Fermentation and Anaerobic Respiration by *Rhodospirillum rubrum* and *Rhodopseudomonas capsulata*

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*Rhodospirillum rubrum* and *Rhodopseudomonas capsulata* were able to grow anaerobically in the dark either by a strict mixed-acid fermentation of sugars or, in the presence of an appropriate electron acceptor, by an energy-linked anaerobic respiration. Both species fermented fructose without the addition of accessory oxidants, but required the initial presence of bicarbonate before fermentative growth could begin. Major products of *R. rubrum* fermentation were succinate, acetate, propionate, formate, hydrogen, and carbon dioxide; *R. capsulata* produced major amounts of lactate, acetate, succinate, hydrogen, and carbon dioxide. *R. rubrum* and *R. capsulata* were also capable of growing strictly through anaerobic, respiratory mechanisms. Nonfermentable substrates, such as succinate, malate, or acetate, supported growth only in the presence of an electron acceptor such as dimethyl sulfoxide or trimethylamine oxide. Carbon dioxide and dimethyl sulfide were produced during growth of *R. rubrum* and *R. capsulata* on succinate plus dimethyl sulfoxide. Molar growth yields from cultures grown anaerobically in the dark on fructose plus dimethyl sulfoxide were 3.8 to 4.6 times higher than values obtained from growth on fructose alone and were 56 to 60% of the values obtained from aerobic, respiratory growth with fructose. Likewise, molar growth yields from anaerobic, respiratory growth conditions with succinate plus dimethyl sulfoxide were 51 to 54% of the values obtained from aerobic, respiratory growth with succinate. The data indicate that dimethyl sulfoxide or trimethylamine oxide as a terminal oxidant is approximately 33 to 41% as efficient as O₂ in conserving energy through electron transport-linked respiration.

*Rhodospirillum rubrum* and *Rhodopseudomonas capsulata*, both purple nonsulfur photosynthetic bacteria, are capable of growth in anaerobic, light conditions and aerobic, dark conditions (20). Recent reports indicate that they can also grow anaerobically in darkness. Growth dependent on the anaerobic, dark fermentation of pyruvate has been demonstrated in strains of *R. rubrum* (12, 19). Uffen (17) found that the products of pyruvate fermentation were acetate, formate, carbon dioxide, and hydrogen; Schön and Biedermann (13) reported that propionate, acetate, and carbon dioxide were formed under anaerobic, dark incubation conditions by cultures of *R. rubrum* with pyruvate or fructose, but increases in cell mass were limited to about one doubling. Prolonged, strictly fermentative growth of a photosynthetic bacterium on sugars has not been previously demonstrated.

Yen and Marrs (24) and Madigan and Gest (9) reported that *R. capsulata* was capable of anaerobic, dark growth on sugars but only with the addition of an accessory oxidant, such as dimethyl sulfoxide (DMSO) or trimethylamine-N-oxide (TMAO), which was reduced during growth. End products of fructose catabolism in the presence of TMAO were acetate, formate, carbon dioxide, trimethylamine, and, upon exhaustion of the TMAO, lactate (8). Madigan et al. (8) concluded that the required oxidant functioned only as an electron sink for the disposal of reducing equivalents generated by a “facilitated fermentation” mechanism rather than as a terminal electron acceptor for electron transport-driven phosphorylation. However, this differed from results obtained with two nonphotosynthetic bacteria, *Proteus* and an unidentified strain, DL-1, with which DMSO or TMAO appeared to be the terminal electron acceptor in an anaerobic, oxidative phosphorylation process (16, 25).

In this communication we report on the ability of *R. rubrum* and *R. capsulata* to grow fermentatively on sugars in the absence of added oxidant or, alternatively, to grow on nonfermentable substrates in the presence of DMSO or TMAO. Analyses of growth, substrate utilization, and product formation revealed that *R.
rubrum and R. capsulata are capable of anaerobic, dark growth utilizing either fermentative or anaerobic, respiratory mechanisms.

MATERIALS AND METHODS

Organisms. R. rubrum G9 and R. capsulata B10 were used unless otherwise specified. A spontaneous rifampin-resistant mutant of G9 was obtained by incubating G9 under anaerobic, dark conditions on RCVB medium containing 80 mM DMSO and 0.1 mg of rifampin per ml. Other strains used were from our culture collection. A second copy of R. capsulata B10 was obtained from Michael Madigan; both copies yielded similar data.

Media and growth conditions. RCVB medium without a carbon source (11), amended to include 11.8 mg of FeSO₄·7H₂O, was used as the basal medium. Though not essential, 0.05% yeast extract stimulated growth and was included in the basal medium where noted. Carbon sources were 30 mM sodium malate, 35 mM sodium succinate, 22 mM sodium acetate, or 11 mM fructose. All were filter sterilized and added aseptically to the autoclaved medium. Where indicated, DMSO and TMAO were added to a final concentration of 80 and 40 mM, respectively. For fermentation studies, 0.05 to 0.1% (w/vol) filter-sterilized sodium bicarbonate was added to the medium; this increased the initial pH to 7.2 to 7.3. All other media had an initial pH of 6.7 to 7.0.

