G. Gottschalk.** 1983. Betaine: new oxidant in the Stickland reaction and methanogenesis from betaine and L-alanine by a *Clostridium sporogenes-Methanosarcina barkeri* coculture. Appl. Environ. Microbiol. **45:**474–483.
27. **Ragsdale, S. W.** 1991. Enzymology of the acetyl-CoA pathway of CO_2 fixation. Crit. Rev. Biochem. Mol. Biol. **26:**261–300.
28. **Shaw, W. V.** 1967. The enzymatic acetylation of chloramphenicol by extracts of R factor-resistant *Escherichia coli*. J. Biol. Chem. **242:**687–693.
29. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. **43:**737–755.
30. **Stupperich, E., P. Aulkemeyer, and C. Eckerskorn.** 1992. Purification and characterization of a methanol-induced cobamide-containing protein from *Sporomusa ovata*. Arch. Microbiol. **158:**370–373.
31. **Thomas, P. E., D. Ryan, and W. Levin.** 1976. An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate-polyacrylamide gels. Anal. Biochem. **75:**168–176.
32. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244:**4406–4412.
33. **Wohlfarth, G., G. Geerligs, and G. Diekert.** 1990. Purification and properties of a NADH-dependent 5,10-methylenetetrahydrofolate reductase from *Peptostreptococcus productus*. Eur. J. Biochem. **192:**411–417.
34. **Yang, H., and H. L. Drake.** 1990. Differential effects of sodium on hydrogen- and glucose-dependent growth of the acetogenic bacterium *Acetogenium kivui*. Appl. Environ. Microbiol. **56:**81–86.