Evidence for an Inducible Nucleotide-Dependent Acetone Carboxylase in *Rhodococcus rhodochrous* B276

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Received 30 December 1998/Accepted 24 February 1999

The metabolism of acetone was investigated in the actinomycete *Rhodococcus rhodochrous* (formerly *Nocardia corallina*) B276. Suspensions of acetone- and isopropanol-grown *R. rhodochrous* readily metabolized acetone. In contrast, *R. rhodochrous* cells cultured with glucose as the carbon source lacked the ability to metabolize acetone at the onset of the assay but gained the ability to do so in a time-dependent fashion. Chloramphenicol and rifampin prevented the time-dependent increase in this activity. Acetone metabolism by *R. rhodochrous* was CO₂ dependent, and ¹³CO₂ fixation occurred concomitant with this process. A nucleotide-dependent acetone carboxylase was partially purified from cell extracts of acetone-grown *R. rhodochrous* by DEAE-Sepharose chromatography. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis suggested that the acetone carboxylase was composed of three subunits with apparent molecular masses of 85, 74, and 16 kDa. Acetone metabolism by the partially purified enzyme was dependent on the presence of a divalent metal and a nucleoside triphosphate. GTP and ATP supported the highest rates of acetone carboxylation, while GTP, UTP, and CTP supported carboxylation at 10 to 50% of these rates. ATP did not support acetone carboxylation. Acetoacetate was determined to be the stoichiometric product of acetone carboxylation. The longer-chain ketones butanone, 2-pentanone, 3-pentanone, and 2-hexanone were substrates. This work has identified an acetone carboxylase with a novel nucleotide usage and broader substrate specificity compared to other such enzymes studied to date. These results strengthen the proposal that carboxylation is a common strategy used for acetone catabolism in aerobic acetone-oxidizing bacteria.

A variety of bacteria are capable of utilizing acetone as a growth-supporting substrate. Studies involving these bacteria have provided evidence leading to several proposed pathways for bacterial acetone metabolism (for a recent review, see reference 10). Collectively, these pathways have considered the initial conversion of acetone to occur by one of two strategies. For most aerobic bacteria, the initial step in acetone metabolism has been proposed to be catalyzed by an O₂-dependent acetone monooxygenase to produce hydroxymacetone, although no acetone monooxygenase activity has been demonstrated in vitro (9, 16, 26, 27). For facultatively or strictly anaerobic bacteria, the initial step in acetone metabolism has been proposed to occur via a CO₂-dependent carboxylation forming acetoacetate or an acetoacetyl derivative (7, 12, 14, 18–20, 22). Recently, a CO₂-dependent pathway of acetone metabolism was identified in *Xanthobacter* strain Py2, an obligately aerobic bacterium (24). Thus, at present it is ambiguous which strategy is predominant in aerobic acetone-metabolizing bacteria. In light of this, it was of interest to investigate acetone metabolism in an obligately aerobic bacterium phylogenetically distinct from *Xanthobacter* strain Py2. *Rhodococcus rhodochrous* (formerly *Nocardia corallina*) B-276 is a gram-positive obligate aerobe that is capable of growth on a variety of carbon sources, including alkanes and alkenes (11). In the present study, *R. rhodochrous* is reported for the first time to utilize acetone as a growth-supporting substrate. Evidence is presented that acetone is metabolized by *R. rhodochrous* in a CO₂-dependent manner by a nucleotide-dependent acetone carboxylase. This enzyme appears to catalyze a reaction identical with that of the *Xanthobacter* strain Py2 and *Rhodobacter capsulatus* acetone carboxylases, previously studied in the purified and partially purified forms, respectively (6, 23). However, distinct differences are apparent, most notably in the nucleotide usage and substrate specificity of the *R. rhodochrous* acetone carboxylase. Presented here is an initial description of both the in vivo and in vitro metabolism of acetone in *R. rhodochrous*. This work is of interest because it provides further evidence that carboxylation may be a significant strategy for acetone metabolism by aerobic bacteria. Additionally, the demonstration of novel in vitro requirements by the *R. rhodochrous* acetone carboxylase helps to expand the very limited knowledge of bacterial acetone-degrading proteins and their catalytic requirements.