Media were prepared by boiling the liquid for 10 min in a round-bottomed flask under a stream of nitrogen which had been passed over heated copper filings. Resazurin, a redox indicator, and cysteine-HCl, a reducing agent, were found to inhibit growth and were therefore not utilized further. Portions (10 ml) of the medium were dispensed anaerobically into rubber-stoppered anaerobe tubes (18 by 142 mm; Bellco Glass, Inc.) by the Hungate technique (6). Tubes were then sterilized in an autoclave press (Bellco Glass, Inc.). Larger batch cultures (100 ml) for time course determinations were likewise prepared by boiling with gassing. All additions and transfers were done in an anaerobic glove box (Coy Manufacturing) containing an atmosphere of 90% N₂ and 10% H₂ (<5 ppm O₂). Inoculated liquid media were placed in a dark incubator maintained at 30°C.

Plates were poured either in the anaerobic chamber or aerobically; the latter were incubated for at least 1 day in the chamber before inoculation. For anaerobic, dark studies, inoculated plates were incubated in a darkened GasPak jar (BBL Microbiology Systems) containing an activated H₂ and CO₂ generator envelope. Jars were retained in the anaerobic chamber to ensure anaerobic conditions. For anaerobic, light studies, plates were incubated in the anaerobic chamber and were exposed to 34 W/m² from 40-W incandescent showcase lamps (General Electric Co.). All plates were maintained at 30°C.

Determinations of molar growth yield and carbon balance. Basal medium containing limiting concentrations of succinate, fructose, or acetate was used. Where indicated, carbon balances were determined with [U-¹⁴C]succinate or [U-¹⁴C]acetate with specific activities of 37,500 and 25,000 dpm/µmol, respectively. For carbon balance determinations, 0.05% yeast extract was included in the medium. Flasks containing 75 ml of medium were autoclaved aerobically and placed in an anaerobic chamber for at least 24 h prior to inoculation. After the addition of substrate and cells (initial absorbance at 660 nm ≈ 0.01), 10-ml samples were removed for substrate analysis, and the flasks were stoppered. For anaerobic, dark studies, cultures were stored in incubators maintained at 30°C. Anaerobic, light cultures were grown at 30°C in an illuminated cabinet in which they were exposed to 32 W/m² from 40-W incandescent showcase lamps. For aerobic, dark studies, cultures were incubated in a darkened oscillating water bath at 30°C. All cultures were harvested at maximum growth.

For hydrogen determinations, serum-stoppered Wheaton vials (39-ml volume) containing 10 ml of culture fluid were used. Vials were gassed with ultrapure N₂ after inoculation.

To determine incorporation of ¹⁴C into cell material, cells were grown on basal medium with 0.05% yeast extract (for carbon balances) or without yeast extract (for molar growth yields) and with limiting concentrations of [U-¹⁴C]fructose, [U-¹⁴C]acetate, or [U-¹⁴C]succinate (New England Nuclear). Specific activities for fructose, acetate, and succinate were 49,000, 25,000, and 37,500 dpm/µmol, respectively. Substrates were filter sterilized separately and added to 15 ml of medium at a concentration of 100,000 to 150,000 dpm/ml for fructose, 500,000 dpm/ml for acetate, and 100,000 to 175,000 dpm/ml for succinate.

Survey of anaerobic, dark growth. Strains were “patched” onto agar plates of appropriate minimal media and placed in desiccators which were repetitively flushed and evacuated with ultrapure N₂. Just prior to sealing the desiccators, a small test tube containing 3 ml of a frozen sodium ascorbate solution (85 mg/ml) was added to an empty petri dish along with 5 ml of a 10 µM CuCl₂ solution. A frozen slurry of NaNHCO₃ was added to a second dish with 5 ml of 5 N HCl. After replacement of the internal atmosphere, the ascorbate solution thawed and mixed with the CuCl₂, scavenging any trace amounts of O₂ remaining; the bicarbonate slurry thawed, mixed with the acid, and generated 5% CO₂ in the atmosphere.

Analysis of growth. Growth of cells was monitored turbidimetrically by measuring the absorbance of the cultures at 660 nm with a Spectronic 21 colorimeter (Bausch & Lomb, Inc.). Viable cell counts were determined by plating appropriate dilutions of cultures onto plates of malate or fructose medium and incubating duplicate plates under anaerobic and aerobic atmospheres. For dry weight measurements, 10 ml of culture suspension was centrifuged; the pellet was washed twice with distilled water, placed in a preweighed aluminum dish, and heated to constant weight at 100°C. For the survey of anaerobic, dark growth, strains were visually scored after two successive subcultures.

