The Metabolism of Aromatic Ring Fission Products by *Bacillus stearothermophilus* Strain IC3

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Bacillus stearothermophilus IC3 degraded the *meta* cleavage product of catechol, 2-hydroxymuconic semialdehyde, to pyruvate and acetaldehyde via the 4-oxalocrotonate pathway. The pathway was identical to those previously delineated in several mesophilic organisms. However, all the enzymes showed activity at 55 °C and other properties (substrate specificities and effects of metal ions) also differed from those displayed by the mesophilic enzymes. All enzymes of this *meta* cleavage pathway, except the 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase and 4-hydroxy-2-oxovalerate aldolase activities, were induced by growth on phenol.

INTRODUCTION

A number of thermophilic bacteria are able to utilize aromatic compounds as carbon and energy sources. A thermophilic *Bacillus* was able to metabolize benzoate and *p*-hydroxybenzoate, via gentisate, to pyruvate and fumarate (Buswell & Twomey, 1974; Buswell & Clark, 1976). *Thermus* strains utilizing phenylacetate as a carbon source have also been observed (Degryse *et al.*, 1978). The metabolism of phenol by unidentified thermophiles (Egorova, 1942, 1946) and *Bacillus stearothermophilus* strains (Buswell, 1974, 1975; Buswell & Twomey, 1975; Golovacheva & Oreshkin, 1975) has also been widely described.

An earlier report (Adams & Ribbons, 1988) established that a number of thermophilic bacilli were able to grow on aromatic compounds, including benzoate, *p*-hydroxybenzoate, phenylacetate, *p*-hydroxyphenylacetate, L-phenylalanine and phenol. One of these organisms, *Bacillus stearothermophilus* IC3, metabolized phenol to 2-hydroxymuconic semialdehyde via catechol. In this paper we describe the further metabolism of 2-hydroxymuconic semialdehyde to pyruvate and acetaldehyde.

METHODS

Isolation, growth media and growth conditions of Bacillus stearothermophilus IC3. These were as described by Adams & Ribbons (1988).

Chemicals. Most chemicals were obtained from major manufacturers (Aldrich, Lancaster Synthesis, Sigma). Authentic 4-oxalocrotonate and 4-methyl-2-oxobutyrolactone were synthesized according to the methods of Lapworth (1901), and Rossi & Schinz (1948), respectively, by Dr John Rossiter of this department. 2-Hydroxymuconic semialdehyde and its methyl substituted analogues were generated *in situ* by the action of a catechol 2,3-dioxygenase present in strain IC3 (Adams & Ribbons, 1988) upon catechol and methyl catechols. 4-Hydroxy-2-oxovalerate was prepared by mild alkaline hydrolysis of 4-methyl-2-oxobutyrolactone (Dagley & Gibson, 1965).

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Abbreviations: HOD, 2-hydroxy-6-oxohepta-2,4-dienoate; HODH, 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase; HMS, 2-hydroxymuconic semialdehyde; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMMS, 2-hydroxy-5-methylmuconic semialdehyde; OC, 4-oxalocrotonate.

The preparation of 2-oxopent-4-enoate was based upon the methods of Collinsworth *et al.* (1973). A 10 ml solution containing 250 μ mol Tris buffer (pH 8.2), 0.5 mmol DL-allylglycine, 25 units D-amino acid oxidase and 1250 units catalase, was incubated at 25 °C for 30 min. The solution was then acidified to pH 1.5 with HCl, and the product extracted twice with 25 ml diethyl ether. The ether was removed and the precipitate resuspended in 5 ml dilute HCl (pH 1.5). The solutions were stored at -20 °C and used within 1 week.

The following enzymes were obtained from Sigma: aldehyde dehydrogenase (EC 1.2.1.5) from bakers' yeast; D-amino acid oxidase (EC 1.4.3.3) from porcine kidney; catalase (EC 1.11.1.6) from bovine liver; formate dehydrogenase (EC 1.2.1.2) from *Pseudomonas oxalaticus*; NADase (EC 3.2.2.5) from *Neurospora crassa*; and pyruvate dehydrogenase from bovine heart.

