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Styrene Formation by the Decomposition by *Pichia carsonii* of *trans*-Cinnamic Acid Added to a Ground Fish Product

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It is not well known how the formation of styrene by microorganisms can occur in foods. In this study, we described and characterized the production of styrene by a yeast isolated from chikuwa fish paste. The styrene was not detected in fresh and normal food products nor in the food package's plastic film. The food containing styrene contained cinnamic acid as an antimicrobial agent and spice, and it was contaminated by 5.4×10^6 CFU of a yeast per gram. On the basis of morphological and biochemical features, the yeast isolated was determined to be a strain of *Pichia carsonii*, now designated strain CHI. Strain CHI, which was able to grow on cinnamic acid, had the ability to form styrene from *trans*-cinnamic acid via *trans-p*-coumaric and caffeic acids. The MIC of *trans*-cinnamic acid against strain CHI was 230 μg/ml. Strain CHI thrived well at pH 5.0 and 26.0°C and was tolerant to 20% NaCl. Styrene was subsequently produced in ground fish meat containing cinnamic acid into which strain CHI had been inoculated. The yeast was found to be an environmental contaminant in food processing plants of the chikuwa manufacturer.

A primary objective during food distribution is to preserve the integrity of the product and to ensure its safety throughout the entire distribution system. The efforts of industry, academia, and regulatory and public health agencies have been directed towards a better understanding of the nature of the problem and the development of prevention and control strategies (19). The growth of microorganisms in foods is affected by environmental factors such as temperature, pH, water activity, and atmosphere and by the presence or absence of preservatives. There is, at present, increased consumer pressure to reduce the amount of additives in foods as well as a general preference for fresh, "natural" food. The handling of foods should be done with strict adherence to storage temperature recommendations (4°C or below) and should maintain package and product integrity. However, psychrotolerant microbes are capable of growing on foods at the refrigeration temperature to which they are normally exposed during storage, transport, and marketing (1, 13, 19).

Many foods are odorous in themselves, but aromas of various foods are occasionally detected in processed foods after preservation. It has been reported that aromas are caused by microorganisms and packaging materials. Many workers (7, 16, 20, 29) reported that the aromas of foods were caused by ethyl acetate, which a yeast, *Hansenula anomala*, produced. Tatsuno (26) and Yamashita et al. (30) reported that small amounts of styrene were readily transferred to food from polystyrene containers. Recently, we found that a petroleumlike aroma, different from that of ethyl acetate, was produced in chikuwa (a kind of fish paste). The aroma resulted in an abnormal and unpleasant taste. The compound associated with the obnoxious odor was identified as styrene. The permissible dose of styrene in foods has not been determined. According to Japanese environmental law (11), however, the maximum permissible exposure to styrene is 50 ppm in the air. Since this unusual formation of a hydrocarbon in foods has not been described previously (with the exception of styrene production by *Torulopsis candida* in seasoned herring roe [23]), we undertook to establish the origin of styrene associated with chikuwa fish paste.

MATERIALS AND METHODS

Occurrences of cases. Three reports were made by consumers who bought the packed and processed food, chikuwa (a stick-shaped fish cake), associated with a petroleumlike aroma or an obnoxious odor in Hiroshima, Osaka, and Kyoto, in September 1990. The food was manufactured by a company in Hyogo, Japan. The food was refrigerated at 4°C or below by consumers and used within recommended dates. The process of making chikuwa involves three steps. First, the pureed fish is blended with a binding agent and molded around a stainless steel rod. Second, the fish paste is steamed. Third, the resulting fish stick is grilled to color the outer layer brown. The fish product becomes firm when cooled, closely resembling bologna in texture. The ingredients of chikuwa are pureed white fish (such as cod, porgy, or shark), a binding agent such as starch to help mold the fish paste, salt, seasoning, and food additive P, an antimicrobial agent. Food additive P consists of sodium acetate (37.5%), cinnamic powder (20.0%, half of which is cinnamic acid), fumaric acid (15%), phytic acid (6%), vitamin B_1 (1.8%), and dextrin (19.7%).