Measurement of carbon dioxide. For nonradioactive experiments, two identical cultures containing 100 ml of medium were used for each experiment. After inoculation in the glove box, both flasks were stoppered and removed from the chamber. One flask was analyzed immediately; the other was analyzed after growth of the bacteria. The CO₂ present in the cultures was trapped by piercing the rubber stopper with two syringe needles and flushing the headspace with N₂ into two sequential 1 N NaOH traps. The medium was
acidiﬁed to pH < 2 by adding 1 ml of 50% H2SO4. After at least 30 min of sparging, the contents of the traps were combined and samples were withdrawn for analysis. Excess BaCl2 was added to the samples, which were then titrated with 0.1 N HCl, with phenolphtha- lein as an endpoint indicator. Samples of NaOH through which no CO2 had been bubbled were also titrated, and the values were subtracted from those cultures evolving CO2 (10). The initial amount of CO2 present in the culture was subtracted from the amount of CO2 present after growth of the bacteria. 

CO2 formed from radioactive substrates was determined by acidiﬁying the culture medium with H2SO4 and sweeping the headspace gas with N2 into two sequential phenethylalnine-methanol (1:1, vol/vol) traps. Radioactivity trapped in this way was assumed to be due solely to 14CO2, the quantity of which was calculated from the specific activity of 14C-labeled substrates.

Quantitation of substrates and products. Culture samples were centrifuged at 20,000 × g for 10 min at 4°C and decanted. The supernatant was frozen at −20°C for later assay. Fructose and lactic acid were assayed by the methods of Spiro (14) and Barker and Summerson (1), respectively. Trimehylamine was determined by the method of Dyer (5). Volatile and nonvolatile organic acids were extracted from the spent medium (6) and were identiﬁed by gas chromatography, with the use of internal and external standards. A stainless-steel column (1/4 in. by 6 ft, 3 mm by 1.8 m) containing 15% SP2200–1% H3PO4 on Chromosorb W AW (Supelco, Inc.) was operated at 130°C with a thermal conductivity detector. DMSO, which was extracted with the nonvolatile organic acids, could also be quantitated on this column. Dimethyl sulﬁde concentrations were determined by gas chromatography using a Teflon column (3 mm by 1.8 m) of Carbopack B–1.5% XE60–1.0% H3PO4 (Supelco, Inc.) and a ﬂame ionization detector. Serum-stopped Wheaton bottles containing culture ﬂuid or standard dimethyl sulﬁde (Aldrich Chemical Co.) were heated for 20 min at 65°C; 0.1-ml samples of gas were injected into the column at 40°C.

Hydrogen was measured in a 1.5-ml water-jacketed chamber ﬁtted with a 5331 Clark-type electrode (Yellow Springs Instrument Co.) as described by Wang et al. (21). Bacteriochlorophyll was determined spectral- ly after exhaustive extraction of cells with acetone/ methanol (2).

Incorporation of 14C into cell material. Cells were harvested by centrifugation, washed twice with distilled water, boiled for 10 min in 1 N NaOH, and cooled. After the addition of one drop of 30% H2O2, the hydrolyzed cells were neutralized with HCl and phosphate buffer (pH 7.0). Radioactivity of samples was determined by using a model LS9000 liquid scintillation counter and MP scintillation cocktail (Beckman Instruments, Inc.). All samples were corrected for self-absorption by an external standardization method (H-number).

RESULTS

Survey for anaerobic, dark growth. Initially, 96 distinct strains of 12 species of the family Rhodospirillaceae were surveyed to determine their ability to grow anaerobically in darkness.

Included were 44 strains of Rhodopseudomonas capsulata, 20 of R. sphaeroides, 10 of P. palustris, 4 of R. gelatinosa, 1 of R. viridis, 5 of R. sulfidophila, 4 of Rhodospirillum rubrum, 1 strain each of R. fulvum, R. tenue, R. molischianum, and R. photometricum, and 4 of Rhodobacter capsulata.

We found that nine strains, including R. capsulata SP7 and R. rubrum FR1, grew fermentatively on a combination of glucose, fructose, and ribose (each at 2 g/liter). With sodium lactate (4 g/liter) as carbon source and CO2 or sulfate as potential electron acceptors, no fermentative or respiratory growth by any strain was observed. On lactate plus DMSO media, 77% of R. capsulata strains and 45% of R. sphaeroides strains grew readily, but strains of other species did not. TMAO was the most widely utilized electron acceptor (excluding O2, but including nitrate), supporting growth on lactate by 91% of R. capsulata strains, 90% of R. sphaeroides strains, 30% of R. palustris strains, 50% of R. gelatinosa strains, 25% of R. rubrum strains, and 100% of R. sulfidophila strains.