Cell-free extracts. These were prepared from 1 litre cultures that had been harvested in the mid-exponential growth phase. The cells were washed twice in 50 mM-potassium phosphate buffer (pH 7.0) at room temperature, and then resuspended in 5 ml fresh buffer containing 10% (v/v) acetone and 0.5 μ mol phenylmethylsulphonyl fluoride. The resuspended cells were then sonicated for 15 s, at an amplitude of 16 μ m, with intermittent cooling to 4 °C, for a total of 3 min. The extracts were subsequently clarified by centrifugation at 35000 g for 40 min at 4 °C. The cleared extracts were stored at 4 °C until used. For all assay procedures the extracts were diluted in 50 mM-potassium phosphate buffer (pH 7.0). Protein concentrations were measured by the method of Bradford (1976), using Bio-Rad Protein Assay Reagent. Bovine serum albumin was used as a standard.

Determination of enzyme activities. All enzyme rates shown are the mean of at least two results. All assays were done with cell-free extracts, at protein concentrations of 0.3–0.5 mg ml⁻¹, diluted in 50 mM-phosphate buffer (pH 7.0). 2-Hydroxy-6-oxohepta-2,4-dienoate hydrolase (HODH) was assayed by following the disappearance of 2-hydroxymuconic semialdehyde (HMS), 2-hydroxy-6-oxohepta-2,4-dienoate (HOD) or 2-hydroxy-5-methylmuconic semialdehyde (HMMS) at 375, 388 and 382 nm respectively. The molar absorption coefficients (l mol⁻¹ cm⁻¹) at pH 7.0 were calculated as: HMS, 27900; HOD, 7600; HMMS, 20200 (Adams & Ribbons, 1988). 2-Hydroxymuconic semialdehyde dehydrogenase (HMSD) was assayed in the same manner as HODH, except that NAD was added to a concentration of 1 mM; the rate of degradation before the addition of NAD was subtracted from the value obtained.

4-Oxalocrotonate tautomerase was assayed by measuring the initial rate of decrease in absorbance at 292 nm on addition of 4-oxalocrotonate. Rates are expressed as the rate of decrease in absorbance at 292 nm (mg protein)⁻¹ min⁻¹, corrected for a spontaneous tautomerization of 3.3 absorbance units min⁻¹.

4-Oxalocrotonate decarboxylase activity was assayed by following the disappearance of the substrate. The total 4-oxalocrotonate concentration was obtained by adding 100 μ l 10 M-NaOH to 1 ml of the reaction mixture and reading the absorbance at 350 nm. The molar absorption coefficient of authentic 4-oxalocrotonate was 20800 units cm⁻¹ at this wavelength.

2-Oxopent-4-enoate hydratase activity was determined by following the disappearance of the substrate. The substrate concentration was determined by HPLC analysis after the addition of 20 μ l concentrated HCl to 100 μ l of sample. All rates were corrected for a spontaneous disappearance of 2-oxopent-4-enoate. Due to the instability of the substrate, previous workers have expressed the activity of this enzyme in units of absorbance at 265 nm (mg protein)⁻¹ min⁻¹. The hydratase activities have therefore been converted into the same units. 4-Hydroxy-2-oxovalerate aldolase was assayed by converting the residual substrate to its lactone (Dagley & Gibson, 1965) and measuring this by HPLC analysis. The HPLC analyses of 2-oxopent-4-enoate and 4-methyl-2-oxobutyrolactone were done using a mobile phase of 20% (v/v) propan-2-ol and 8 mM-orthophosphoric acid. A Spherisorb 10 C18 reverse phase column was used. With a UV monitor operating at 270 nm, and a mobile phase flow rate of 2.5 ml min⁻¹, 2-oxopent-4-enoate had a retention time of 3.45 min. Using a monitoring wavelength of 230 nm and a flow rate of 1 ml min⁻¹, the 4-methyl-2-oxobutyrolactone had a retention time of 4.18 min.