Microbiological analysis. The foods associated with a petroleumlike aroma were obtained from a consumer. Handling of foods was done promptly and with strict adherence to low temperatures to avoid recontamination. After sampling and appropriate dilution, viable counts were made in

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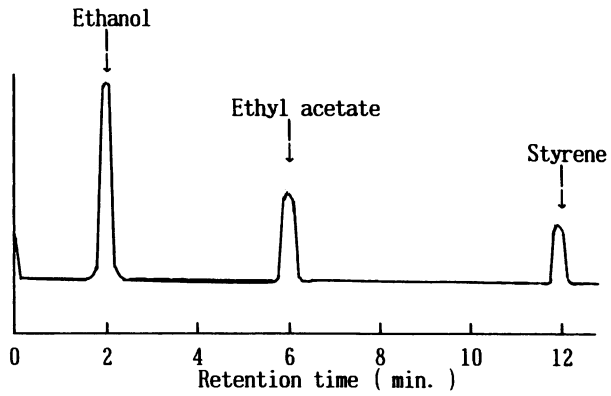

FIG. 1. Gas chromatogram of food associated with petroleum-like aroma. Authentic standards are indicated by vertical arrows. GC was done on 20% PEG 20M and Chromosorb 101 glass column with a flame ionization detector and N_2 at 30 to 50 ml min^{-1}. See Materials and Methods for details.

duplicate from two consecutive 10-fold dilutions. Yeast growth was measured by performing viable plate counts with Sabouraud dextrose agar (Oxoid Ltd., Basingstoke, England) and potato dextrose agar (Difco Laboratories, Detroit, Mich.) supplemented with chloramphenicol (50 μg/ml). Plates were incubated for 5 days at 25°C. Total bacterial counts were also done, using the standard method agar for plate counts (Nissui Ltd., Tokyo, Japan) and blood agar (Difco) under aerobic and anaerobic conditions, respectively. These incubations were done for 48 h at 36°C.

Analysis of odor compounds. The analytical method for identifying odor-causing compounds (styrene, ethanol, and ethyl acetate) in food (chikuwa) and culture media was developed by using gas chromatography (GC) (22). For extraction of the compounds, cells of the isolates were cultured in a liquid medium (see below) and removed from the culture fluid by membrane filtration (pore size, 0.45 μm; Millipore Products Division, Bedford, Mass.). The filtrate was extracted with *n*-hexane. Chikuwa was also homogenized with *n*-hexane, and then the *n*-hexane layer was separated. Each extract was injected into the GC column. The GC apparatus used consisted of a Hitachi 263-50 gas chromatograph (Hitachi Ltd., Tokyo, Japan) equipped with a flame ionization detector (Hitachi). In order to detect styrene, the column used was a glass column (2 m by 3 mm) packed with 20% polyethylene glycol (PEG) 20M (60/80 mesh) and with Chromosorb 101 (60/80 mesh) (Wako Pure Chemical Industries Ltd., Osaka, Japan) to detect ethanol and ethyl acetate. GC operating conditions were as follows: injector, column, and detector temperatures for PEG 20M were 200, 110, and 210°C, respectively, and for Chromosorb 101 were 180, 140, and 210°C, respectively. The carrier gas was N_2 at a flow rate of 30 to 50 ml min^{-1}. Additionally, to determine concentrations of compounds, cultures were periodically extracted with *n*-hexane, and the extracts were analyzed by GC as described above. The concentration of each compound in food and culture medium was calculated by use of linear calibration curves of authentic standards from the chromatographic peak area.

Chemicals. Food additive P was obtained from Y. Kasei Co., Tokyo, Japan. Styrene monomer, sodium acetate, fumaric acid, and thiamine hydrochloride were obtained from Wako. Phytic acid was obtained from Sigma Chemical Co., St. Louis, Mo. Dextrin was obtained from Difco.