Subsequently, we found that the growth capabili- ties of photosynthetic bacteria under anaerobic, dark conditions are more widespread than the survey indicated. With optimized media and adherence to proper anaerobic techniques, R. capsulata B10 and MSG, R. rubrum G9 (and S-1), and R. sphaeroides R-26 and 2.4.16, all strains that originally tested negative for fermentative growth on sugars, were determined to be competent. Likewise, R. rubrum G9 (and S-1) initially exhibited no growth utilizing DMSO or TMAO but later proved positive.

R. capsulata B10 and R. rubrum G9 were selected as representative strains for more detailed studies of anaerobic, dark growth.

Growth of R. rubrum and R. capsulata on sugars. With repetitive subculturing, R. rubrum and R. capsulata grew anaerobically in the dark on fructose as the sole carbon and energy source. No accessory oxidant was required. Viable cells per milliliter and number of generations of growth for one subculture are shown in Table 1. Fructose supported sustained fermentative growth of R. rubrum, which exhibited a doubling time of 24 h and yielded 3.5 × 108 viable cells per ml at maximum growth; R. capsulata had a doubling time of 26 h and yielded 2.1 × 108 viable cells per ml. Ribose (13 mM) or xylose (13 mM) supported a low level of fermentative growth of R. capsulata with repetitive subculturing, giving doubling times of 76 and 104 h, respectively. Neither glucose nor sucrose promoted fermentative growth of R. capsulata, although both can be utilized by cultures aerobically in the dark or anaerobically in the light. Neither sugar inhibited fermentative
growth on fructose, however. Glucose, sucrose, ribose, and xylose were not fermented by *R. rubrum* (data not shown).

Both strains required the initial presence of bicarbonate before fermentative growth could begin. Maximum growth rates and yields for *R. rubrum* were obtained with 11 mM fructose and 0.05% sodium bicarbonate at an initial pH of 7.2; *R. capsulata* grew maximally when supplemented with 0.1% sodium bicarbonate. No growth occurred when fructose (or bicarbonate) was eliminated from the medium. Both species grew fermentatively in minimal media without added yeast extract; however, the doubling time was shorter and the cell yield was greater in media containing 0.05% yeast extract. Final pH values of the fermentative cultures ranged from 4.5 to 5.0.

When *R. rubrum* and *R. capsulata* were grown anaerobically on fructose in the presence of DMSO, cell doubling times decreased and cell yields increased relative to strictly fermentative values (Fig. 1). Doubling times of cells grown on fructose plus DMSO were 10 h for *R. rubrum* and 6 h for *R. capsulata*. Similar growth rates and yields were observed when DMSO was replaced by TMAO. No bicarbonate was required for DMSO- or TMAO-dependent growth. The final pH of the DMSO-containing cultures was 5.0 to 5.5; TMAO-containing cultures had a final pH of 7.2 to 7.6.

**Growth of *R. rubrum* and *R. capsulata* on nonfermentable substrates.** Typical respiratory

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**TABLE 1. Growth of *R. capsulata* and *R. rubrum* under anaerobic, dark conditions**

<table>
<thead>
<tr>
<th>Organism and conditions</th>
<th>Maximum absorbance at 660 nm</th>
<th>Viable cells/ml at maximum growth</th>
<th>No. of generations</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. capsulata</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>0.36</td>
<td>2.2 x 10^8</td>
<td>6.2</td>
</tr>
<tr>
<td>Ribose</td>
<td>0.15</td>
<td>5.9 x 10^7</td>
<td>4.3</td>
</tr>
<tr>
<td>Xylose</td>
<td>0.07</td>
<td>3.8 x 10^7</td>
<td>3.7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.02</td>
<td>2.0 x 10^7</td>
<td>2.7</td>
</tr>
<tr>
<td>No carbon</td>
<td>0.02</td>
<td>2.2 x 10^7</td>
<td>2.9</td>
</tr>
<tr>
<td>Fructose + DMSO</td>
<td>0.97</td>
<td>9.2 x 10^8</td>
<td>7.8</td>
</tr>
<tr>
<td>Succinate + DMSO</td>
<td>0.78</td>
<td>8.3 x 10^8</td>
<td>7.7</td>
</tr>
<tr>
<td>Acetate + DMSO</td>
<td>0.74</td>
<td>6.0 x 10^8</td>
<td>7.2</td>
</tr>
<tr>
<td>Malate + DMSO</td>
<td>0.32</td>
<td>2.5 x 10^8</td>
<td>6.0</td>
</tr>
<tr>
<td>Lactate + DMSO</td>
<td>0.69</td>
<td>6.0 x 10^8</td>
<td>7.2</td>
</tr>
<tr>
<td>Propionate + DMSO</td>
<td>0.47</td>
<td>5.4 x 10^8</td>
<td>7.1</td>
</tr>
<tr>
<td>Succinate + TMAO</td>
<td>0.27</td>
<td>2.3 x 10^8</td>
<td>5.8</td>
</tr>
<tr>
<td>Malate + TMAO</td>
<td>0.17</td>
<td>1.0 x 10^8</td>
<td>4.6</td>
</tr>
<tr>
<td>No carbon + DMSO</td>
<td>0.09</td>
<td>4.3 x 10^7</td>
<td>3.4</td>
</tr>
<tr>
<td>No carbon + TMAO</td>
<td>0.07</td>
<td>3.6 x 10^7</td>
<td>3.2</td>
</tr>
<tr>
<td><em>R. rubrum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>0.52</td>
<td>3.5 x 10^8</td>
<td>6.9</td>
</tr>
<tr>
<td>No carbon</td>
<td>0.01</td>
<td>1.4 x 10^7</td>
<td>2.1</td>
</tr>
<tr>
<td>Fructose + DMSO</td>
<td>0.74</td>
<td>6.0 x 10^8</td>
<td>7.6</td>
</tr>
<tr>
<td>Fructose + TMAO</td>
<td>0.68</td>
<td>5.6 x 10^8</td>
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</tr>
<tr>
<td>No carbon + DMSO</td>
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<tr>
<td>No carbon + TMAO</td>
<td>0.05</td>
<td>3.0 x 10^7</td>
<td>3.3</td>
</tr>
</tbody>
</table>