Identification of intermediates. 4-Oxalocrotonate (OC) was isolated and identified as follows: a 50 ml phosphate buffer (pH 7.0) solution containing 5 μ mol HMS, 10 mg cell-free extract protein, 50 μ mol NAD and 250 μ mol EDTA was incubated at 55 °C until the yellow colouration had disappeared. The solution was then acidified to pH 1.5 with concentrated HCl and the precipitate removed by centrifugation. The product was then extracted from the supernatant three times with 50 ml ethyl acetate. After drying over anhydrous MgSO₄ and removal of the ethyl acetate by rotary evaporation, the precipitate was dissolved in ethanol. Adding 50 μ l of this solution to 1 ml phosphate buffer (pH 7.0) gave a spectrum with a peak at 292 nm that disappeared within 5 min, resulting in the production of a new peak within the 235–240 nm region. On addition of 100 μ l 10 M-NaOH a peak with a wavelength of 350 nm appeared. These spectral characteristics are consistent with those reported for 4-oxalocrotonate (Sala-Trepat & Evans, 1971a).

2-Oxopent-4-enoate was analysed by HPLC analysis. A 10 ml phosphate buffer solution containing 5 mg cell-free extract was incubated at 55 °C. The transient appearance of an intermediate with the same retention time as authentic 2-oxopent-4-enoate (3.45 min) was observed. The intermediate, like the authentic compound, was also unstable in alkaline solution.

HPLC analysis was also used to monitor 4-hydroxy-2-oxovalerate. A phosphate buffer solution (1 ml) containing 0.5 mg cell-free extract protein, 1 μ mol MgSO₄ and 100 μ l of the 2-oxopent-4-enoate preparation was

incubated at 55 °C. After 1 min the product reached its maximum concentration (22.1 mM), and thereafter slowly disappeared.

Pyruvate was determined enzymically by a method based upon that of Brown & Perham (1976). Cell-free extract solutions (1 ml) were held at 100 °C for 30 min to destroy any thermophilic NADH oxidase activity. The samples were clarified by centrifugation and 0.5 ml was added to a cuvette. To the sample was added: NAD, 1 µmol; thiamin pyrophosphate, 0.2 µmol; MgSO₄, 2.6 µmol; pyruvate dehydrogenase, 0.25 units. The samples were made up to 1 ml with 50 mM-phosphate buffer (pH 7.0) and incubated at 30 °C. The pyruvate concentrations were determined by following the production of NADH as absorbance at 340 nm.

Acetaldehyde was determined by the method of Lundquist (1958). The NADH oxidase activity was destroyed in the same manner as for pyruvate measurements. A 0.5 ml sample was added to a cuvette together with: NAD, 1 µmol; L-cysteine, 1 µmol; aldehyde dehydrogenase, 0.25 units. The samples were made up to 1 ml with 50 mM-phosphate buffer (pH 7.0), and incubated at 25 °C. Acetaldehyde concentrations were also measured by following NADH production as absorbance at 340 nm.

RESULTS

Table 1 shows the rates of disappearance of the ring fission products of catechol and the methylcatechols and the effects of NAD upon these rates. Chemical and enzymic analyses were consistent with formate being a product of HMS metabolism in the absence of NAD. A 4 ml solution of 50 mM-phosphate buffer containing 2 mg cell-free extract protein was incubated with HMS for 1 h at 55 °C. During this time, 224 nmol HMS (calculated from the decrease in absorbance at 375 nm) was metabolized. A total of 180 nmol formate was produced when assayed by the method of Grant (1948), a yield of 80%.

Formate was also detected enzymically using formate dehydrogenase. A 3 ml sample containing 1.2 mg cell-free extract protein in phosphate buffer metabolized 48 nmol HMS with the production of 42 nmol formate, an 88% yield.

