Octene Epoxidation by a Cold-Stable Alkane-Oxidizing Isolate of Pseudomonas oleovorans

ROBERT D. SCHWARTZ

Corporate Research Laboratories, Esso Research and Engineering Company, Linden, New Jersey 07036

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The isolation of an alkane-oxidizing strain of Pseudomonas oleovorans which maintains its viability at 5 C is described. This strain epoxidizes 1-octene at a rate five times that of the parent strain. The most efficient substrates for induction of the epoxidase are C\textsubscript{7}, C\textsubscript{8}, and C\textsubscript{9}, although C\textsubscript{10} to C\textsubscript{14} also serve as growth substrates and inducers. The greater rate may be attributed to an enhanced general stability of the cells as opposed to a modification of the enzyme system involved.

As part of our program on the enzymatic oxidation of hydrocarbons (HC) we have been investigating the inducible oxygenase system(s) responsible for the hydroxylation and epoxidation reactions carried out by a strain of Pseudomonas oleovorans (3–12; B. J. Abbott and C. T. Hou, manuscript in preparation). To facilitate shake-flask and fermentor operations, it was desirable to learn about the stability of this strain relative to the storage of cultures to be used as inocula. This report summarizes the results of cold-storage experiments and the subsequent isolation from the refrigerated cultures of a cold-stable, alkane-oxidizing, 1-octene-epoxidizing variant.

MATERIALS AND METHODS

Bacteria. The parent strain, P. oleovorans 1 RAM, is a recent single clone isolate from a culture originally purchased several years ago from M. Coon (University of Michigan, Ann Arbor). The cold-stable variant has been designated P. oleovorans TF4-1. Cultures were stored on nutrient agar (NA) slants to which a few drops of octane were added.

Media and growth conditions. Three-hundred-milliliter baffled shake flasks, containing 100 ml of minimal P medium (Table 1) supplemented with alkane (to 1% vol/vol) as sole source of carbon and energy, was used for growth of the organisms. NA petri dishes were used for titering by the spread plate method. All media were autoclaved, except that, when HC was to be included, it was filter sterilized and added to P\textsubscript{1} medium at the time of use. Shake flasks were incubated on a water-bath shaker operated at 350 rpm and 30 C (New Brunswick Scientific Co., New Brunswick, N.J.). Cultures were harvested, titered, and assayed for the production of 1,2-epoxoctane when the optical density (OD) at 660 nm reached 1.0 to 3.0. Petri plates were also incubated at 30 C, and colonies were counted after 24 to 48 h of growth.

The following HC were 59 mol % pure and were purchased from Phillips Petroleum Co., Bartlesville, Okla.: pentane (C\textsubscript{5}), hexane (C\textsubscript{6}), heptane (C\textsubscript{7}), octane (C\textsubscript{8}), nonane (C\textsubscript{9}), decane (C\textsubscript{10}), undecane (C\textsubscript{11}), dodecane (C\textsubscript{12}), tridecane (C\textsubscript{13}), tetradecane (C\textsubscript{14}), pentadecane (C\textsubscript{15}), hexadecane (C\textsubscript{16}), heptadecane (C\textsubscript{17}), octadecane (C\textsubscript{18}), 1-octene.

Isolation of cold-stable variant. To initiate the experiment, an NA slant of P. oleovorans 1 RAM was washed with P\textsubscript{1} medium, and the washings were used to inoculate a shake-flask containing C\textsubscript{8} as substrate. When grown to an OD of 1 to 3, the viability of the culture was determined and then it was refrigerated at 5 C. At various times, samples were removed from the refrigerated culture for viability estimation and use as inocula for additional C\textsubscript{8} cultures. The initial culture was designated MF (master flask), and subsequent cultures derived from it were designated TF (transfer flask). Following growth, a portion of each TF culture was titered and assayed for epoxide production. Two of these cultures, TF4 and TF4-1, were also tested for maintenance of viability at 5 C and were used for subsequent transfers.