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* Media contained 0.05% yeast extract. Bicarbonate was added in absence of DMSO or TMAO.

b Number of generations = ln N2 - ln N1/ln 2, where N2 represents the number of cells at maximum growth and N1 represents the number of cells at initial growth.
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substrates, such as malate, succinate, and acetate, supported anaerobic growth of *R. rubrum* and *R. capsulata* only in the presence of an electron acceptor such as DMSO (Fig. 2 and Table 1). Doubling times of *R. rubrum* were 8 h for malate plus DMSO, 9.5 h for succinate plus DMSO, and 18 h for acetate plus DMSO. Comparable values for *R. capsulata* were 10, 10, and 14 h, respectively. Lactate (16 mM) and propionate (26 mM) also supported growth of both strains in the presence of DMSO. *R. capsulata* exhibited doubling times of 10 h for lactate and 26 h for propionate. Gas chromatographic analyses of cultures confirmed that the sole product of DMSO reduction was dimethyl sulfide. The final pH of the cultures was 6.5 to 7.0.

Several other potential electron acceptors were tested for their ability to support anaerobic, dark growth of *R. rubrum* or *R. capsulata* on malate. Growth of both strains was appreciable with 40 mM TMAO as electron acceptor, but was inhibited during the latter stages of the growth cycle by the alkalinity of the trimethylamine produced. Approximately 20 mM trimethylamine was produced during growth, resulting in a final pH of 9.0. Nitrate, sulfate, thiosulfate, or sulfite did not support growth of either strain; methionine sulfoxide gave only moderate growth.

Hydrogen, which contaminated the headspace during inoculation in the glove box, was determined not to be essential. No change occurred in the growth of either *R. rubrum* or *R. capsulata* on DMSO-containing succinate, malate, or acetate medium when the gas phase contained ultrapure N₂ (99.99%) instead of the glove box atmosphere.

To discount the possibility that anaerobic, dark growth of *R. rubrum* and *R. capsulata* cultures was actually due to the selection of a mutant cell line with aberrant metabolic properties, photosynthetically or aerobically grown cultures, not previously exposed to the anaerobic, dark regimen, were diluted, plated on fructose or malate, and incubated under several growth conditions. Results showed that similar numbers of cells of *R. rubrum* could grow on fructose anaerobically in the light, aerobically in the dark, or fermentatively in the dark. Likewise, cells of both strains could grow equally well on malate plates incubated aerobically in the dark, anaerobically in the light, or anaerobically in the dark with DMSO.

**Long-term anaerobic, dark growth of *R. rubrum***. After periods of prolonged anaerobic, dark growth, *R. rubrum* lost its photosynthetic pigment in the same manner as that described by Uffen et al. (18) for pyruvate-grown *R. rubrum* S-1. Bacteriochlorophyll concentrations were 3 nmol/g (wet weight) of cells after growth on malate plus DMSO. For comparison, 1,870 nmol of bacteriochlorophyll per g (wet weight) of cells was formed under anaerobic, light conditions. Two types of colonies were isolated from the G9 parent: pigmented (blue-green) and nonpigmented (orange), corresponding to Uffen's cell types C and G1 obtained after prolonged pyruvate fermentation (18). Results similar to Uffen's were seen when nonpigmented cells of *R. rubrum* were incubated photosynthetically; approximately 2 of 10⁶ cells formed pigmented colonies. However, when dark-grown (malate plus DMSO), nonpigmented colonies were placed in the light, blue-green papillae formed within or on the periphery of about 90% of the colonies. No significant change could be found in the growth rate of the two colony types under anaerobic, dark conditions on fructose, fructose plus DMSO, or malate plus DMSO.