**MATERIALS AND METHODS**

**Chemicals.** Nucleotides and antifoam 289 were purchased from Sigma Chemicals. NaH¹³CO₃ (99% ¹³C atom) and all volatile organic compounds were purchased from Aldrich Chemicals. NaH¹⁴CO₃ (specific activity, 54.4 mCi of ¹⁴C mmol⁻¹) was purchased from ICN Radiochemicals, Irvine, Calif. Ascarite II was purchased from Thomas Scientific, Swedesboro, N.J. All other chemicals used were of analytical grade.

**Bacteria and growth conditions.** Cultures of *R. rhodochrous* B276 (ATCC 31338) were grown at 30°C in either shake flasks or a carboy. The growth medium was a mineral salts medium (28) in which NaNO₃ (2 g/liter) was substituted for NH₄Cl as the primary nitrogen source. The carbon sources for growth were either glucose (1% [w/vol]), isopropanol (40 mM), or acetone (40 mM). Shake flask cells were harvested by centrifugation (8,600 g), resuspended into 50 mM phosphate buffer (pH 7.2), and frozen at −80°C for storage. Batch fermentation was carried out in a 45-liter glass carboy with forced aeration, containing 39 liters of minimal salts medium, antifoam 289 (0.1% [v/vol]), and acetone (40 mM). Five liters of 48-h *R. rhodochrous* shake flask cultures grown on identical media served as the fermentor inoculum. Air was replenished at 24-h intervals, and acetone levels were monitored by gas chromatography. After reaching an A₅₀₀ of approximately 2.5, cells were harvested by tangential-flow filtration with a Pellicon system (Millipore Corp.) followed by centrifugation (8,600 g). Cell paste was drop frozen in liquid nitrogen and stored at −80°C.

**Gas chromatography.** Gas chromatography (flame ionization detector) was performed by using a Shimadzu GC-8A interfaced with a Shimadzu CR601 integrator. Assays using 9- or 3-ml sealed serum vials involved injection of 100- or 30-μl gas-phase samples, respectively. Unless otherwise indicated, N₂ was
used as a carrier gas, the column packing material consisted of Porapak Q, and the injector temperature was 200°C. Conditions for assaying the following individual compounds are given in parentheses after the compound name(s): acetone (200°C, 0.5-3 by 45-cm column) to remove acetone (100°C), acetate (200°C, 0.3- by 45-cm column at 130°C), 2-pentanone and 3-pentanone (100 KPa; 0.3- by 45.5-cm column at 150°C), and 2-hexanone (100 KPa; 0.3- by 45.5-cm column at 160°C). Liquid-phase sampling was performed to acetoacetate quantification by using the following conditions: carboxyl carrier gas at 200°C, a 0.3- by 45.5-cm column at 120°C, and an injector temperature of 230°C.

**Induction of acetone-metabolizing activity.** *Rhodococcus* cultures which had been grown for several generations on either glucose, isopropanol, or acetone were used to inoculate shake flasks with a total volume of 3% (vol/vol) reaching *A*<sub>540</sub> values ranging from 1.25 to 1.96, cells were harvested by centrifugation (7,800 *g* for 10 min) followed by ultracentrifugation (140,000 *g* for 30 min) to a pellets. For further purification, cell extracts (125 ml) were passed through a French pressure cell three times at 125,000 KPa. The cell pellet was resuspended in 1.5 volumes of buffer (50 mM Tris-HCl [pH 8.0], MgCl<sub>2</sub> (5 mM), NH<sub>4</sub>Cl (100 mM), propionaldehyde, or propylene oxide). At desired times, 1 ml of liquid was removed from assay vials and analyzed for acetoacetate as described previously (1).

**RESULTS**

**Induction of acetone-degrading activity in *R. rhodochrous*.** *R. rhodochrous* B276 is capable of growth with short-chain aliphatic alkanes and alkenes as carbon sources, including propane (11). Since other propane oxidizers are capable of growth on isopropanol or acetone (5, 9), we investigated the possibility that these compounds could be utilized as carbon and energy sources for *R. rhodochrous* (5). Both isopropanol and acetone were found to support the growth of *R. rhodochrous*, with exponential-phase doubling times of approximately 3 h.

In order to determine whether the genes encoding the acetone-degrading enzyme of *R. rhodochrous* are expressed constitutively or induced during a specific condition, the ability of *R. rhodochrous* to metabolize acetone was investigated after growth with various carbon sources. As shown in Fig. 1, acetone and isopropanol-grown *R. rhodochrous* readily consumed acetone from the onset of exposure, and this activity was not prevented by the inclusion of chloramphenicol and rifampin, which are protein and RNA synthesis inhibitors, respectively.