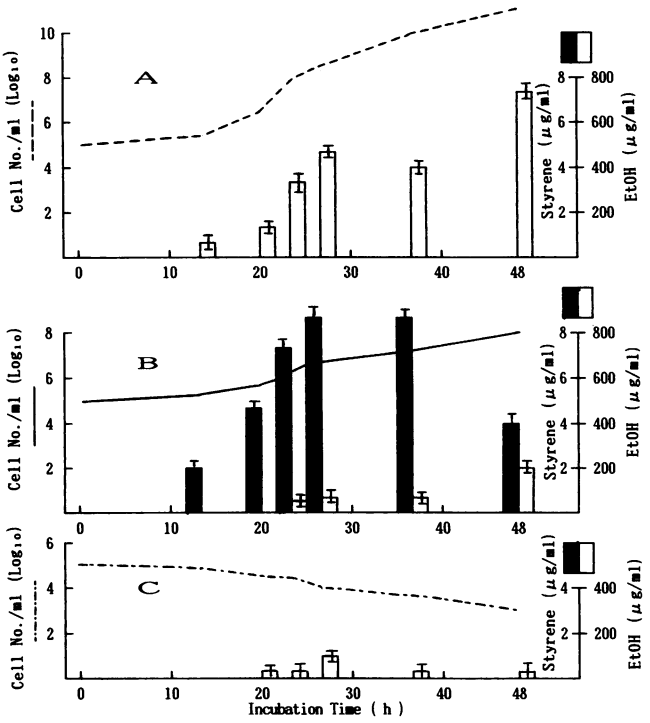

FIG. 2. Time course of styrene production by strain CHI. Fifty milliliters of GYP medium without food additive P (A) or with 0.1% (B) or 0.2% (C) food additive P was poured into a 100-ml Erlenmeyer flask. The flask was sealed tightly and incubated at 25°C during the reaction. Open bars, ethanol (EtOH); solid bars, styrene. Standard deviations of triplicate samples are indicated.

trans-Cinnamic acid was obtained from Kanto Chemical Co., Tokyo, Japan. DNAs originating from *Escherichia coli* strain B type VIII and *Micrococcus lysodeikticus* type XI were purchased from Sigma.

Measurement of growth. Growth of the cultures was monitored turbidimetrically at 500 nm with a Coleman Junior II model 6/20 spectrophotometer (Coleman Instrument Co., Maywood, Ill.). Ten-milliliter portions of potato dextrose broth (Difco) in tubes were adjusted to pHs from 3.0 to 11.0 with HCl and NaOH and were inoculated with 0.2 ml of an overnight culture of each isolate. The optimal growth temperature was determined on the potato dextrose broth with a temperature gradient incubator model TN-3 (Toyo Kagaku Sangyo Co. Ltd., Tokyo, Japan) at temperatures in the range from 4 to 50°C.

Formation of odor by isolates. The GYP medium used to determine the odor-causing agent contained the following (in grams per liter): glucose (Wako), 20; yeast extract (Difco), 1; and polypeptone (BBL Microbiological Systems, Cockeysville, Md.), 5. The isolates from chikuwa were first grown on GYP medium with or without food additive P for 48 h at 25°C, and odors were tested by sniffing. After incubation for 5 days at 25°C, this culture was used to inoculate the production medium to a final amount of 2% (vol/vol). Inoculum and production cultures were prepared in 100-ml Erlenmeyer flasks containing 50 ml of medium supplemented with the following substances (per liter) instead of food additive P: *trans*-cinnamic acid, 0.1 g; phytic acid, 60 mg; dextrin, 0.2 g; sodium acetate, 0.38 g; thiamine HCl, 18 mg; and fumaric acid, 0.15 g. The broth was analyzed by GC as described above.

TABLE 1. Styrene odor by strains CHI and ITA in GYP medium supplemented with various substances[a]

Test no.	Supplement							Styrene odor[c] produced by strain:	
	FAP[b]	Cinnamic acid	Phytic acid	Dextrin	Sodium acetate	Thiamine	Fumaric acid	CHI	ITA
1								−	−
2	●							6+	6+
3		●	●	●	●	●	●	6+	6+
4		●	●	●		●	●	6+	4+
5		●		●	●	●	●	6+	5+
6		●			●	●	●	6+	6+
7		●			●		●	5+	6+
8		●				●	●	5+	4+
9		●					●	5+	4+
10		●				●		6+	5+
11		●			●			5+	5+
12		●		●				6+	5+
13		●	●					6+	6+
14		●						5+	6+
15			●	●	●	●	●	−	−

[a] Isolates were inoculated into GYP medium supplemented with the substances denoted by filled circles.
[b] FAP, food additive P.
[c] The odor produced in each test was sniffed and expressed as positive intensity by three panelists after 24 h of cultivation at 25°C. −, negative (no odor). The odor was confirmed as styrene by GC. See Materials and Methods for details.

Identification of isolates. The isolates were identified by using the methods described in standard taxonomic manuals (1, 4, 14). DNAs were purified by using the method of Marmur (17), and moles percent G+C contents were estimated by the spectroscopic method of Ulitzur (27) with DNAs from *E. coli* B and *M. lysodeikticus* as reference standards. *Saccharomyces cerevisiae* IFO 0205 and *Pichia carsonii* IFO 0946 were also used in this study as reference yeast strains.

