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Oxidation of Aromatic Acids by a Facultative Thermophilic Bacillus sp.

By J. A. BUSWELL AND J. S. CLARK

Department of Biology, Paisley College of Technology, Paisley PA1 2BE, Strathclyde

(Received 5 December 1975; revised 10 May 1976)

INTRODUCTION

Aerobic catabolism of benzoate and p-hydroxybenzoate is widely reported in several genera of bacteria. Oxidative breakdown of benzoate most frequently involves the formation of catechol prior to aromatic ring-fission whereas p-hydroxybenzoate is usually hydroxylated in the C-3 position to yield protocatechuate. Further conversion of these dihydroxylated products proceeds by either intradiol (ortho-fission) or extradiol (meta-fission) cleavage of the benzene nucleus (Dagley, 1971).

An earlier report (Buswell & Twomey, 1974) indicated that gentisic acid was an intermediate in the degradation of benzoate by a thermophilic bacterium isolated from soil. We now describe the novel conversion of p-hydroxybenzoate to gentisate by a facultative thermophilic strain of Bacillus, and the subsequent induction, during growth on benzoate and p-hydroxybenzoate, of enzymes which catalyse gentisate dissimilation.

METHODS

Bacteria. The organism was isolated from coke-oven washings (Ravenscraig Steelworks Motherwell, Lanarkshire) by selective enrichment at 55 °C in a semi-defined basal medium which contained p-hydroxybenzoate as the major carbon and energy source. It is facultatively thermophilic with upper and lower temperature limits for growth of 62 °C and 30 °C, respectively. It is considered to be a Bacillus sp. because young cultures consisted of Gram-positive slender rods of different lengths and aged cultures contained oval endospores, located terminally or subterminally, which swell the vegetative cell.

Media. The basal growth medium contained (g l⁻¹): K₂HPO₄, 0.5; NH₄Cl, 1.0; MgSO₄.7H₂O, 0.02; yeast extract (Oxoid), 0.1; aromatic growth substrate, 1.0; 1 ml trace element solution; adjusted to pH 7.2 with 2 M-NaOH. The trace element mixture consisted of (g l⁻¹): NaCl, 10.0; CaCl₂.2H₂O, 6.5; MnSO₄.4H₂O, 0.4; ZnSO₄.7H₂O, 0.01; CuSO₄.5H₂O, 0.04; CoCl₂.6H₂O, 0.04; NaMoO₄.2H₂O, 0.05; FeSO₄.4H₂O, 0.4. Sodium fumarate (2 g l⁻¹) replaced the aromatic substrate for the growth of non-induced bacteria.

 Cultures. Bacterial cultures (1 l) were grown in baffled 2 l Erlenmeyer flasks at 55 °C (± 0.5°C) in an orbital incubator shaker (model IH460, Gallenkamp) operated at 110 rev./min. Bacteria were harvested during the exponential phase of growth by centrifugation (20000 g; 10 min; 25 °C) and washed with 0.1 M-Tris-HCl buffer, pH 7.5. Bacterial growth was determined by measuring the culture turbidity with an EEL nephelometer (blue filter) and referring readings to a standard curve relating bacterial dry weight (dried at 120 °C to constant weight)
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to turbidity. Stock cultures were maintained on slopes of basal medium solidified with 2% (w/v) Difco agar.

Cell extracts. Washed pellets of bacteria were suspended in 4 vol 0·1 M-Tris-HCl buffer, pH 7·5, and broken by five 1 min periods of sonication, with intermittent cooling to 5 °C, in a MSE model 150 W ultrasonic disintegrator. The suspension was then clarified by centrifuging at 35 000 g for 1 h and the supernatant was used for enzyme assays.

Assays. Oxygen uptake by washed bacterial suspensions was measured at 50 °C by conventional Warburg manometry. Reaction mixtures contained: 250 µmol Tris-HCl buffer, pH 7·5; 3 µmol substrate; and bacterial suspension (9 to 11 mg dry wt) in 3 ml water. 2,5-Dihydroxybenzoate 1,2-dioxygenase (EC 1.13.11.4; gentisate 1,2-dioxygenase) was assayed by measuring oxygen consumption with a Clark oxygen-electrode (Rank, Bottisham, Cambridgeshire) and by measuring the increase in extinction at 334 nm caused by maleylpyruvate formation (Crawford, Hutton & Chapman, 1975). Assay mixtures contained: 250 µmol Tris-HCl buffer, pH 7·5; 0·4 µmol gentisate; and enzyme (dialysed for 12 h against distilled water at 4 °C) in 3·0 ml. When no further increase in extinction at 334 nm occurred, maleylpyruvate hydrolase activity was determined by adding undialysed extract to the reaction cuvette and measuring the rate of decrease in extinction at 334 nm. Reaction mixtures were kept at 50 °C in a constant-temperature cuvette housing heated by circulating water. Both dialysed and undialysed extracts were activated with Fe2+ before assay by incubating 1 ml extract (12 to 16 mg protein) with 0·25 ml 0·01 M-ferrous ammonium sulphate for 20 min at 25 °C. Maleylpyruvate formation was calculated using the molar extinction coefficient reported by Crawford et al. (1975). Enzymic production of pyruvate from gentisate was determined spectrophotometrically using lactate dehydrogenase and reduced nicotinamide adenine dinucleotide (NADH) as previously described (Collinsworth, Chapman & Dagley, 1973). When hydrolysis of maleylpyruvate was complete, as indicated by zero extinction at 334 nm, the reaction cuvettes were cooled to 20 °C before 2 units of lactate dehydrogenase (Sigma) and 1 µmol NADH were added. Values given in Table 2 for oxygen uptake, and for maleylpyruvate and pyruvate formed, are the mean of three determinations each initiated with different concentrations of gentisate.