The product of HMS degradation in the presence of NAD was isolated and identified by its spectral characteristics as 4-oxalocrotonate. To establish the stoichiometry of the reaction, cuvettes containing 50 nmol HMS, 0.4 mg cell-free extract, 1 µmol NAD and 5 µmol EDTA in 1 ml phosphate buffer were incubated at 55 °C until the semialdehyde had been completely metabolized. On completion of the reaction 100 µl NaOH (10 M) was added. A new peak at 350 nm was observed, corresponding to 52 nmol 4-oxalocrotonate, a yield of 104%.

The metabolism of 4-oxalocrotonate was observed spectrophotometrically by following a decrease in absorbance at 292 nm and an increase in absorbance at 235–240 nm. This is consistent with previous reports of a tautomerization between the enol and keto forms of this compound (Sala-Trepat & Evans, 1971*a*). The reaction occurs spontaneously, with a rate of decrease in absorbance at 292 nm of 3.3 units min⁻¹ (55 °C, pH 7.0). The cell-free extract of phenol-grown cells increased this rate of tautomerization by 400 units (mg protein)⁻¹ min⁻¹ (Table 2). The keto form of OC was further degraded by cell-free extracts. This reaction was stimulated 11-fold by 1 mM-Mg²⁺ and completely inhibited by 5 mM-EDTA. A transient product of the reaction was identified as 2-oxopent-4-enoate by HPLC analysis.

Authentic 2-oxopent-4-enoate yielded 4-hydroxy-2-oxovalerate in the presence of cell-free extracts from phenol-grown cells. The appearance of this product was again transient. The reaction was stimulated 36-fold by 1 mM Mg²⁺ and Mn²⁺ (Table 3). The metabolism of 4-hydroxy-2-oxovalerate proceeded by an aldolase reaction, preliminary experiments indicating that pyruvate and acetaldehyde were the reaction products. Triplicate incubations of phosphate buffer solutions (1 ml) containing cell-free extract protein (0.5 mg) and 4-hydroxy-2-oxovalerate (0.5 µmol) were incubated at 55 °C for 30 min. The incubations were done in sealed microfuge tubes to prevent the escape of acetaldehyde (boiling point 21 °C). The solutions were then re-assayed for 4-hydroxy-2-oxovalerate, pyruvate and acetaldehyde. On average, 78 nmol 4-hydroxy-2-oxovalerate were degraded and 38 nmol pyruvate and 22 nmol acetaldehyde were produced. In further experiments, the maximum amount of synthetic 4-hydroxy-2-oxovalerate metabolized was 40%.

The enzymes involved in the degradation of HMS to pyruvate and acetaldehyde were assayed in cell-free extracts prepared from glucose- and phenol-grown cells (Table 1–3). All of the

Table 1. *Rates of disappearance of ring fission products*

Rates are expressed as $\text{nmol (mg protein)}^{-1} \text{ min}^{-1}$. All extracts were treated with 0.056 units NADase for 30 min at 37 °C. NAD was added to a final concentration of 1 mM. When NADP was added to the NADase-treated phenol-grown cell-free extracts, to a final concentration of 1 mM, the rate of HMS disappearance increased to $11.4 \text{ nmol (mg protein)}^{-1} \text{ min}^{-1}$. ND, Not determined.

Ring fission product	Phenol-grown cells				Glucose-grown cells			
	Without NAD		With NAD		Without NAD		With NAD	
	Rate	%	Rate	%	Rate	%	Rate	%
2-Hydroxymuconic semialdehyde	3.34	5	67.6	100	2.1	3	2.1	3
2-Hydroxy-6-oxo-hepta-2,4-dienoate	4.04	6	4.3	6	ND	—	ND	—
2-Hydroxy-5-methyl-muconic semialdehyde	1.02	2	57.7	85	ND	—	ND	—

Table 2. *Activities of 4-oxalocrotonate-metabolizing enzymes*

Tautomerase rates are expressed in absorbance units $(\text{mg protein})^{-1} \text{ min}^{-1}$ and decarboxylase rates as $\text{nmol (mg protein)}^{-1} \text{ min}^{-1}$. Spontaneous disappearance of 4-oxalocrotonate was not observed in the absence of cell-free extracts. EDTA was added to a final concentration of 5 mM, and MgSO_4 , 1 mM. ND, Not determined.