RESULTS

Odors and microbiological analysis in food. Although about 310,000 chikuwas were manufactured by the company in Hyogo, they had already sold out. Therefore, they could not be obtained from the retail stores or distribution system. Three claims were made from consumers of chikuwa with a petroleumlike aroma. One of these three cases was studied by us. The odor-causing compounds were identified by GC. Gas chromatograms of the product with petroleumlike aroma and authentic standards are presented in Fig. 1. In the case of the normal product, only a peak corresponding to ethanol was detected (data not shown). However, in the case of the product with petroleumlike aroma, three peaks were found. The first peak was ethanol, the second was ethyl acetate, and the third was at the same retention time as styrene (Fig. 1). Ethanol, ethyl acetate, and styrene were contained in the product at 2,550, 93, and 44 ppm, respectively. The odor of the product with petroleumlike aroma was very similar to that of an authentic styrene as determined by sensory tests. Another two cases were analyzed by Adachi and co-workers and by Tsue and co-workers. They also detected styrene (personal communication). These results implicated styrene as one of the causative compounds of the odor. However, styrene was not detected in the packaging materials of the final product. The microbiological analysis resulted in a yeast being isolated directly from the 1:10 food homogenate; there was 5.4×10^6 CFU of the yeast per gram of food. No other microorganisms were detected. A yeast which was morphologically very similar to the strain isolated from the food was isolated from the cold room (capacity, 300 liters; regulatory temperature, about 10°C) used for thawing the frozen ground fish meat at the manufacturing company. The isolates from chikuwa with petroleumlike aromas and from the cold room were designated strains CHI and ITA, respectively.

Tolerance to *trans*-cinnamic acid. Potato dextrose broth media that contained different concentrations of *trans*-cinnamic acid and food additive P were inoculated with strain CHI. The highest cinnamic acid concentration at which growth was observed was 200 μg/ml. The MICs of cinnamic acid and food additive P were 230 and 1,500 μg/ml, respectively.

Formation of odor by isolates. When strain CHI was inoculated into the GYP medium with food additive P, a petroleumlike aroma was produced. The odor was compared with that of an authentic styrene by three panelists and was determined to be very similar to that of an authentic styrene. The odor compounds were also identified as styrene by GC. Styrene was produced whenever strain CHI was inoculated into the medium with food additive P. The same results were also obtained with the ground fish meat containing food additive P. The formation of styrene was characterized by an initial population of approx. 10^5 CFU of strain CHI per ml that progressively grew to 10^8 CFU/ml during the course of the 48-h incubation, as shown in Fig. 2. Ethanol was produced only in the medium without food additive P (Fig. 2A), whereas styrene, of which the maximum amount was obtained after incubation for between 28 and 38 h, was found in the medium with 0.1% food additive P (equivalent to 100 μg of cinnamic acid per ml) (Fig. 2B). In the medium with 0.2% food additive P, only a small amount of ethanol was produced, and growth was inhibited (Fig. 2C). However, even when the ground fish meat with 0.5% food additive P (equivalent to 500 μg of cinnamic acid per g) was inoculated with the yeast at 9.7×10^5 CFU/g and incubated at 25°C for 24 h, the formation of styrene was observed, and the yeast grew to 8.9×10^6 CFU/g (data not shown). From the results of the experiments using components of food additive P (Table 1), we concluded that styrene was formed only in a

TABLE 2. Characteristics of isolated strains, *P. carsonii*, and *S. cerevisiae*[a]

