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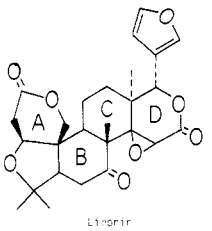
A Species of Bacterium-Producing Constitutive Enzymes for Limonoid Metabolism

Shin Hasegawa* and A. Douglas King, Jr.

A species of bacterium, capable of metabolizing limonoids, was isolated from soil by enrichment with 3-furoic acid as a single carbon source. It was classified as *Corynebacterium fascians* (NRRL B-15096). Unlike other limonoid-metabolizing bacteria identified thus far, this organism produced constitutive enzymes for limonoid metabolism. Cells grown on carbon sources other than limonoids such as fructose and galactose produced limonoate dehydrogenase (limonoate:NAD oxidoreductase).

Bitterness due to limonin in certain citrus juices is one



Limonin

of the major problems of the citrus industry worldwide and has significant economic impact. In recent years, several processes have been developed for reduction of limonin bitterness of citrus juices. Such processes are (1) a metabolic debittering process which involves exposure of intact fruit to 20 ppm of ethylene gas for 3 h and then holding them for several days in air (Maier et al., 1973), (2) a selective adsorption of limonin from the juice using cellulose acetate beads (Chandler and Johnson, 1979), (3) the use of limonin bitterness suppressing agents like neodiosmin in the juice (Guadagni et al., 1976), or (4) the use of enclosure complex forming agents with limonin like β-cyclodextrin in the juice (Misaki et al., 1981). There is also a preharvest approach—spraying citrus trees with inhibitors of limonoid biosynthesis (Hasegawa et al., 1977; Casas et al., 1980). However, none of them is totally satisfactory.

Recently, limonin-debittering processes using immobilized bacterial cells have been developed (Vaks and Lifshitz, 1981; Hasegawa et al., 1982; Hasegawa and Pelton, 1983). These processes have several advantages over the others and have potential for practical applications. For the biological processes, several species of bacteria, each capable of metabolizing limonoids, namely, *Arthrobacter globiformis* (Hasegawa et al., 1972b), *Pseudomonas* 321-18 (Hasegawa et al., 1974b), *Bacterium* 342-152-1 (Hasegawa and Kim, 1975), and *Acinetobacter* sp. (Vaks and Lifshitz, 1981), have been isolated from soil. The limonoid-metabolizing enzymes produced by these species of bacteria are all induced enzymes. They can be induced by the presence of limonoids in the growth media.

We have recently isolated from soil a species of bacterium which produces constitutive enzymes for limonoid metabolism. This paper shows the production of limonoid-metabolizing enzyme activity in this bacterium grown on carbon sources other than limonoids.

MATERIALS AND METHODS

Isolation and Characterization of the Bacterium. The bacterium was isolated from soil by enrichment with 3-furoic acid as a single carbon source. After purification the organism was classified as *Corynebacterium fascians* (NRRL B-15096) by following standard procedures (Buchanan and Gibbons, 1974; Lennette et al., 1974) and the use of the API 20 E Gram-negative bacteria identification system (Analytab Products, Plainview, NY).

Growth of Cells. The substate, 500 mL of a mineral salt medium (Hasegawa et al., 1972b), 0.2% nutrient broth, and 0.4% carbon source of interest, was placed in a 2.8-L Fernbach flask and inoculated with 10 mL of a 48-h culture of *C. fascians*. Incubation was at 25 °C on a shaker, and growth was followed by measuring the increase in optical density of the medium at 600 nm. After incubation, cells were harvested by centrifugation, washed with 0.5 M phosphate buffer at pH 7.0, and frozen until used for enzyme analysis.

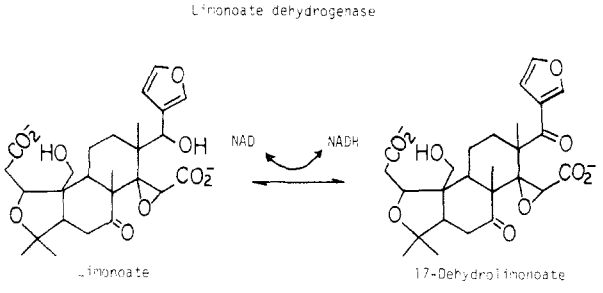
Identification of Metabolites. Silica gel G plates were used for thin-layer chromatography (TLC). Plates were developed with toluene–EtOH–H_2O–HOAc (200:47:15:1, upper layer), cyclohexane–EtOAc (2:3), and CH_2Cl_2–MeOH (97:3). Chromatograms were revealed by spraying with 50% H_2SO_4 and heating or by spraying with Ehrlich's reagent and exposing to HCl gas (Dreyer, 1965). NMR spectra were run at 100 MHz in $CDCl_3$ with a JEOL JNN-PS-100 spectrometer.