Preparation of resting cell suspensions. The general procedures of Abbott and Hou (manuscript in preparation) were used. Following growth, 100 ml of the cell suspension was washed twice by centrifugation with 100 ml of 0.1 M phosphate buffer, pH 7, to which two drops of Triton X-100 had been added. The final cell pellet was suspended in buffer to yield an OD of 1.8 at 660 nm. The viable titer of this standard cell preparation was 2 x 10\textsuperscript{9} to 4 x 10\textsuperscript{9} ml. To a test tube (16 by 150-mm) were added 5 ml of the standard cell preparation and 0.05 ml of 1-octene. The suspension was mixed on a Vortex mixer and...
incubated at 30 C with shaking. Cell viability in the assay tube was routinely determined at the beginning and end of each experiment. At various times samples were removed and assayed for the production of 1,2-epoxyoctane.

**Epoxide assay.** The gas chromatographic procedure of May and Abbott (J. Biol. Chem., in press) was used to quantitate the amount of 1,2-epoxyoctane produced. A 1-ml sample of resting cell suspension was extracted with 1 ml of hexane to which 2-octanol (0.25 mg/ml) had been added as an internal standard. The emulsion formed was broken by centrifugation, and the upper hexane layer was analyzed for 1,2-epoxyoctane using a Perkin-Elmer model 900 gas chromatograph equipped with a flame ionization detector. The column was 20 feet by ½ inch (about 60.96 by 0.3 cm) stainless-steel packed with 10% carbowax 20 M on 80/100 Chromosorb W (Applied Sciences Laboratories, State College, Pa.). An isothermal temperature of 180 C was maintained, and the carrier gas flow was 35 ml of helium/min. The 1,2-epoxyoctane was quantitated by measuring the ratio of the epoxide peak area to the peak area of 2-octanol.

**RESULTS**

The derivation of the various cultures is shown in Fig. 1. The increase in the ability of the culture to survive at 5 C after successive subculturing is shown in Fig. 2. The parent culture, MF, exhibited an initial exponential decay in viability during 1 week of storage followed by a leveling off in viable cell number. The variant, TF4-1, showed essentially no change in number of viable cells during 479 h of storage.

All of the cultures, with the exception of MF, were tested for their ability to carry out the epoxidation of 1-octene. The results of this assay are shown in Table 2. TF4 and the cultures derived from it produced 3 to 6 times as much 1,2-epoxyoctane in 1 h than did TF3, which did not differ in epoxide production from *P. oleovorans* 1 RAM.

The question of greater epoxide yield being caused by maintenance of culture viability, as opposed to a modification of the enzyme system, was answered by assaying for epoxide during a 5-h period and measuring the change in viable cell count during this period. The results of a typical experiment are shown in Fig. 3. During the course of the reaction the titer of *P. oleovorans* 1 RAM decreased about 10-fold, whereas that of the derivative, TF4-1, did not change. Apparently, the increase in epoxide production is due to maintenance of culture viability.

TF4-1 was used for the subsequent experiments. Assays were performed at various viable cell concentrations to determine the optimum for epoxide production. From curves similar to

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**TABLE 1. Composition of *P.* minimal media**

| Compound         | Amt   |
|------------------|-------|
| \((\text{NH}_4)_2\text{HPO}_4\) | 10.0 g |
| \(\text{K}_2\text{HPO}_4\)       | 5.0 g  |
| \(\text{Na}_2\text{SO}_4\)       | 0.5 g  |
| \(\text{CaCl}_2\) (50 g/L)       | 1.0 ml |
| Salts “B”         | 10.0 ml|
| \(\text{MgSO}_4\cdot7\text{H}_2\text{O}\) | 40.0 g |
| \(\text{FeSO}_4\cdot7\text{H}_2\text{O}\) | 2.0 g  |
| \(\text{MnSO}_4\cdot\text{H}_2\text{O}\) | 1.6 g  |
| \(\text{NaCl}\)          | 2.0 g  |
| Distilled water    | 1 liter|
| Distilled water    | 1 liter|
| Distilled water    | 1 liter|
| Agar*             | 15 g   |

* From Difco.
TABLE 2. Yield of 1,2-epoxyoctane from 1-octene after 1 h by octane-grown cells

| Culture | Epoxide (µg/ml) |
|---------|----------------|
| TF3     | 20.5           |
| TF4     | 68.7           |
| TF4-1   | 121.5          |
| TF4-2   | 97.4           |
| TF4-1-1 | 74.9           |
| TF4-1-2 | 72.1           |
| TF4-1-3 | 83.8           |

FIG. 3. Epoxide yield versus time versus viability. C₅-grown cells. P. oleovorans 1 RAM (●), P. oleovorans TF4-1 (○).

those shown in Fig. 3 the rates of 1,2-epoxyoctane production were calculated. The results of such experiments (Fig. 4) indicate an optimum of about 3 x 10⁹ cells/ml.