Isolation of gentisate. Bacteria grown in the presence of p-hydroxybenzoate were harvested during the mid-exponential phase of growth and washed once with 0·1 M-Tris-HCl buffer, pH 7·5. In a baffled 2 l Erlenmeyer flask, 200 ml 0·1 M-Tris-HCl buffer, pH 7·5, 1 mm 2,2'-bipyridyl and bacteria grown with p-hydroxybenzoate (approx. 5 mg dry wt ml-1) were equilibrated by shaking for 20 min at 55 °C, and 10 ml 0·1 M-sodium p-hydroxybenzoate pH 7·0 was added. When oxidation ceased, as indicated for similar reaction mixtures in Warburg flasks, second and third additions of 10 ml were made at 1·5 h intervals. After removing the bacteria by centrifuging, the supernatant was acidified to pH 2 with 6 M-HCl and any resultant precipitate was removed by further centrifuging. Excess 2,2'-bipyridyl was converted into its iron chelate by adding ferrous sulphate to the supernatant which was then extracted three times with 200 ml ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure. The solid residue was taken up in 4 ml diethyl ether, applied to the top of a silica-gel (Reeve Angel Scientific, London) column (2 × 35 cm) and eluted with petroleum ether (b.p. 40 to 60 °C)/diethyl ether/formic acid (35:15:1, by vol.). To achieve further separation from unchanged p-hydroxybenzoate, fractions which contained the accumulated product were concentrated under reduced pressure, applied to a similar column and eluted as before. The relevant fractions were evaporated to dryness and the solid residue twice recrystallized from water.
Table 1. Oxidation of aromatic compounds by washed suspensions of bacteria grown on benzoate and p-hydroxybenzoate

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Benzoate</th>
<th>m-Hydroxy-</th>
<th>p-Hydroxy-</th>
<th>Gentisate</th>
<th>Protocatechuate</th>
<th>Catechol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoate</td>
<td>1·50</td>
<td>0·03</td>
<td>0·04</td>
<td>0·68</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>p-Hydroxybenzoate</td>
<td>ND</td>
<td>1·36</td>
<td>1·46</td>
<td>1·26</td>
<td>0·45*</td>
<td>0·56*</td>
</tr>
</tbody>
</table>

ND, Not detected
* Not observed in the presence of chloramphenicol.

Chromatography was carried out on silica-gel chromatography plates with fluorescent indicator (Eastman Chromagram sheets, type 13181). The plates were developed in either (i) benzene/dioxan/acetic acid (90:25:4, by vol.) or (ii) petroleum ether (b.p. 40 to 60 °C)/diethyl ether/formic acid (35:15:1, by vol.), and visualized by illuminating with ultraviolet light and by spraying with Gibb’s reagent (2 % 2,6-dichloroquinone-4-chloroimide in ethanol).

Infrared spectra were recorded in Nujol with a Perkin–Elmer model 700 spectrophotometer.

RESULTS AND DISCUSSION

Washed suspensions of bacteria grown in the presence of benzoate readily oxidized benzoate and gentisate but not catechol or protocatechuic acid (Table 1). Rapid oxygen uptake by suspensions of the intact bacterium grown with p-hydroxybenzoate was observed with m-hydroxybenzoate, p-hydroxybenzoate and gentisate (Table 1). Significant uptake also occurred with catechol and protocatechuate without any apparent lag period, but this was abolished when chloramphenicol (300 μg) was incorporated in the reaction mixtures. Oxygen consumption on the other substrates was unaffected by chloramphenicol which suggested that a rapid induction of enzymes metabolizing catechol and protocatechuate occurred in the Warburg flasks. In vessels containing catechol, a yellow compound appeared which had spectral characteristics identical with those described for 2-hydroxymuconic semialdehyde, the product formed by meta-cleavage of the aromatic ring. The aromatic-oxidizing enzymes must be inducible since bacteria grown with fumarate did not attack benzoate, m-hydroxybenzoate or p-hydroxybenzoate and oxidized gentisate at a much reduced rate compared with bacteria grown in the presence of the specific aromatic compounds.