The supernatant from a culture of *C. fascians* which had been incubated with 1 g of sodium limonoate in 500 mL

U.S. Department of Agriculture, Agricultural Research Service, Fruit and Vegetable Chemistry Laboratory, Pasadena, California 91106.

of the mineral salt medium for 3 days was acidified with HCl to pH 2 and allowed to stand overnight. It was then extracted twice with CH_2Cl_2, and the extracts were combined and evaporated to give 320 mg. TLC analysis showed two major components, one of which was limonin. The second compound was acidic and Ehrlich's reagent negative. The extract was therefore dissolved in EtOAc and extracted twice with 5% $KHCO_3$. The $KHCO_3$ extracts were combined, washed with EtOAc, acidified with HCl, and extracted with CH_2Cl_2. The CH_2Cl_2 extract was washed with H_2O and evaporated to give 210 mg. The acidic fraction was dissolved in CH_2Cl_2, and methylated with CH_2N_2, and crystallization from MeOH gave 150 mg of the major component.

Limonoate Dehydrogenase. Limonoate dehydrogenase was purified from cell extracts by fractionation on a DEAE-cellulose column by the procedure of Hasegawa et al. (1972b). This preparation was used for identification of the enzyme.



Limonoate dehydrogenase activity was used as a measure of limonoid-metabolizing activity of cells grown on various media. Crude extracts of cells for assay of dehydrogenase activity were obtained as follows. Frozen cells were suspended in 50 mL of 0.1 M potassium phosphate buffer at pH 7.0 containing 10^{-3} M dithiothreitol and disrupted in an ice bath with a Branson J-22 sonifier. The suspension was centrifuged at 20000*g* for 15 min and the supernatant was brought to 0.9 saturation with solid $(NH_4)_2SO_4$. The mixture was then centrifuged at 20000*g* for 15 min, and the recovered precipitate was dissolved in a minimal portion of 0.1 M potassium phosphate buffer at pH 7.0 and used for enzyme analysis.

Enzyme Assay. Limonoate dehydrogenase was assayed by following the increase in absorption at 340 nm due to the formation of NADH. Activity was assayed at 25 °C in 1 mL of a reaction mixture contaiing 10^{-2} M sodium limonoate, 0.1 M Tris buffer at pH 8.2, 5×10^{-4} M NAD, and enzyme. One unit of limonoate dehydrogenase activity was defined as the amount which catalyzes the production of 1 μmol of 17-dehydro metabolite/min under the conditions used.

RESULTS AND DISCUSSION

A continuing survey of limonoid-metabolizing microorganisms resulted in the isolation of bacterium from soil by enrichment with 3-furoic acid which is a precursor of the limonoids. It was identified as *fascians* as follows. Colonies are circular, entire, convex, and orange-white with a colony diameter on nutrient agar of 1–3 mm after 4 or more days. The cells are straight or club-shaped, 0.3–0.5 × 1–3 μm, Gram-positive, aerobic, grow at 37 °C, are nonmotile and no spores were detected. The cells are catalase positive, utilize citrate as the sole carbon source, and have an active urease. Utilization of sugars as indicated by use of the API strip were negative but glucose, sucrose, lactose, and galactose were utilized and fructose was rapidly utilized and acid produced in fermentation tubes. The following tests were negative: β-galactosidase,

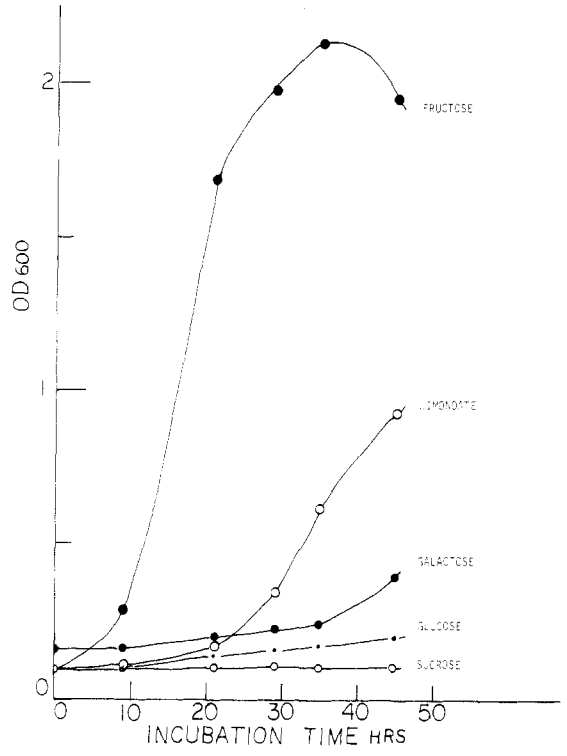


Figure 1. Growth of *C. fascians* on limonoate and other carbohydrate media. The cultural conditions are described in the text.

arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase, and tryptophan deaminase; acetoin, indole, and H_2S were not produced nor was gelatin liquefied. This culture was thus designated *C. fascians*. It has been deposited in the USDA ARS Culture Collection and designated NRRL B-15096. This organism also grew well on a limonoate–mineral salt medium. The major metabolite isolated from its growth medium was an acidic and Ehrlich's-negative compound. R_fs of its methylated compound with three solvent systems and its NMR spectrum were identical with those of authentic methyl ester of 17-dehydrolimonoate A-ring lactone, the major metabolite of limonoate A-ring lactone produced by *A. globiformis* (Hasegawa et al., 1972b). This showed that this bacterium metabolizes limonoid through the 17-dehydrolimonoid pathway. This was confirmed by the purification from cell extracts of limonoate dehydrogenase which catalyzes the first step of the 17-dehydrolimonoid pathway, that is, the conversion of limonoate to 17-dehydrolimonoate. The enzyme was purified approximately 150-fold by $(NH_4)_2SO_4$ precipitation followed by a DEAE-cellulose column chromatography. The enzyme required NAD and sulfhydryl groups for its catalytic action like other limonoate dehydrogenases isolated from *A. globiformis* (Hasegawa et al., 1972b) and *Bacterium* 342-152-1 (Hasegawa and Kim, 1975). Its activity was optimal at pH 8.0–9.0.

There were also several minor metabolites in its limonoate growth medium. They were Ehrlich's-positive compounds which differed from any of the metabolites of the deoxylimonoid pathway previously established (Hasegawa et al., 1972a, 1974a).

The organism also grew on simple sugars and produced limonoate dehydrogenase enzyme. Figure 1 shows the results of its growth on various carbon sources. The organisms grew best on fructose followed by limonoate and galactose. It grew poorly on glucose and did not grow on sucrose. However, when 0.2% nutrient broth was added to 0.4% sugar media, the organism grew very well on li-

Table I. Limonoate Dehydrogenase Activity of *C. fascians* Grown on Various Carbon Sources[a]

substrates	cell yields, g	limonoate dehydrogenase: total activity, units	limonoate dehydrogenase: activity/g of cells, units
limonoate	3.22	7392	2296
fructose	2.06	1546	750
galactose	2.20	2957	1344

[a] Growth media: 0.2% nutrient broth, 0.4% substrate of interest, and mineral salts in 500 mL.

monoate, galactose, or glucose, and the rates of growth on these carbon sources became very similar to that grown on fructose.

Table I shows the results of a typical experiment on analyses of limonoate dehydrogenase activity of cells grown on limonoate, galactose, or fructose. The activity was highest in cells grown on limonoate followed by galactose and fructose. An experiment similar to the above was done in triplicate with similar results in each case. The enzyme activity of *C. fascians* grown on limonoate was higher than that of either *A. globiformis* (Hasegawa et al., 1972b), *Bacterium* 342-152-1 (Hasegawa and Kim, 1975) or *Pseudomonas* 321-18 (Hasegawa et al., 1974a) when their cells were prepared under similar conditions. *C. fascians* grown on galactose produced limonoate dehydrogenase activity equaling that of *A. globiformis*, but higher than that of either Bacterium 342-152-1 or *Pseudomonas* 321.

The limonoate dehydrogenase of *C. fascians* was produced without the need for a limonoid inducer in the medium. This clearly showed that the organism produces constitutive limonoid-metabolizing enzymes. This is advantageous from a practical viewpoint because cells which possess limonoid-metabolizing enzymes can be produced conveniently and relatively cheaply by using inexpensive carbon sources. At present none of the citrus limonoids is commercially available.

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Registry No. Limonin, 1180-71-8; limonoic acid, 22153-41-9; 17-dehydrolimonoic acid, 38837-66-0; 3-furoic acid, 488-93-7; fructose, 57-48-7; galactose, 59-23-4; limonoate dehydrogenase, 37325-58-9.

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Objective Measurement of Aroma Quality of Golden Delicious Apples as a Function of Controlled-Atmosphere Storage Time

Guido A. Willaert, Patrick J. Dirinck,* Herman L. De Pooter, and Niceas N. Schamp

Flavor quality of Golden Delicious apples as a function of controlled-atmosphere storage time is studied by using aroma analysis as an objective criterium. For representative aroma isolation, a fast headspace concentration technique, which isolates the volatiles released during maceration of the fruits, is used. As flavor quality is a dynamic process, the evolution of volatiles as a function of ripening after removal from controlled atmosphere is followed for six periods during the storage season. Results objectively indicate an important decrease in aroma quality after long controlled-atmosphere storage (CA storage).

The volatile compounds of apples have been studied by several authors and over 250 volatile components have been identified (Van Strating and de Vrijer, 1973). The most important flavor compounds in apples are esters, alcohols, and aldehydes. According to Flath, the components directly associated with the characteristic Delicious apple-like aroma are ethyl 2-methylbutyrate, 1-hexanal, and *trans*-2-hexenal (Flath et al., 1967). Williams and co-workers established the importance of 4-methoxy-

State University of Gent, Faculty of Agricultural Sciences, Department of Organic Chemistry, Coupure Links 653, B-9000 Gent, Belgium.

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