In contrast to these results, *R. rhodochrous* cells cultured with glucose as the carbon source lacked the ability to consume acetone at the onset of the assay but gained the ability to do so in a time-dependent fashion (Fig. 1). The addition of chloramphenicol and rifampin to glucose-grown cells prevented this time-dependent increase in acetone-consuming activity (Fig. 1). These results suggest that the acetone-metabolizing enzyme in *R. rhodochrous* is not constitutively synthesized but instead is synthesized in response to growth on either acetone or isopropanol.

**CO<sub>2</sub>-dependent acetoacetate metabolism and acetone-dependet**

**14CO<sub>2** fixation in cell suspensions of acetone-grown *R. rhodochrous*.** The study of acetone metabolism in the aerobic bacterium *Xanthobacter* strain Py2 had validated carboxylation as a potential strategy for aerobic acetone utilizers (23, 24). To investigate the possibility that acetone might be metabolized by carboxylation in *R. rhodochrous*, two experiments using whole-cell suspensions were performed. Together, these experiments...
investigated the effects of CO₂ enrichment and depletion on acetone consumption, and the substrate-dependent fixation of ¹⁴CO₂.

Assay vials containing cell suspensions supplemented with CO₂ and NaHCO₃ consumed acetone at rates 40% greater than those containing no added carbonate species (Fig. 2). When either of two CO₂-trapping agents (6 M KOH or ascarite, a silicate carrier containing adsorbed NaOH) was added to microcentrifuge tubes placed inside assay vials, the consumption of acetone was almost completely prevented (Fig. 2). To verify that the decreased acetone consumption rates observed in the presence of the hydroxide-based traps were due to CO₂ depletion, HClO₄ was added to one of the traps (at 65 min) to liberate CO₂ trapped as K₂CO₃. As shown in Fig. 2, the addition of HClO₄ resulted in an immediate increase in the rate of acetone consumption, verifying the requirement of CO₂ for acetone metabolism by *R. rhodochrous*.

In order to determine if CO₂ is a cosubstrate of acetone metabolism, the abilities of acetone and other organic compounds to stimulate ¹⁴CO₂ fixation into whole cells were investigated. Three isomeric compounds (acetone, propionaldehyde, and epoxypropane) and glucose were tested in this regard. Of the isomeric compounds, acetone and propionaldehyde were readily consumed by acetone-grown *R. rhodochrous*, while epoxypropane was not (Fig. 3). Glucose was also metabolized by acetone-grown cells, and at a rate comparable to those of acetone and propionaldehyde, as evidenced by the increased rate of O₂ consumption observed when glucose was added to resting-cell suspensions (data not shown). As shown in Fig. 3, acetone consumption occurred concomitantly with the fixation of ¹⁴CO₂ into acid-stable cell products. In contrast, the consumption of propionaldehyde and glucose (data not shown) did not support levels of ¹⁴CO₂ fixation above the background rate.

**Requirements for and optimization of in vitro acetone carboxylase activity.** In vitro acetone carboxylase activity has been demonstrated for two bacteria: *Xanthobacter* strain Py2 and *R. capsulatus* (6, 23, 24). In both cases acetone carboxylase activity required the addition of ATP. The carboxylation of acetone is an endergonic reaction (ΔG°' = +17.1 kJ/mol), so it is not surprising that a source of energy would be required to support the reaction. For all other CO₂-dependent acetone-degrading bacteria that have been studied, no in vitro acetone carboxylase activity has been successfully reconstituted (12, 20). It was therefore of interest to determine whether, and under what conditions, in vitro acetone degradation activity could be measured for *R. rhodochrous*.

No acetone degradation activity could be detected in cell extracts of *R. rhodochrous* in the absence of either CO₂ or a nucleoside triphosphate. While low rates of CO₂- and ATP-dependent acetone degradation activity (less than 0.2 mU mg⁻¹) were observed in cell extracts, significantly higher rates (1.5 to 3.0 mU mg⁻¹) were observed when GTP was included in the assay in place of ATP. The addition of the cations Mg²⁺ and NH₄⁺ was found to have a stimulatory effect on activity, but they could not themselves replace the requirement of GTP.
or ATP. All of the acetone degradation activity was recovered in the soluble fraction after removal of membranes by centrifugation at 140,000 × g. The addition of membranes to the soluble fraction did not affect acetone degradation activity.

