Bioconversion of Cinnamic Acid to Acetophenone by a Pseudomonad: Microbial Production of a Natural Flavor Compound

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A mutant derivative of a novel pseudomonad isolated from the soil accumulated acetophenone when supplied with cinnamic acid. The microorganism has been identified as an unclassified Pseudomonas sp., similar to Pseudomonas acidovorans. Mass spectrum analysis of the product acetophenone derived from catabolism of cinnamic acid in the presence of $^{18}$O or H$_2^{18}$O supported the conclusion that cinnamic acid degradation is initiated by addition of water to the double bond of the side chain, followed by dehydrogenation to generate 3-keto-3-phenylpropionic acid. The intermediate 3-keto-3-phenylpropionic acid is accumulated in cultures of the mutant during active cinnamic acid catabolism. However, this intermediate is unstable so a portion of it spontaneously decarboxylates to form acetophenone. Neither 3-keto-3-phenylpropionic acid nor acetophenone is a preceding intermediate in cinnamic acid degradation. Isolation of the novel strain and mutant provide the rudiments for a process to produce natural acetophenone by bioconversion of natural cinnamic acid.

The use of microbial fermentations by humans began in the food industry, first to preserve and enhance milk (e.g., cheese and yogurt) or beverages (e.g., wine and beer). This historical fact is responsible for the general view that microbial fermentations or bioconversions are natural. With the increased health consciousness that has arisen in America and Europe, consumers are increasingly choosing foods that are comprised of all natural ingredients. Many flavoring agents are the products of organic syntheses and are not considered natural. These syntheses are chosen because the process technology is well known and because of their generally low costs. With the increasing consumer demand for natural ingredients, and concomitant increase in product costs, microbes may be enlisted more frequently in the future to carry out biosyntheses and bioconversions of natural products to produce natural food ingredients.

We describe here the development of a process for the bioconversion of cinnamic acid, an abundant, natural, flavorless compound, to acetophenone. Acetophenone is useful as a component of flavors and fragrances; it is found naturally as a component of honey, plums, and strawberries. Bacteria were enriched from the soil for the ability to catabolize cinnamic acid and screened for use of a catalytic pathway through benzoic acid. Cells of isolates were mutagenized, selected for normal growth with benzoic acid as the primary source of carbon, and screened for impaired growth on agar media in which cinnamic acid was the primary available source of carbon. Some mutants of one of the isolates accumulated acetophenone and an unstable aromatic compound that spontaneously degraded to acetophenone when supplied with cinnamic acid as substrate. We propose that the unstable aromatic compound is 3-keto-3-phenylpropionic acid (3-KPP) and, although it was not expected, that this unstable compound is an intermediate in the pathway for the degradation of cinnamic acid.

**MATERIALS AND METHODS**

**Media.** Nutrient broth and broth were from Difco Laboratories, Detroit, Mich. Minimal medium salts contained the following, per liter: KH$_2$PO$_4$, 1.5 g; K$_2$HPO$_4$, 1.5 g; (NH$_4$)$_2$SO$_4$, 0.5 g; NaCl, 0.25 g; FeSO$_4$·7H$_2$O, 0.4 mg; MgCl$_2$, 15 mg. Filter-sterilized concentrated stocks of monosodium glutamate, cinnamic acid (pH 7 with NaOH), or benzoic acid (pH 7 with NaOH) were added to a final concentration of 0.5 g/liter as indicated in the text. Some media were further supplemented with yeast extract (0.5 g/liter; Difco) to stimulate growth rate or Bacto-Agar (15 g/liter; Difco), as indicated. Flasks were incubated on an orbital shaker rotating at 300 rpm, except where otherwise indicated.

**Isolation of the parent strain.** Bacterial strains were isolated from soil samples obtained from a local residential yard by first enriching for growth with cinnamic acid as the primary carbon source in liquid minimal medium and then selecting for growth of colonies on the same medium with 1.5% agar. Purified isolates were screened for simultaneous adaptation (7) to benzoic acid after growth on cinnamic acid, using an oxygen electrode chamber (Yellow Springs Instruments Co., Yellow Springs, Ohio), assuming that benzoic acid would be an intermediate in cinnamic acid degradation for microorganisms utilizing a pathway in which initial attack is on the 3-carbon side chain. An isolate designated 132 was found to be adapted to benzoic acid after growth on cinnamic acid and is the subject of the present research.