The effect of growth substrate on epoxide production is shown in Table 3. Each result is the average of at least three experiments at cell concentrations of 2 x 10⁹ to 4 x 10⁹/ml for each substrate. Of the n-alkanes supporting growth, C₅, C₆, and C₇ are the most efficient for induction of the epoxidase. No growth was observed with C₁₃ to C₁₈ after 22 days.

The growth conditions chosen were arbitrary, no attempt was made to optimize substrate concentration, temperature, etc., and no detailed growth curves were constructed. Nevertheless, it can be said that growth rates on C₇ to C₁₉ are similar, but are considerably lower on C₈, C₁₀, C₁₁, and C₁₂. The extent of growth is similar on C₆ to C₁₂.

Attempts to grow TF4-1 in P₁ broth with 1-octene as substrate were unsuccessful. However, growth did occur when 1-octene was supplied as a vapor to cells spread on P₁ minimal agar. To test for the epoxidation of 1-octene, the cells were washed from the plates and a resting cell suspension was prepared. The 1-octene-grown cells epoxidate 1-octene at about three-fourths the rate of cells grown on C₁₀ in a similar fashion.

DISCUSSION

The isolation of a cold-stable, alkane-oxidizing variant of P. oleovorans which carries out the epoxidation of 1-octene at a rate five times that of the parent is described. The only

TABLE 3. Effect of growth substrate on rate of epoxidation of 1-octene. TF4-1

| Substrate | 1,2-Epoxoctane produced (µg per ml per h) |
|-----------|------------------------------------------|
| C₁        | 33                                       |
| C₂        | 61                                       |
| C₃        | 96                                       |
| C₄        | 96                                       |
| C₅        | 109                                      |
| C₆        | 76                                       |
| C₇        | 79                                       |
| C₈        | 79                                       |
| C₉ to C₁₈ | NG*                                      |

* No growth after 22 days.

FIG. 4. Effect of viable cell concentration on the production of 1,2-epoxyoctane from 1-octene. C₅-grown TF4-1.
selective pressure used in the isolation procedure was low temperature (5 C). For our present purposes, the use of this isolate will both facilitate the storage of inocula and provide a culture with greater epoxidative activity.

Although viability is maintained, preliminary studies indicate that growth does not occur at 5 C and little of the induced epoxidase activity remains in the stored cultures. The latter is probably due to the degradation or inactivation of the induced enzymes during storage at 5 C relative to freshly harvested cells. Similarly, Ensign observed changes in the endogenous metabolism of Arthrobacter crystallopoietes without concomitant loss of viability for 30 days, although the conditions of long-term starvation at 30 C were used rather than 5 C as described here (1, 2).

The epoxidation of 1-octene by a strain of P. aeruginosa has been reported previously (3, 10, 12). In those studies the epoxide was reported not to be further metabolized. With our strains of P. oleovorans the rate of product formation appears to be linear, also suggesting that 1,2-epoxyoctane is not further metabolized. That this may not necessarily be the case has been suggested by recent studies showing that 1,2-epoxyoctane can be rapidly metabolized by the cells but that this further metabolism is inhibited by the presence of the substrate, 1-octene (B. J. Abbott and C. T. Hou, personal communication). In the present study 1-octene was always in excess, perhaps thus precluding the further metabolism of the epoxide.

The linearity of the reaction with the parent strain P. oleovorans 1 RAM, even though the cells were dying (Fig. 3), perhaps indicates that two populations were present, one stable and the other nonstable, the former being present in lower concentrations and responsible for the epoxidative activity.

The greater 1,2-epoxyoctane production by TF4-1 may be attributable to a cell wall or membrane modification which has resulted in a more stable cell, as opposed to a modification of the enzyme system per se.

Cells grown on C4 to C12 epoxidate 1-octene; C4 to C6 are the best inducers of the epoxidase. The correlation between the ability of an al-kane to serve as growth substrate (presumably via hydroxylation) and the rate at which the resting cells epoxidate 1-octene indicates that the enzyme system responsible for hydroxylation and epoxidation are the same. Recent in vitro studies using the purified hydroxylase system from P. oleovorans tend to support this conclusion (6).

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