In order to further characterize the acetone carboxylase activity, cell extracts were fractionated by DEAE anion-exchange chromatography. Acetone carboxylase activity was recovered (>70% recovery) in the fractions eluting between 260 and 320 mM KCl (fourfold enrichment over cell extracts). Unfortunately, further attempts to purify the acetone carboxylase activity (e.g., by hydrophobic interaction, gel filtration, or Q-Sepharose anion-exchange chromatography) resulted in large losses of activity. These losses could not be prevented by the addition of stabilizing agents (glycerol, EDTA, or metal ions) or protease inhibitors. In addition, activity could not be restored by pooling together various fractions resolved by the chromatographic separations. Therefore, all subsequent studies of the acetone carboxylase activity made use of the active fractions partially purified by DEAE chromatography.

As observed for cell extracts, acetone carboxylation by the partially purified acetone carboxylase was CO₂ and nucleotide dependent. Table 1 compares the acetone carboxylation rates supported by several nucleoside triphosphates. GTP and ITP supported the highest specific rates of acetone carboxylation, while ATP did not support detectable acetone carboxylase activity (Table 1). XTP, CTP, and UTP also supported acetone carboxylase activity, although at lower rates than either GTP or ITP (Table 1). No stimulation of activity was observed when ATP and GTP were added to assays simultaneously (i.e., ATP did not stimulate the GTP-dependent reaction). Acetone carboxylation was not supported by inorganic pyrophosphate or nucleoside diphosphates. As observed for cell extracts, activity was stimulated by the addition of Mg²⁺ and NH₄⁺. No other factors, including biotin and the low-molecular-weight components of the cell extract resolved by using ultrafiltration versus a 30,000-M₅0 cutoff membrane, had any effect on acetone carboxylase activity. Avidin, a potent inactivator of biotin-dependent carboxylases, did not inhibit acetone carboxylase activity.

Characterization of acetocetate as the product of in vitro acetone carboxylation. Acetocetate has been shown to be the product of acetone carboxylation by the acetone carboxylase from *Xanthobacter* strain Py2 (24), and there is strong evidence that either acetocetate or an acetocetyl derivative is the product in anaerobic acetone-utilizing bacteria (10). Based on these precedents, we expected that acetocetate would be the stoichiometric product of CO₂-dependent acetone metabolism in *R. rhodochrous*. This prediction was verified by using ¹³C NMR spectroscopy and gas chromatography to identify and quantify acetocetate produced from in vitro acetone metabolism. Figure 4A shows the ¹³C NMR spectrum obtained after sodium borohydride reduction. The resonance at 174.7 ppm is identical in chemical shift to the resonance of the C-1 carbon atom of acetoacetate. (B) Spectrum of the product of acetocetate carboxylation after sodium borohydride reduction. The resonance at 180.3 ppm is identical in chemical shift to the resonance of the C-1 atom of β-hydroxybutyrate. (C) Spectrum of a sample prepared from an assay mixture containing all components except GTP. (D) Spectrum of the sample in panel C after sodium borohydride reduction. The resonance at 77.0 ppm in each spectrum is due to chloroform, which was used as the reference. The resonances at approximately 160 ppm (panel A, 160.1 ppm; panel B, 165.6 ppm; panel C, 160.4 ppm; panel D, 166.7 ppm) are due to the presence of NaH¹³CO₃ in the samples. The shifts in position of the bicarbonate resonances from 160.1 and 160.4 ppm (in panels A and C, respectively) to 165.6 and 166.7 ppm (in panels B and D, respectively) are due to the change in pH resulting from the addition of sodium borohydride. The additional resonances at 170.9 ppm in panel B and 171.0 ppm in panel D are due to formate formed by the sodium borohydride reduction of a portion of the NaH¹³CO₃ present in these samples.