**Mutant isolation and characterization.** Log-phase cells of strain 132 were mutagenized by treatment with N-methyl-N'-nitro-N-nitrosoguanidine for 30 min at 5 μg/ml (6). Mutants were isolated by spreading on minimal salts agar with benzoic acid as the sole available source of carbon and screened by replica plating on two minimal agar medium plates with benzoic acid or cinnamic acid as the sole available source of carbon. Mutants which grew well on benzoic acid and poorly on cinnamic acid were purified, and the phenotype was confirmed by repeated comparison of the growth of the parental strain with that of the mutant strain on cinnamic acid and benzoic acid. Several mutants accumulated a pleasant-smelling aromatic compound identified as acetophenone (see below) when cultured on cinnamic acid-containing media. Mutant strain 132.6 accumulated the most acetophenone and has been deposited with the American Type Culture Collection, Rockville, Md., under accession number 53716. Strain 132.6 was identified by the American Type Culture Collection as an unclassified Pseudomonas sp.
which resembles *Pseudomonas acidovorans* and *P. testosteroni*.

**Analytical methods.** For initial determination of acetophenone and cinnamic acid concentrations in culture broths, we used the reagent 2,4-dinitrophenylhydrazine in a colorimetric assay (3) and the \( A_{360} \), respectively. Analyses were also performed by reversed-phase high-performance liquid chromatography (HPLC). After conversion of 3-KPP to acetophenone was observed (see Results), samples were routinely diluted twofold or more into methanol, filtered through a 0.22-µm filter, and warmed to 37°C for 1 to 2 h prior to chromatography to drive any 3-KPP to acetophenone. HPLC separation was isocratic on a 5-µm spherical C\(_18\) column (250 mm by 4.6 mm; catalog no. 68-2207-076; Isco, Lincoln, Neb.) eluted with 52% methanol in 0.5% acetic acid at a flow rate of 1 ml/min. Analytes were detected with an in-line spectrophotometer (model V4; Isco) with data acquisition and peak integration, using an IBM PC computer running the ChemResearch program of Isco.

Gas chromatography-mass spectrometry (GC-MS) analyses used a Varian 3400 instrument with a 15-m-long DB-5 coated capillary column and helium carrier flowing at 1.1 ml/min; temperature was programmed to hold at 50°C for 1 min and then increased 20°C/min for 10 min and finished with 5 min at 250°C. A 700 series ion trap detector (Finnigan Mat, San Jose, Calif.) was used to generate both total ion chromatograms and mass spectra for cinnamic acid and acetophenone peaks.

**Biotransformation in the presence of isotopic oxygen.** Cells of strain 132.6 were prepared by growth in a shaken culture containing 1 g of monosodium glutamate and 0.5 g of yeast extract per liter of minimal salts broth and frozen in 1-ml aliquots at −70°C in the presence of 5% glycerol. Single aliquots from a common cell preparation were used for each isotopic bioconversion; all isotopic bioconversions and controls were done simultaneously and in parallel. Aliquots were thawed, a measured volume (see below) was pelleted by centrifugation, the supernatants were thoroughly removed, and the cells were suspended in an equal volume of minimal medium salts containing cinnamic acid (0.5 g/liter) as the sole added source of carbon.

For the \( ^{18} \text{H}_2\text{O} \) experiments, 0.25-ml volumes of a single batch of minimal salts-cinnamic acid broth were lyophilized to dryness and suspended in \( ^{18} \text{H}_2\text{O} \) (catalog no. 550486, 97 to 99% \( ^{18} \text{O} \); ICN Biomedicals, Inc., Cambridge, Mass.) or deionized tap water (i.e., >99% \( ^{16} \text{H}_2\text{O} \)) prior to their use for suspension of the packed cells.

For \( ^{18} \text{O} \) experiments and controls, the resuspension of packed cells in 1 ml of the minimal salts-cinnamic acid broth took place in an anaerobic glove box chamber (<2 ppm of \( \text{O}_2 \) [<2 µl/liter]). The cell suspensions were placed in 25-ml glass test tubes that had been prepared as follows: crimp-sealed tubes were alternately evacuated and filled with nitrogen, repeating the evacuation-flush cycle five times. Finally, 5 ml of nitrogen was removed from each tube and replaced with 5 ml of oxygen gas (catalog no. 550483, 98 to 99% \( ^{16} \text{O}_2 \); ICN Biomedicals). A control tube was filled with normal room air (ca. 21% \( ^{18} \text{O}_2 \)) following the fifth evacuation.