Further support for the idea that gentisate has an intermediate role in p-hydroxybenzoate dissimilation was provided by its isolation from reaction mixtures in which washed suspensions of bacteria had been incubated with p-hydroxybenzoate in the presence of 2,2'-bipyridyl. Addition of 2,2'-bipyridyl is known to inhibit the further degradation of gentisate and alkyl-substituted gentisates which accumulate from the oxidation of alkylphenols by a non-fluorescent Pseudomonas sp. (Hopper & Chapman, 1971). The compound which accumulated in the reaction mixture had the u.v. absorption spectrum of gentisate, the same $R_F$ value as authentic gentisate on t.l.c. plates developed in benzene/dioxan/acetic acid ($R_F = 0·10$) or petroleum ether/diethyl ether/formic acid ($R_F = 0·17$), and gave the same colour reactions with 5 % aqueous FeCl₃ (blue) and Gibb’s reagent (red). Its i.r. spectrum was essentially the same as that of authentic gentisate; and when exposed to dialysed cell-free extracts which contained gentisate 1,2-dioxygenase activity, the compound was converted...
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Table 2. Enzyme activities in cell-free extracts and stoichiometry of gentisate oxidation after growth on benzoate and p-hydroxybenzoate

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Specific activity [μmol min⁻¹ (mg protein)⁻¹]</th>
<th>O₂ consumed (mol/mol gentisate)</th>
<th>Maleylpyruvate formed (mol/mol gentisate)</th>
<th>Pyruvate (mol/mol gentisate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gentisate 1,2-dioxygenase</td>
<td>Maleylpyruvate hydrolase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoate</td>
<td>0.20</td>
<td>0.096</td>
<td>0.95</td>
<td>0.98</td>
</tr>
<tr>
<td>p-Hydroxybenzoate</td>
<td>1.88</td>
<td>1.10</td>
<td>0.93</td>
<td>1.09</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.02</td>
<td>0.002*</td>
<td>†</td>
<td>†</td>
</tr>
</tbody>
</table>

* Lower limit of detection.
† Not measured

...to an intermediate which had u.v. spectral characteristics identical to maleylpyruvate, the product formed after ring-fission of gentisate.

Cell-free extracts of bacteria grown in the presence of benzoate or p-hydroxybenzoate oxidized gentisate but not catechol, protocatechuate or 2,3-dihydroxybenzoate. Two metabolic pathways for gentisic acid dissimilation have been described. The initial product of ring-cleavage, maleylpyruvate, may undergo a reduced glutathione (GSH)-dependent isomerization to fumarylpyruvate which is then hydrolysed to pyruvic and fumaric acids (Tanaka et al., 1957; Lack, 1959; Crawford et al., 1975). Alternatively, maleylpyruvate is hydrolysed to maleate and pyruvate without prior cis, cis to cis, trans isomerization (Hopper, Chapman & Dagley, 1968, 1971; Crawford, 1975 b). Oxidation of gentisate by dialysed extracts of bacteria grown with p-hydroxybenzoate or benzoate resulted in the accumulation of a product which showed identical spectral characteristics (λmax, 330 nm at pH 7.15, which disappeared on acidification and shifted to 335 nm at pH 14) with those described for maleylpyruvate (Lack, 1959). One mole of oxygen was consumed and one mole of maleylpyruvate formed for each mole of gentisate oxidized (Table 2). Low levels of gentisate 1,2-dioxygenase activity were also detectable in extracts of fumarate-grown bacteria but this activity was increased about 10-fold and 90-fold by growth in the presence of benzoate and p-hydroxybenzoate respectively. When undialysed extract was added to reaction curvettes which contained accumulated maleylpyruvate, the extinction at 334 nm rapidly fell to zero. The rate of decrease was not enhanced on addition of 0.5 μmol GSH indicating direct hydrolysis of maleylpyruvate to pyruvate and maleate. One mole of gentisate yielded one mole of pyruvate (Table 2), but unequivocal proof of direct conversion must await the identification of maleate as the other product of hydrolase activity.

Intramolecular migration of isotopes of hydrogen, halogen atoms and methyl groups, the ‘NIH shift’ takes place during hydroxylation of various aromatic compounds by both microbial and mammalian systems (Guroff et al., 1967). A similar shift of an alkyl side-chain occurs during the enzymic conversions of p-hydroxyphenylpyruvate and p-hydroxyphenylacetate to homogentisate (La Du & Zannoni, 1955, 1956; Hagar, Gregerman & Knox, 1957; Blakley, 1972; Hareland et al., 1975). Hydroxylation-dependent side-chain migration and β-oxidation was also suggested as an alternative route in 4-hydroxyphenylpropionate dissimilation by certain species of Bacillus (Crawford & Chapman, 1975). The implication of gentisate as an intermediate in p-hydroxybenzoate catabolism indicates a further unusual example of this phenomenon.

It was noted recently (Crawford, 1975 b) that aromatic degradation by bacteria of the genus Bacillus may occur by novel catabolic pathways (Ensign & Rittenberg, 1964; Wall-
noeffer & Engelhardt, 1971; Spokes & Walker, 1974; Crawford, 1975a; Buswell, 1975): additional evidence for this is suggested in our report.

We thank Drs P. A. Williams and D. J. Hopper for helpful correspondence and Mr P. Anderson for recording the infrared spectra. A grant-in-aid for equipment from The Royal Society to J. A. Buswell is gratefully acknowledged.

REFERENCES