![FIG. 4. ¹³C NMR identification of acetocetate as the product of in vitro GTP-dependent acetocetate carboxylation.](https://example.com/fig4.png)

**FIG. 4.** ¹³C NMR identification of acetocetate as the product of in vitro GTP-dependent acetocetate carboxylation. (A) Spectrum of the product of acetocetate carboxylation. The resonance at 174.7 ppm is identical in chemical shift to the resonance of the C-1 carbon atom of acetocetate. (B) Spectrum of the product of acetocetate carboxylation after sodium borohydride reduction. The resonance at 180.3 ppm is identical in chemical shift to the resonance of the C-1 atom of β-hydroxybutyrate. (C) Spectrum of a sample prepared from an assay mixture containing all components except GTP. (D) Spectrum of the sample in panel C after sodium borohydride reduction. The resonance at 77.0 ppm in each spectrum is due to chloroform, which was used as the reference. The resonances at approximately 160 ppm (panel A, 160.1 ppm; panel B, 165.6 ppm; panel C, 160.4 ppm; panel D, 166.7 ppm) are due to the presence of NaH¹³CO₃ in the samples. The shifts in position of the bicarbonate resonances from 160.1 and 160.4 ppm (in panels A and C, respectively) to 165.6 and 166.7 ppm (in panels B and D, respectively) are due to the change in pH resulting from the addition of sodium borohydride. The additional resonances at 170.9 ppm in panel B and 171.0 ppm in panel D are due to formate formed by the sodium borohydride reduction of a portion of the NaH¹³CO₃ present in these samples.

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**TABLE 1. Effects of various nucleoside triphosphates on CO₂-dependent acetone degradation by the partially purified acetone carboxylase from *R. rhodochrous* B276**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>No added CO₂ (μmol·mg⁻¹)</th>
<th>With CO₂ (μmol·mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ATP</td>
<td>1 ± 0.2</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>GTP</td>
<td>2 ± 1</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>TTP</td>
<td>2 ± 0.2</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>XTP</td>
<td>2 ± 0.2</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>CTP</td>
<td>0.2 ± 0.3</td>
<td>6.0 ± 0.4</td>
</tr>
<tr>
<td>UTP</td>
<td>0.2 ± 0.3</td>
<td>6 ± 0.4</td>
</tr>
</tbody>
</table>

* All assays were performed in duplicate and contained a nucleoside triphosphate (20 mM), glycerol (1.5% [vol/vol]), and partially purified acetone carboxylase (1.03 mg) in addition to the standard assay mixture. One unit of activity is defined as 1 μmol of acetone degraded per min.

* Vials contained 50 mM carbonate species, added as 43 mM KHCO₃ plus 7 mM CO₂.
to that of acetone, while 2-pentanone, 3-pentanone, and 2-hexanone were degraded at rates that were 70, 40, and 42%, respectively, of the acetone-dependent rate. These results are dramatically different from those obtained for the *Xanthobacter* strain Py2 acetone carboxylase, which was unable to degrade 2-pentanone, 3-pentanone, or 2-hexanone (23). 2-Butanone was degraded by the *Xanthobacter* enzyme, but at a rate 60% lower than that of acetone (23).

Identification of inducible polypeptides in *R. rhodochrous* extracts and comparison of *R. rhodochrous* and *Xanthobacter* strain Py2 acetone carboxylase polypeptides. SDS-PAGE analysis of cell extracts prepared from acetone- or isopropanol-grown *R. rhodochrous* cells shows the presence of two polypeptides produced at high levels, with apparent molecular masses of 74 and 85 kDa (Fig. 5, lanes 4 and 5). These polypeptides are not visible in extracts prepared from propylene- or glucose-grown cells, which lack acetone degradation activity (Fig. 5, lanes 2 and 3). The presence of acetone carboxylase activity in either *Xanthobacter* strain Py2 or *R. capsulatus* also leads to the high-level production of polypeptides with apparent molecular weights similar to those observed here for *R. rhodochrous* (6, 23).

The present work expands our base of knowledge concerning bacterial acetone metabolism by demonstrating that an aerobic hydrocarbon-oxidizing actinomycete, *R. rhodochrous* B276 (formally *N. corallina*), metabolizes acetone via a CO$_2$-dependent process analogous to those described for *Xanthobacter* strain Py2 and anaerobic acetone-utilizing bacteria. This finding supports the idea that carboxylation may be a common pathway for acetone metabolism in both aerobic and anaerobic bacteria. Previous studies of acetone metabolism in *Mycobacterium vaccae* JOB5 (27) and four gram-positive enrichment cultures (26) led to the hypothesis that aerobic acetone metabolism involves an initial monooxygenase-catalyzed hydroxylation producing acetol (hydroxyacetone). Acetol is proposed to undergo further oxidation to pyruvate or cleavage to acetaldehyde and formaldehyde (26, 27). However, acetone monooxygenase activity has not been demonstrated for any aerobic acetone-oxidizing bacteria, and this proposed route remains speculative.