For both sets of experiments, tubes were incubated with gentle shaking (ca. 100 rpm) at 25 to 30°C; a quantity of cinnamic acid equivalent to the initial quantity (0.5 g/liter) was added to each set of tubes twice (at 20 and 27 h of incubation), resulting in a cumulative input of 1.5 g of cinnamic acid per liter of broth. After incubation of the cultures for 70 h (total), the tubes were opened, and the broths were immediately removed and filtered through a 0.22-µm-pore-size membrane filter to remove the cells. The filter effluents were collected in 1.5-ml microcentrifuge tubes and stored frozen at −20°C until the time of GC-MS analysis.

**RESULTS**

**Isolation of mutant 132.6.** Cinnamic acid-degrading microorganisms were isolated from soil samples by enrichment and selection for utilization of cinnamic acid as the primary carbon source. Isolates were screened for evidence that the degradation of cinnamic acid was initiated by attack on the 3-carbon side chain. An isolate designated strain 132 was found to be adapted to benzoic acid after adaptation to cinnamic acid. To obtain mutants impaired in their ability to perform any step in the degradation of the cinnamic acid side chain, mutagenized cells of strain 132 were screened for impaired growth when cinnamic acid was the sole available carbon source and for normal growth when benzoic acid was the sole available carbon. Some mutant derivatives produced a pleasant aroma when grown on cinnamic acid-containing plates and broths. One of those, designated strain 132.6, was selected for further characterization.

**Identification of acetophenone in broth.** HPLC chromatograms of broth samples from mutant strain 132.6 had two major UV-absorbing peaks with retention times of 5 and 9 min (Fig. 1A) in addition to the residual substrate peak (retention time, 10.5 min) when sampled during active cinnamic acid utilization. Broths sampled after continued incubation following depletion of the cinnamic acid had a single major peak with a retention time of 9 min (data not shown). Repeated injection of the methanolic sample used for Fig. 1A

![FIG. 1. Detection of two aromatic metabolites of cinnamic acid and spontaneous conversion of the 5-min analyte to the 9-min analyte. HPLC analysis of a single sample of broth from a culture of the mutant strain 132.6 taken during active cinnamic acid catabolism and chromatographed shortly after sample preparation (A) and 1 (B) and 2 (C) h after the initial injection. Insets show the structures of cinnamic acid (A; retention time, ca. 10 min), 3-KPP (B; inferred to be the analyte with a retention time of ca. 5 min), and acetophenone (C; inferred to be the analyte with a retention time of ca. 9 min).](http://aem.asm.org/article/1/1/624/1/fig1.jpg)
showed that the analyte responsible for the 5-min peak was quantitatively converted to the analyte responsible for the 9-min peak with time (Fig. 1B and C and Table 1). The analyte responsible for the 9-min peak had a pleasant odor, formed an orange-red precipitate when reacted with acidic 2,4-dinitrophenylhydrazine, and was radioactive when [3-14C]cinnamic acid was fed (data not shown). These observations demonstrated that the 9-min analyte had a carbonyl oxygen and was derived from cinnamic acid. Acetophenone standard had a retention time and odor identical to those of the 9-min analyte (data not shown). When other broths were analyzed later by GC-MS, a single peak with the retention time and mass spectrum of acetophenone was observed (see below). We infer that the unstable analyte with a retention time of 5 min in HPLC analysis is 3-KPP by analogy with the conversion of acetoacetic acid to acetone plus carbon dioxide (ketogenesis in diabetics) and based on the observation of the decarboxylation of 3-KPP to acetophenone in the analysis of horse urine (5). No evidence of 3-KPP was seen in GC analyses, presumably due to the high chromatography temperature and the instability of that compound.

**TABLE 1.** Quantitative conversion of the analyte with a retention time of 5 min to the analyte with a retention time of 9 min

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Corresponding chromatogram</th>
<th>Relative areas under peaks 5 min</th>
<th>Relative areas under peaks 9 min</th>
</tr>
</thead>
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<tr>
<td>0.0</td>
<td>Fig. 1A</td>
<td>0.94</td>
<td>0.50</td>
</tr>
<tr>
<td>0.5</td>
<td>Not shown</td>
<td>0.82</td>
<td>0.68</td>
</tr>
<tr>
<td>1.0</td>
<td>Fig. 1B</td>
<td>0.71</td>
<td>0.83</td>
</tr>
<tr>
<td>1.5</td>
<td>Not shown</td>
<td>0.66</td>
<td>0.89</td>
</tr>
<tr>
<td>2.0</td>
<td>Fig. 1C</td>
<td>0.51</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*Areas under the peaks were determined from the chromatograms shown in Fig. 1 (times zero and 1.0 and 2.0 h) and two additional chromatograms (not shown) from analyses run at 0.5 and 1.5 h after the initial injection (0.0 h).