Characteristic	Strain CHI[b]	Strain ITA[c]	*P. carsonii*	*S. cerevisiae*
Anaerobic growth[d]	–	–	–	w[e] (5)
Aerobic growth[d]	+ (3)	+ (3)	+ (3)	+ (3)
Growth[f] at:				
4°C	+ (14)	+ (14)	+ (14)	+ (3)
25°C	+ (2)	+ (2)	+ (2)	+ (1)
36°C	–	–	–	+ (1)
40°C	–	–	–	–
NaCl tolerance[f]:				
10%	+ (2)	+ (3)	+ (5)	+ (13)
20%	+ (30)	+ (32)	+ (32)	–
Pellicle[f]	–	–	+ (10)	–
Pseudo/true hyphae	–	–	–	–
Chlamydospores	–	–	–	–
Ascospores	Spherical	Spherical	Spherical	Spherical
Germ tube	–	–	–	–
Capsule	–	–	–	–
Urease	–	–	–	–
KNO_3 utilization	–	–	–	–
Arbutin degradation	+ (3)	+ (5)	+ (3)	–
Assimilation of:				
D-Glucose	+ (4)	+ (4)	+ (4)	+ (2)
D-Xylose	–	–	–	+ (4)
Maltose	+ (4)	+ (7)	+ (4)	+ (4)
Trehalose	+ (5)	+ (6)	+ (5)	–
Raffinose	+ (4)	+ (6)	–	+ (4)
Cellobiose	+ (6)	+ (6)	+ (6)	–
Lactose	–	–	–	–
D-Galactose	+ (5)	+ (6)	+ (6)	+ (4)
Sucrose	+ (4)	+ (6)	+ (6)	+ (2)
Inositol	–	–	–	–
Dulcitol	–	–	–	–
Starch	–	–	–	–
Fermentation of:				
D-Glucose	–	–	–	+ (2)
D-Xylose	–	–	–	–
Maltose	–	–	–	+ (4)
Trehalose	–	–	–	–
Raffinose	–	–	–	+ (5)
Cellobiose	–	–	–	–
Lactose	–	–	–	–
D-Galactose	–	–	–	+ (4)
Sucrose	–	–	–	+ (2)
Inositol	–	–	–	–
Dulcitol	–	–	–	–
G+C content (mol%)	39.9	40.8	39.1	36.3

[a] Numbers within parentheses show days of incubation.
[b] Isolated from chikuwa with aroma.
[c] Isolated from cold room of manufacturing company.
[d] On agar plate.
[e] Weak growth.
[f] In broth.

medium containing cinnamic acid, which was confirmed by GC. These results indicated that the yeast isolated from chikuwa associated with petroleumlike aromas (strain CHI) converted the cinnamic acid in food additive P to styrene. The same results were also obtained for strain ITA (Table 1).

Characterization of isolates. Strains CHI and ITA grew in potato dextrose broth media at temperatures in the range from 4 to 29°C and at pHs between 4.0 and 10.5. The optimum temperature and pH were 26°C and pH 5.0. The phenotypic characteristics of the isolated strains of the yeast, as well as their G+C contents, are shown in Table 2. Strains CHI and ITA exhibited quite similar characteristics. Estimates of the G+C content were 39.9 ± 2.5 mol% for strain CHI and 40.8 ± 2.5 mol% for strain ITA. The characteristics of both strains were identical to those of *P. carsonii* IFO 0946, except for pellicle formation in broth and the assimilation of raffinose. They were identified as *P. carsonii* CHI and ITA on the basis of their morphological and biochemical properties.

DISCUSSION

The mass production of wholesome, nutritious, and safe food products in industrialized countries is based on manufacturing, packaging, storage, and distribution technologies. At the same time, however, extra handling of food products creates additional risks associated with increased spoilage and incidence of microbes. It has been known that the odors of foods are sometimes caused by ethyl acetate, which is produced by a yeast, *Hansenula anomala* (7, 16, 20, 29). However, contamination of food with styrene produced by yeasts is rare. Sato et al. (23) reported that styrene was produced by a yeast, *Torulopsis candida*, in seasoned herring roe, although the precursor was not clear. There is no other paper indicating the microbial formation of styrene in foods. Within the food industry, yeasts play important roles in both production and spoilage. In higher plants, cinnamic acid is an intermediate in the isoflavonoid biosynthesis (8, 12). Cinnamic acid is widely used as a natural preservative or spice in various foods (18). This paper describes the formation of styrene from *trans*-cinnamic acid by a yeast, *P. carsonii* CHI, contaminating chikuwa with petroleumlike aromas. The same yeast was also isolated from the cold room of used for thawing the raw material the frozen ground fish meat, at the manufacturing company. From these results, we concluded that the food was contaminated with the yeast during thawing and that the yeast converts cinnamic acid, added as an antimicrobial agent, to styrenes. Styrene is readily transferred to food from polystyrene containers (26, 30), but in this case styrene was not detected from any other material such as packaging films, fish meat, and normal food products. The yeast's growth was inhibited with 0.2% food additive P medium (Fig. 2); however, the yeast grew with 0.5% food additive P in ground fish meat (data not shown). This result indicates that the effect of food additive P as an antimicrobial reagent may be lost in ground fish meat.