The metabolism of acetone by *R. rhodochrous* B276 may be relevant to the pathway of propane metabolism, and possibly to that of longer-chain saturated-hydrocarbon metabolism, in this bacterium, although this has not been investigated in the present work. Propane catabolism in hydrocarbon-oxidizing *Mycobacterium* strains has been shown to proceed by oxidation to isopropanol and then acetone (16, 27). Isopropanol and acetone are potential intermediates in the pathway of propane metabolism in *R. rhodochrous*, based on the present work showing that they support the growth of the bacterium. Interestingly, *R. rhodochrous* B276 is capable of growth using short-chain unsaturated hydrocarbons (e.g., propylene and 1-butylene) as carbon sources as well. The pathway of propylene metabolism has been characterized for *R. rhodochrous* B276 (2) and *Xanthobacter* strain Py2 (1, 25) and shown to proceed through epoxypropane and acetooacetate as intermediates, as shown in reactions 2 and 3:

\[
\text{propylene} + O_2 + \text{NADH} + \text{H}^+ \rightarrow \text{epoxypropane} + \text{H}_2\text{O} + \text{NAD}^+ \quad (2)
\]

\[
\text{epoxypropane} + \text{NADPH} + \text{CO}_2 + \text{NAD}^+ \rightarrow \text{acetooacetate}
\]
The isomeric compounds epoxypropane and acetone, intermediates of unsaturated and saturated C3 hydrocarbon metabolism, respectively, are thus metabolized by carboxylation reactions that produce the common central intermediate acetocacetate.

While the carboxylation of epoxypropane and acetone form the same product, the reactions are catalyzed by distinct enzymes induced under different growth conditions. In addition, the cofactor requirements for the two reactions are dramatically different. In both _R. rhodochrous_ and _Xanthobacter_ strain Py2, epoxypropane carboxylation is catalyzed by a complex four-component enzyme system that requires NADPH and NAD+ as cofactors (3, 4). In contrast, acetone carboxylation requires energy input in the form of nucleoside triphosphate hydrolysis, as illustrated by the stoichiometry of reaction 1 for the _Xanthobacter_ strain Py2 acetone carboxylase.

The initial characterization of acetone carboxylase from _R. rhodochrous_ highlights similarities and differences with regard to the corresponding enzymes studied in _Xanthobacter_ strain Py2 and _R. capsulatus_. With regard to similarities, in all three cases acetone carboxylase activity correlates with the inducible, high-level production (10 to 20% of cell protein) of two polypeptides with apparent molecular masses of 70 to 75 and 80 to 85 kDa on SDS-PAGE (Fig. 5) (6, 23). A polypeptide with a molecular mass of 21 kDa constitutes the third subunit of the _Xanthobacter_ acetone carboxylase, which has an α2β2γ2 quaternary structure (23). A polypeptide of similar molecular weight, present in a stoichiometric ratio to the larger polypeptides, can be observed in the partially purified acetone carboxylase of _R. rhodochrous_ (Fig. 5). While a corresponding small peptide was not specifically noted for the partially purified acetone carboxylase of _R. capsulatus_, there appears to be a significant amount of protein at the dye front, present just under the 20-kDa marker protein, in the SDS-PAGE system of Birks and Kelly (see Fig. 4 in reference 6) if the acetone carboxylase of _R. capsulatus_ does indeed contain a similar peptide, it would indicate that the three acetone carboxylases of diverse bacteria contain a conserved subunit structure of (αβγ)2.

With regard to differences between the three acetone carboxylase systems studied in vitro, the nucleoside triphosphate specificity of the _R. rhodochrous_ enzyme is significantly different from that found for the _Xanthobacter_ strain _Rhodochrous_ acetone carboxylase (23). A distinguishing feature of the _R. rhodochrous_ acetone carboxylase is its ability to use other ketones (butanone, 2-pentanone, 3-pentanone, and 2-hexanone) as substrates at rates comparable to that with acetone. By comparison, only acetone and butanone (46% of the acetone consumption rate) were found to be substrates for the _Xanthobacter_ acetone carboxylase (23). Birks and Kelly have reported that butanone was not a substrate for the acetone carboxylase activity of _R. capsulatus_ (6). Thus, the _R. rhodochrous_ acetone carboxylase has a broader substrate specificity than the other acetone carboxylases studied.