Identification of the origin of the carbonyl oxygen of acetophenone. Detection of the two unexpected metabolites of cinnamic acid spurred our interest in the relationship of the cinnamic acid degradation pathway of strain 132 to other reported pathways. The compound 3-KPP has one oxygen atom more than its precursor, cinnamic acid; thus, identification of the origin of the carbonyl oxygen introduced during conversion of cinnamic acid to acetophenone by strain 132.6 would allow inference of whether the initiating reaction was by hydration or by an oxygenase. Cultures of the mutant strain 132.6 were prepared to allow bioconversion of cinnamic acid to acetophenone under controlled atmospheric conditions wherein 18O2 and H216O or, separately, 16O2 and H218O would be the predominant available sources of atomic oxygen. Bioconversion in the presence of isotopic oxygen in the form of H218O resulted in the shift in m/z of acetophenone from 120 to 122 and a shift of +2 for its oxygen-containing ions (Fig. 2), proving that the carbonyl oxygen of acetophenone was derived from addition of water to the substrate cinnamic acid. The sample containing 18O2 and H218O (Fig. 2B) appears identical to the controls in which no isotopically enriched oxygen was present (Fig. 2A).

Comparison of the parent and mutant strains in shaken culture. Strains 132 and 132.6 were inoculated into flasks containing nutrient broth supplemented with cinnamic acid, and the flasks were incubated with shaking at 30°C and sampled occasionally for determination of turbidity, pH, and cinnamic acid and acetophenone concentrations (Fig. 3).

**FIG. 2.** MS analyses of acetophenone formed from cinnamic acid by strain 132.6 and isolated by GC. Bioconversion was in the presence of H216O and 18O2 (A), H218O and 16O2 (B), and H218O and 16O2 (C). Inflected structures of four dominant ions are shown in panel A. Oxygen-containing ions are highlighted with arrows in panel C.

Growth of strains 132 and 132.6 was similar initially, but became dissimilar when disappearance of cinnamic acid became rapid (ca. 19 to 20 h). At 22 to 24 h the turbidity of the 132 culture continued upward, approaching an optical density of 2, while the 132.6 culture leveled near 1.5 and
increased very slowly thereafter. The flasks were supplemented with additional cinnamic acid (to 2 g/liter concentration) at 24 h, after depletion of that present initially. The apparent acetophenone concentration (3-KPP plus acetophenone) reached a maximum near 45 h and then declined to a stable final concentration. The pH increased steadily throughout these and other (not shown) experiments and ultimately may have limited growth and acetophenone production in some experiments. When flasks similar to those described in Fig. 3 were repeatedly fed with cinnamic acid, the pH ultimately reached as high as 9.0 and cinnamic acid utilization ceased in spite of the presence of ample residual cinnamic acid (data not shown).

The final molar efficiencies for conversion of cinnamic acid to acetophenone were 0.4% for parent strain 132 and 10.4% for the overproducing mutant, strain 132.6, at termination of the experiment shown in Fig. 3. The acetophenone concentration was 806 mg/liter at 45.5 h for a peak potential molar conversion efficiency of 39%. In other experiments, the apparent efficiency of conversion ranged from <1 to 33% for the parent strain and from 6 to 65% for the strain 132.6 (data not shown).

No utilization of acetophenone by the parent and mutant strains. Typical culture analyses, such as those described in Fig. 3, commonly showed higher apparent concentrations of acetophenone when significant concentrations of cinnamic acid were present than later in the same fermentation after cinnamic acid was depleted. However, acetophenone concentrations appeared stable for weeks after a final titer was determined. To determine whether acetophenone could be utilized by strains 132 and 132.6, shake flasks containing minimal medium salts were supplemented with 1 g of monosodium glutamate and 0.3 g of acetophenone per liter of broth and inoculated with either strain 132 or 132.6 or left uninoculated. The flasks were incubated in a laboratory shaker, and samples were taken daily for analysis of growth parameters and residual concentrations of acetophenone. Although the concentration of acetophenone decreased slightly during incubation, it decreased at an equivalent rate when inoculated with strain 132 or 132.6 or when uninoculated (Fig. 4). One pair of flasks was supplemented with both cinnamic acid (0.5 g/liter) and acetophenone (0.3 g/liter) prior to inoculation with mutant strain 132.6 or its parent. No evidence of utilization of acetophenone was observed, although the acetophenone was present throughout the time that the cultures completely utilized the cinnamic acid.