A rifampin-resistant mutant of *R. rubrum* was isolated to rule out the possible presence of contaminants in the predominantly nonpigmented cultures. Viable counts from cultures grown in anaerobic, dark growth modes revealed the same number of colonies on plates incubated anaerobically in the dark with or without rifampin. The mutant exhibited the same growth kinetics as its parent on fructose, fructose plus DMSO, or malate plus DMSO.

As the bacteriochlorophyll concentration of *R. rubrum* decreased during prolonged growth on malate plus DMSO, the cells became noticeably orange, indicating the substantial presence of cytochromes. Analyses of cell membranes revealed extremely high concentrations of two low-redox-potential, membrane-bound, c-type cytochromes. More complete characterizations
of these cytochromes will be reported elsewhere.

Molar growth yields of *R. rubrum* and *R. capsulata*. The molar growth yields (micrograms [dry weight] of cells per micromole of substrate) of *R. rubrum* and *R. capsulata* growing under various conditions are shown in Table 2. Carbon substrate was limiting in all cases, and the dry weight was proportional to the amount of substrate that disappeared from the medium. No organic acid intermediates could be detected at the cessation of growth when O₂, DMSO, TMAO, or light was present. Growth yields from anaerobic, dark conditions on fructose plus DMSO were 3.8 to 4.6 times higher than yields obtained from anaerobic, dark growth on fructose alone and were 56 to 60% of yields obtained from respiratory growth on fructose plus O₂. Likewise, yields from anaerobic, respiratory growth on succinate plus DMSO were 51 to 54% of yields obtained from respiratory growth on succinate plus O₂.

The percentage of substrate assimilated into cell material varied with the growth conditions (Table 2). With *R. rubrum* 69% of the substrate utilized was assimilated into cell material during photosynthetic growth on fructose, whereas 8% was assimilated during fermentative growth.

**Products of growth.** A fermentation balance of *R. rubrum* revealed that major amounts of acetate, propionate, formate, succinate, CO₂, and H₂, along with smaller amounts of butyrate, were produced during fermentative growth on fructose (Table 3). Oxidized carbon waste products were compensated by formation of H₂, resulting in an oxidation-reduction balance of 1.11.

The addition of DMSO to the fructose medium significantly altered the products of fructose metabolism (Table 3). Only acetate and CO₂ were recovered as products (other than dimethyl sulfide) and accounted for 102% of the carbon dissimilated by *R. rubrum*. However, the concentration of acetate present in the medium was time dependent when *R. rubrum* was grown under conditions of limiting fructose. The acetate "pool" formed during early stages of growth was subsequently oxidized, with concomitant DMSO reduction, as fructose became exhausted (Fig. 3).

When *R. rubrum* was grown on succinate plus DMSO (Table 3), CO₂ was the major labeled product formed during growth, accounting for 79% of the carbon metabolized. About 10% of the labeled carbon was recovered in the supernatant but was not identified. The low oxidation-reduction balance suggests the presence of an additional oxidized carbon product.

*R. capsulata* produced CO₂, lactate, acetate, H₂, and succinate in addition to a small amount of butyrate during fermentative growth on fructose (Table 4). These products accounted for 107% of the carbon metabolized. In contrast, the major products from growth on fructose plus

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**Table 2. Molar growth yields of *R. rubrum* and *R. capsulata***

<table>
<thead>
<tr>
<th>Conditions</th>
<th><em>R. rubrum</em></th>
<th><em>R. capsulata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Substrate assimilated into cells (%)</td>
<td>Molar growth yield</td>
</tr>
<tr>
<td>Fermentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose + YE</td>
<td>8.1</td>
<td>18.8</td>
</tr>
<tr>
<td>Fructose</td>
<td>8.0</td>
<td>15.0</td>
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<tr>
<td>Anaerobic respiration</td>
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<tr>
<td>Fructose + DMSO</td>
<td>19.9</td>
<td>57.5</td>
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<tr>
<td>Succinate + DMSO</td>
<td>20.2</td>
<td>21.5</td>
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<tr>
<td>Succinate + TMAO</td>
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<tr>
<td>Acetate + DMSO</td>
<td>NDd</td>
<td>ND</td>
</tr>
<tr>
<td>Aerobic respiration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose + O₂</td>
<td>37.1</td>
<td>102.8</td>
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<tr>
<td>Succinate + O₂</td>
<td>31.2</td>
<td>42.2</td>
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<tr>
<td>Acetate + O₂</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Photosynthesis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>69.4</td>
<td>-e</td>
</tr>
<tr>
<td>Succinate</td>
<td>65.2</td>
<td>-e</td>
</tr>
</tbody>
</table>

*a* Cell carbon was determined by incorporation of [U-¹⁴C]fructose or [U-¹⁴C]fructose into cell material.