It is intriguing that 3-pentanone is a substrate for the acetone carboxylase, due to the lack of a terminal carbon alpha to the carbonyl, a feature which was thought to be required for carboxylation. Instead, the symmetrical 3-pentanone contains two methylene carbon atoms flanking the carbonyl, both of which are sites representing greater steric hindrance to carboxylation relative to a terminal position. Nonetheless, 3-pentanone was metabolized at rates 40% of that observed for acetone. It is not known at present whether 3-pentanone or the other longer-chain ketones will support the growth of _R. rhodochrous_.

It should be noted that the specific activities measured in cell extracts and for the partially purified preparation of _R. rhodochrous_ acetone carboxylase are significantly lower than the rates observed in whole-cell suspensions. Typical rates for acetone degradation in whole-cell suspensions were in the range of 70 to 150 nmol of acetone degraded min⁻¹ mg⁻¹, while the rates in cell extracts were in the range of 1.5 to 3.0 nmol min⁻¹ mg⁻¹. The in vitro rates are thus only about 2% of the maximal rates observed in cell suspensions. At present it is not known why the in vitro rates are so much lower. Possible explanations include inactivation during and after cell lysis, nonoptimal assay conditions, absence or limitation of an unidentified cofactor, and/or the presence of inhibitory components in the assay (e.g., phosphatases or nucleotide hydrolases products). In light of the low activity, some caution must be exercised in definitively ascribing a physiological role to GTP in the acetone carboxylase assay. While acetone carboxylase activity was recovered at a good yield and with the expected fold purification (about fourfold) after a single DEAE-Sepharose column, further attempts to purify the enzyme resulted in low recoveries and decreases in the specific activity of the enzyme. Again, it is not known whether this loss of activity is due to inherent instability of the protein, the loss of a required cofactor, or some other phenomenon. Studies of other acetone-metabolizing bacteria have been similarly hampered by the complete or partial loss of acetone-degrading activity upon cell lysis. For example, no acetone carboxylase activity has to date been suc-
cessfully reconstituted in cell extracts of acetone-grown sulfate reducers, denitrifiers, or fermentative enrichment cultures (12, 20). Schink and coworkers have, however, successfully measured ADP-dependent acetococatalase activity in cell extracts of an acetone-grown denitrifying bacterium (20). In addition, they have demonstrated ADP-dependent 14CO\textsubscript{2} acetococatalase exchange in cell extracts from the same bacterium (12). Strong evidence was provided that both the decarboxylase activity and the exchange activity were catalyzed by the acetone carboxylase (12, 20). As noted earlier, ATP-dependent acetone carboxylase activity has been measured in vitro for \textit{R. capsulatus}. However, as for \textit{R. rhodochrous}, the specific activities in cell extracts of \textit{R. capsulatus} were dramatically lower (100- to 1,000-fold) than those in whole-cell suspensions (6). The only acetone carboxylase that can be reconstituted in vitro at physiologically relevant rates is the enzyme from \textit{Xanthobacter} strain Py2: this enzyme exhibits a specific activity in cell extracts equivalent to that in whole cells and can be purified in a fully active state (23). The only cofactors required by the \textit{Xanthobacter} enzyme are ATP and Mg\textsuperscript{2+}, while K\textsuperscript{+} exerts a stimulatory, but not essential, effect on activity (23). Further avenues need to be explored in order to optimize the in vitro activity of the \textit{R. rhodochrous} acetone carboxylase, with the hope of purifying the enzyme to homogeneity in an active state.

In summary, evidence has been provided for the presence of a nucleotide-dependent acetone carboxylase in the gram-positive actinomycete \textit{R. rhodochrous}. The occurrence of acetone carboxylases in two distantly related aerobic bacteria such as \textit{R. rhodochrous}, a gram-positive actinomycete, and \textit{Xanthobacter} strain Py2, a gram-negative eubacterium, provides further evidence that carboxylation may be a significant strategy of acetone metabolism in aerobic bacteria. The novel nucleotide usage and substrate specificity of the \textit{R. rhodochrous} acetone carboxylase set it apart from the \textit{Xanthobacter} strain Py2 and \textit{R. capsulatus} acetone carboxylases previously studied. This work provides additional insights into the novelty of bacterial acetone-degrading enzymes and their catalytic requirements.

**ACKNOWLEDGMENTS**

This work was supported by National Science Foundation grant MCB9630081.

We thank Miriam Sluis for helpful discussions and technical assistance.

**REFERENCES**