The success of cold-temperature storage in food preservation hinges on the general inability of spoilage and pathogenic microorganisms to proliferate under these restrictive growth conditions. Maintenance of cold temperatures during processing selectively favors psychrotolerant microbes. The growth of these microbes is influenced by numerous factors, including nutrient availability, pH, water activity, and composition of the atmosphere in contact with the surfaces of products. The presence of cold-tolerant microbes may constitute a special health hazard in refrigerated foods. The yeast contamination of ready-to-eat food such as chikuwa is of great concern, especially with regard to food items kept for a long period at refrigeration temperature. Even when initial cell numbers are small, some foods are good substrates for the growth of yeasts and may permit considerable multiplication of a yeast during the storage period. As noted by Palumbo (21), refrigeration can no longer be deemed sufficient to keep foods safe from microbial hazards.

Interestingly, we also observed that *P. carsonii* IFO 0946 converts cinnamic acid to styrene about 2 weeks after incubation of the cells at 25°C, but we did not observe cinnamic acid production by *S. cerevisiae*, *S. ellipsoideus*,

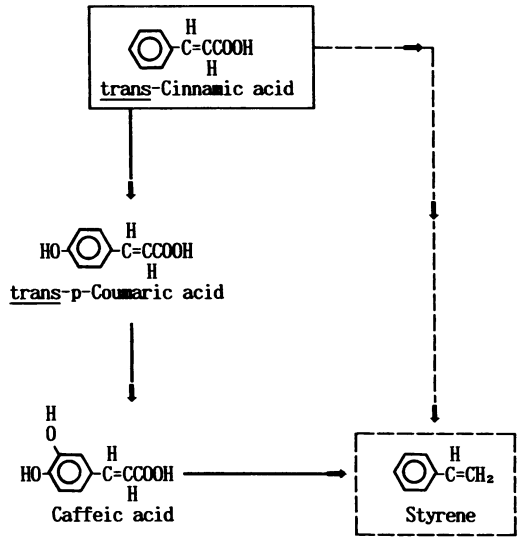

FIG. 3. Proposed pathway for the degradation of *trans*-cinnamic acid by isolates.

S. oviformis, *H. anomala*, or *Candida torresii* (data not shown). Chen and Peppler (2) reported that styrene was formed by the decarboxylation of cinnamic acid by an *S. cerevisiae* mutant (strain 3212). Styrene monomer is a widely used, important material for synthetic resins, such as polystyrene and styrene-butadiene rubber. Styrene can be also found in nature. Finkle et al. (6) have reported that *p*-hydroxycinnamic, *p*-coumaric, and caffeic acids are converted to styrene by enzymatic decarboxylation in the decay of vegetative material. Styrene is also known to be formed from cinnamic and *p*-coumaric acids by molds (3, 8, 10) and yeasts (2). Most studies of the health effects of styrene have been concerned with respiratory and dermal exposure rather than oral exposure, for which the effects are relatively unknown (11). The 50% lethal dose of styrene is 5.0 g/kg of body weight when orally administered to rats (15). Styrene is a depressant of the central nervous system (11), and it is biologically degraded in mammalian tissues (5, 15, 28). Mammalian styrene metabolism has been studied quite extensively in view of the toxic effects that arise from the metabolic activation of styrene. The major pathway of styrene degradation involves styrene oxide as an intermediate (5, 15). Styrene oxide is believed to have carcinogenic properties (28), but it can be detoxified in various ways (9, 24, 25). A yeast isolated from chikuwa, *P. carsonii* CHI, grew with caffeic and *p*-coumaric acids, which were converted to styrene (data not shown). These two compounds are often associated with microbial degradation of cinnamic acid (3, 6, 8, 10). Caffeic and *p*-coumaric acids have both been proposed as initial transformation products in the degradation of cinnamic acid (Fig. 3), although we have been unable to obtain direct evidence for the involvement of these compounds in the metabolism of cinnamic acid in *P. carsonii*. The degradation of styrene remains unexplained. Styrene metabolism is presently being studied in various styrene-degrading bacteria (9, 24, 25). Further research will focus on the elucidation of the initial step in cinnamic acid metabolism and the degradation of styrene in *P. carsonii* CHI described in this paper and in the yeast (*T. candida*) recently isolated by Sato et al. (23).

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