*b* Molar growth yields were calculated by dividing cell dry weight (micrograms per milliliter) by substrate catabolized but not assimilated (micromoles per milliliter). Values shown are averages of at least two experiments.

*c* Cells were grown in media containing 0.05% yeast extract. All other experiments were performed with minimal media.

*d* ND, Not determined.

*e* Molar growth yields cannot be directly calculated as a result of the energy input of light.
DMSO were acetate and CO₂, along with small amounts of lactate, butyrate, and succinate.

When *R. capsulata* was grown on limiting succinate in the presence of DMSO (Table 4), CO₂ was the only labeled product recovered. A time-course study (not shown) under conditions of limiting substrate revealed that acetate was indeed formed during growth, reaching concentrations of 0.69 mol per mol of succinate. However, after the succinate was depleted, the acetate was oxidized to CO₂ with further reduction of DMSO. Analysis of growth on acetate plus DMSO (Table 4) indicated that 94.5% of the carbon catabolized was oxidized to CO₂.

**Calculation of *Y*<sub>ATP</sub>.** Since it is known that *R. capsulata* catabolizes fructose via the Embden-Meyerhof pathway (3), it is possible to estimate the bacterial cell mass produced per mole of ATP synthesized (*Y*<sub>ATP</sub>). The assumptions made are that 2 mol of ATP is generated per mol of fructose fermented to pyruvate and that 1 additional mol of ATP is synthesized per mol of acetate or butyrate produced. It is further assumed that no net ATP synthesis occurs during the formation of succinate from pyruvate; ATP utilization by pyruvate carboxylase is countered by ATP synthesis during the reduction of fumarate to succinate. It should be noted that if either phosphoenolpyruvate carboxylase or "malic enzyme" is used for the carboxylating step the ATP yield may be higher. Calculations from data for *R. capsulata* (Table 4) yield 2.46 mol of ATP per mol of fructose fermented. Since 19 g (dry weight) of cells is formed per mol of fructose catabolized (Table 2), the *Y*<sub>ATP</sub> is 7.7. The additional production of propionate by *R. rubrum* makes a similar calculation more tenuous since the pathway of propionate formation has not been established.

**DISCUSSION**

Results presented herein show that strains of the family *Rhodospirillaceae* are capable of anaerobic, dark growth using energy generated either from the fermentation of sugars to mixed acid products or from nonfermentable organic substrates in electron transport-coupled, anaerobic respiration. That these growth modes can be sustained is demonstrated by the fact that both fermentative and anaerobic, respiratory cultures of *R. rubrum* have been maintained for over 2 years by repeated transfer into either fructose media or malate plus DMSO media.

Pyruvate fermentation by *R. rubrum* was reported by Uffen and Wolfe (19), although they observed no fermentative growth on fructose.

**FIG. 3.** Growth, substrate utilization, and product formation of *R. rubrum* on fructose plus DMSO. Cells were grown anaerobically in the dark at 30°C. Media contained 0.05% yeast extract.
The absence of growth on fructose may be due to the inclusion of cysteine-HCl and resazurin in their growth media. These compounds are often used to ensure strict anaerobiosis, but we found that the addition of cysteine and resazurin to the media resulted in an inhibition of sugar fermentation in both \textit{R. rubrum} and \textit{R. capsulata}.

Our findings on the requirement for bicarbonate for fermentative growth of \textit{R. rubrum} and \textit{R. capsulata} augment those of Schön and Biedermann (13), who correlated propionate production with CO$_2$ fixation during pyruvate fermentation in resting cells of \textit{R. rubrum}. Presumably, carbon dioxide is required for the formation of C4 acids; this has been shown to be true for several microorganisms, including \textit{Bacteroides fragilis}, which ferments glucose to succinate and propionate via the reductive tricarboxylic acid pathway (7). This pathway appears to involve a b-type cytochrome, which mediates an anaerobic, electron transport involved with the reduction of fumarate to succinate (22). Although c- and b-type cytochromes have been detected in fermenting cells of photosynthetic bacteria (J. E. Schultz and P. F. Weaver, to be published), it is not yet known whether propionate and succinate production by these organisms involves electron transport, as is the case in fermenting nonphotosynthetic bacteria.