Enzyme	Addition	Cells grown on:			
		Phenol		Glucose	
		Rate	%	Rate	%
4-Oxalocrotonate tautomerase	None	400	100	0	0
	EDTA	400	100	ND	—
4-Oxalocrotonate decarboxylase	None	88.5	100	2.8	3
	EDTA	0	0	ND	—
	MgSO_4	963	1090	ND	—

Table 3. *Activities of 2-oxopent-4-enoate hydratase and 4-hydroxy-2-oxovalerate aldolase*

All additions were added to final concentrations of 1 mM. Controls without cell-free extracts showed no significant rate of degradation above the background levels. ND, Not determined.

Enzyme	Addition	Cells grown on:			
		Phenol		Glucose	
		Rate	%	Rate	%
2-Oxopent-4-enoate hydratase	None	0.17	100	0	0
	MgSO_4	6.15	3600	ND	—
	MnSO_4	6.19	3600	ND	—
4-Hydroxy-2-oxo-valerate aldolase	None	3.83	100	3.39	89
	NAD	3.58	93	ND	—

enzymes, with the exception of HODH and 4-hydroxy-2-oxovalerate aldolase, were induced by growth on phenol.

DISCUSSION

The degradation of *meta* ring fission products by *B. stearothermophilus* IC3 is consistent with the pathway shown in Fig. 1. Similar pathways have been delineated in pseudomonads (Murray *et al.*, 1972; Hopper & Taylor, 1975), *Alcaligenes eutrophus* (Bayly *et al.*, 1978) and *Azotobacter* species (Sala-Trepat & Evans, 1971*a*).

Fig. 1. Degradation of ring fission products. Numerals represent chemicals as follows: 1, 2-hydroxymuconic semialdehyde; 2, 4-oxalocrotonate (enol form); 3, 4-oxalocrotonate (keto form); 4, 2-oxopent-4-enoate; 5, 4-hydroxy-2-oxovalerate. Letters represent enzymes as follows: A, 2-hydroxymuconic semialdehyde dehydrogenase; B, 4-oxalocrotonate tautomerase; C, 4-oxalocrotonate decarboxylase; D, 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase; E, 2-oxopent-4-enoate hydratase; F, 4-hydroxy-2-oxovalerate aldolase.

In strain IC3, two different enzymes appear to be responsible for the degradation of 2-hydroxymuconic semialdehyde and its 5-methylated derivative. One of these enzymes, which is not NAD dependent, gives formate as a reaction product. The results indicate that the reaction may be catalysed by an HODH. The other product of the reaction of HODH upon HMS has previously been shown to be 2-oxopent-4-enoate (Dagley & Gibson 1965). This is assumed to be the other product in hydrolytic HMS metabolism by strain IC3, but it has not been directly demonstrated as such.

As observed in previous reports, the hydrolase enzyme has a greater activity towards HOD than HMS or HMMS. However, the relative activities of the enzyme from strain IC3 towards HMS and HMMS were much greater than those observed in *B. stearotherophilus* PH24 (Buswell, 1974), *Pseudomonas putida* strains NCIB 10015, NCIB 9865 (Bayly & Di Berardino, 1978) and mt-2 (Duggleby & Williams, 1986), and *Alcaligenes eutrophus* 345 (Bayly *et al.*, 1987).

The alternative route for the metabolism of ring fission products in the thermophile is an NAD-mediated dehydrogenation. The enzyme, HMSD, quantitatively converts HMS into 4-oxalocrotonate. The enzyme also attacks HMMS, but the product was not determined. The ketone product of 3-methylcatechol ring cleavage, HOD, was not a substrate for the dehydrogenase. These results are similar to those observed with the dehydrogenase from several *Azotobacter* species (Sala-Trepat & Evans, 1971*a, b*) and *P. putida* mt-2 (Murray *et al.*, 1972).