**DISCUSSION**

We have observed formation of acetophenone and an unstable metabolite believed to be 3-KPP from cinnamic acid, catalyzed by a novel pseudomonad isolated from the soil. Neither acetophenone, 3-KPP, nor ring-modified congeners of these two compounds have been reported as intermediates in the catabolism of cinnamic acid or its ring-modified congeners, nor were they predicted in prior literature. Thus, the detection of acetophenone and 3-KPP has implications regarding the pathway of cinnamic acid catabolism and provides the basis for an unexpected natural bioconversion process. A mutant derivative was isolated that forms acetophenone more efficiently than the initial isolate, presenting the possibility of a commercial bioconversion process.

Based on the failure of strains 132 and 132.6 to utilize exogenous acetophenone, even while actively catabolizing cinnamic acid, we conclude that acetophenone is not an intermediate in the normal catabolic pathway for cinnamic acid degradation used by the soil isolate, strain 132 (Fig. 5). We conclude, however, that 3-KPP, or the tautomer with which it is in equilibrium, is a normal intermediate in the cinnamic acid degradation pathway of *Pseudomonas* sp. strain 132. This conclusion is based on the above-mentioned observation that acetophenone itself does not appear to be utilized by the cells and the observation that the apparent acetophenone concentration (which represents the sum of the concentrations of 3-KPP and acetophenone) is generally
higher during active cinnamic acid utilization than the final acetophenone concentration. Alternatively, it is possible that both 3-KPP and acetophenone are intermediates but only 3-KPP can be transported into the cells. The putative intermediate 3-KPP differs from the substrate cinnamic acid by the addition of 1 oxygen atom. Since that oxygen is derived from H2O, not O2, there must be a dehydrogenation step following the hydration of cinnamic acid.

Toms and Wood (8) observed that catabolism of ferulic acid (m-methoxy-p-hydroxycinnamic acid) by P. acidovorans required no more oxygen than an equal molar amount of vanillin and further demonstrated that the 2-carbons initially removed from ferulic acid were lost as acetic acid. Based primarily on these observations, Toms and Wood (8) proposed that hydration initiates the degradation of ferulic acid, followed by release of acetic acid and vanillin. Karanth and Reber (4) made concurring observations regarding the release of acetic acid in catabolism of 4-hydroxycinnamic acid by P. testosteroni, another pseudomonad taxonomically related to P. acidovorans and Pseudomonas sp. strain 132. However, neither group reported detection of anything analogous to 3-KPP and assumed that the acetic acid removal step immediately follows hydration. The presence of 3-KPP as an intermediate of cinnamic acid catabolism in culture broths of Pseudomonas sp. strain 132 raises the possibility that the respective ring-modified analogs of 3-KPP are intermediates in ferulic degradation by P. acidovorans and p-hydroxycinnamic acid degradation by P. testosteroni. Microbial and enzymatic bioconversion processes are considered natural; thus, if natural cinnamic acid were used as substrate in a bioconversion process catalyzed by strain 132.6, the product acetophenone would also be natural. No efficient process for the production of natural acetophenone has been reported previously, although acetophenone and related, ring-modified compounds have been detected in biological samples. Chloroacetophenone is formed from catabolism of chlorinated biphenyls by microbes of several genera (1, 2). In addition, when grown on blood agar plates, P. aeruginosa produces a characteristic grapelike odor that has been identified as due to 2-aminoacetophenone (2a); the metabolic origin of 2-aminoacetophenone was not reported. Other examples of low levels of biologically derived acetophenones also exist; however, none of these appear to be derived from catabolism of cinnamic acid or its congeners.

Attempts to define the comparative efficiency of conversion of cinnamic acid to acetophenone were plagued by lack of reproducibility, appearing to range from 0 to 30% molar efficiency with the parent strain 132 to a range of 6 to 65% molar efficiency with the overproducing mutant, strain 132.6 (unpublished observations). Most of this variability can be attributed to the unstable nature of 3-KPP which, if present in a sample, would decarboxylate to acetophenone but can apparently be degraded by two competing pathways in the culture broths. 3-KPP accumulates in cultures of both strains 132 and 132.6 during active cinnamic acid catabolism, but appears transiently, to be either further catabolized by the cells or spontaneously decarboxylated to acetophenone. The highest apparent efficiencies of conversion were seen when residual cinnamic acid was present, presumably continuing to supply the acetophenone precursor. The mutation carried by strain 132.6 affects the distribution of 3-KPP between these alternative paths, probably due to a reduced rate of biological degradation compared with the parental strain. Other factors, such as the pH of the culture broth and conditions of the cells, may also influence this distribution. Isolation of a mutant more tightly blocked in cinnamic acid catabolism or development of a continuous extraction process could be used to attain a higher bioconversion process efficiency.

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LITERATURE CITED