Values obtained for molar growth yields in some cases may be slightly high as a result of the formation of storage polymers, such as poly-$\beta$-hydroxybutyrate or glycogen. Indeed, Madigan et al. (8) reported that as much as 20% of the cell dry weight was poly-$\beta$-hydroxybutyrate when \textit{R. capsulata} was grown under anaerobic, dark conditions on fructose plus TMAO. Such refinements in the growth yield calculations, however, will not significantly alter the present data, which indicate a 360% increase (Table 2) in cell yield for \textit{R. capsulata} when fructose was oxidized with DMSO as opposed to when it was fermented. About 43% of this increase can be attributed to additional substrate-level phosphorylation accompanying the production of 0.42 mol of excess acetate and total oxidation of 0.3 mol of fructose (Table 4).

Using the $Y_{\text{ATP}}$ value of 7.7 for \textit{R. capsulata} grown fermentatively, we calculated that during growth on fructose plus DMSO (data from Table 2 wherein no reduced carbon intermediates remained) 7.6 mol of ATP was formed per mol of fructose dissimilated (58.5 g of cells·mol of fructose$^{-1}$ × mol of ATP·7.7 g of cells$^{-1}$). Since the complete dissimilation of fructose should yield 6 CO$_2$ + 10 NADH + 2 reduced flavin adenine dinucleotide + 4 ATP (from substrate-level phosphorylation), an additional 3.6 ATP must be obtained from oxidation of the reduced nucleotides. Thus, the P/2e$^-$ ratio with DMSO as oxidant is 0.30. Theoretically, if there are two, completely coupled, energy conservation steps in the reduction of DMSO by NADH (one from reduced flavin adenine dinucleotide), the P/2e$^-$ ratio would be 1.83; if one, it would be 0.83; if none, as predicted by Madigan et al. (8), it would be 0.65. Although the ratio obtained is greater than 0.65, the actual number of phosphorylation steps is not readily discernible as a result of apparent uncoupling in vivo. In vitro phosphorylation studies will be the subject of a later report.

Similar calculations for \textit{R. capsulata} growth on fructose plus O$_2$ indicate that 12.7 mol of ATP is synthesized per mol of fructose dissimilated, of which 8.7 mol is generated by electron transport-linked phosphorylation. The P/2e$^-$ ratio to O$_2$ is therefore 0.72, or 2.4 times that of
DMSO-linked oxidations. Corrections for maintenance energy requirements have not been attempted, but are expected to be similar for all growth conditions since the cell generation times are approximately the same.

ATP requirements for biosynthesis of cell materials may be higher for cells growing on succinate or acetate as carbon source and would be reflected in a lower \( Y_{\text{ATP}} \) for these substrates (15). Since neither succinate nor acetate can be fermented with a known yield of ATP, it is not possible to calculate the actual \( Y_{\text{ATP}} \) values. Assuming that they are not greatly different from the value of \( Y_{\text{ATP}} \) for fructose, the \( P/2e^- \) ratios following from the dissimilation of succinate to \( 4 \text{CO}_2 + 5 \text{NADH} + 2 \text{ATP} \) would be 0.21 to DMSO, 0.24 to TMAO, and 0.63 to \( O_2 \).

Acetate is perhaps the most straightforward example of a respiratory substrate. All of the energy for growth must come from electron transport-linked phosphorylation since there is no net substrate-level phosphorylation in its dissimilation to \( 2 \text{CO}_2 + 3 \text{NADH} + \text{flavin adenine dinucleotide} + 2 \text{ATP} \). Calculated \( P/2e^- \) ratios with acetate as substrate are 0.24 to DMSO and 0.73 to \( O_2 \).

In all cases the \( P/2e^- \) ratios for electron transport to DMSO or TMAO are 33 to 41% of those to \( O_2 \). This is consistent with the 40 to 42% energy drop from \( \text{NADH}/\text{NAD}^+ \) (\( E_{\text{m}} = -320 \text{ mV} \)) to DMSO/DMSO (\( E_{\text{m}} = +160 \text{ mV} \) [23]) or to TMAO/TMAO (\( E_{\text{m}} = +130 \text{ mV} \) [as referenced in 16]) relative to that of \( O_2/H_2O \) (\( E_{\text{m}} = +815 \text{ mV} \)).

Our data differ from those of Madigan et al. (8), who found no anaerobic, dark growth of \( R. \text{capsulata} \) on fructose alone or on respiratory substrates with TMAO. Their results suggest that TMAO serves as an electron sink for management of reox balance in a fermentative process (4, 8). Based on our results with DMSO (and, to a lesser extent, TMAO), we believe that the "facilitated fermentation" of \( R. \text{capsulata} \) growing on fructose plus TMAO (or DMSO) is a hybrid form of growth; \( R. \text{capsulata} \) ferments fructose to mixed acids, which are further oxidized by energy-yielding reactions coupled to TMAO (or DMSO) reduction.

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LITERATURE CITED