Previous workers have suggested that the hydrolytic route, where the oxidative route is present, is utilized in the metabolism of compounds which yield HOD and analogous ketones, while the oxidative route is used for the metabolism of compounds which give aldehyde intermediates. When HMSD activity is absent, HMS and its methyl substituted analogues can be metabolized hydrolytically (Bayly & Dagley, 1969). In contrast, HMSD will only metabolize HMS and HMMS. Consequently, organisms such as *Azotobacter vinelandii* that only display low, non-inducible levels of the hydrolase, possibly of an unrelated pathway, cannot grow on compounds such as *o*-cresol that yield HOD (Sala-Trepat & Evans, 1971*a*).

4-Oxalocrotonate was identified as an intermediate in the degradation of catechol by Nishizuka *et al.* (1962). Subsequently, it was shown to be degraded by a tautomerization from the enol to the keto form, followed by a decarboxylation, yielding 2-oxopent-4-enoate (Sala-Trepat & Evans, 1971 *a*). The results in Table 2 show that strain IC3 also possesses an inducible 4-oxalocrotonate tautomerase activity. The fact that this enzyme is necessary for growth, despite the rapid spontaneous rate of tautomerization, has been verified by a number of workers (Wigmore *et al.*, 1974; Barbour & Bayly, 1980; Harayama *et al.*, 1987). The activity of the decarboxylase enzyme, like those reported previously, was stimulated by Mg^{2+} ions and inhibited by EDTA.

Strain IC3 also possesses a 2-oxopent-4-enoate hydratase activity. This activity is stimulated 36-fold by both Mg^{2+} and Mn^{2+} ions. Collinsworth *et al.* (1973) found that a similar enzyme activity in *P. putida* was stimulated 5-fold by Mn^{2+} , no stimulation being observed in the presence of Mg^{2+} . Another *P. putida* 2-oxopent-4-enoate hydratase activity was stimulated 7- and 2-fold by Mn^{2+} and Mg^{2+} ions respectively (Lampel, 1979).

The 2-oxopent-4-enoate hydratase and 4-hydroxy-2-oxovalerate aldolase were found to be stereospecific in other organisms. Collinsworth *et al.* (1973) concluded that L-(S)-4-hydroxy-2-oxovalerate was the biologically active enantiomer in the degradation of aromatic compounds by *P. putida* NCIB 10015. The fact that only 40% of the synthetic 4-hydroxy-2-oxovalerate was metabolized by the thermophilic extracts suggests that the hydratase and aldolase reactions are also stereospecific in *B. stearothermophilus* IC3, although the biologically active enantiomer was not determined.

The results demonstrate that 4-hydroxy-2-oxovalerate was degraded to pyruvate and acetaldehyde. This reaction indicates the presence of an aldolase enzyme similar to that described by Dagley & Gibson (1965). The metabolism of 4-hydroxy-2-oxovalerate was not stimulated by the presence of NAD. This suggests that acetylpyruvate is not an intermediate, as reported for some pseudomonads (Nishizuka *et al.*, 1962).

Most of the degradative enzymes were only present when strain IC3 was grown on phenol. However, the HODH and 4-hydroxy-2-oxovalerate aldolase were present in significant amounts in cell-free extracts prepared from glucose-grown cells. The constitutive but low production of HODH has been noted in *Azotobacter* species (Sala-Trepat & Evans, 1971 *b*). These strains were unable to grow on compounds producing 2-hydroxy-6-oxohepta-2,4-dienoate. The *Azotobacter* strains also possessed a constitutive 4-hydroxy-2-oxovalerate aldolase. This reaction in both the mesophile and *B. stearothermophilus* may be catalysed by a non-specific enzyme which also has different functions within the bacterial cell.

The metabolism of ring fission products by *B. stearothermophilus* IC3 is similar to the metabolism of these compounds in mesophiles, all of the enzymes being functional at 55 °C. However, as no data are available on the heat optima or stability of the enzymes, this would be a useful objective for further studies.

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