

Methionine Degradation by *Pseudomonas fluorescens* UK1 and its Methionine-utilizing Mutant

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SUMMARY

Pseudomonas fluorescens UK1, and a mutant derived from it that can use methionine as its sole source of carbon and nitrogen, were used to study methionine degradation. Radioactive 2-oxomethionine, 2-oxobutyric acid and carbon dioxide comprised 95 % of [^{14}C]methionine consumed by the mutant. Demethiolating activity was detected in both strains when they were grown with methionine.

The ability to grow on methionine depended on the decarboxylation of 2-oxobutyric acid derived from it. This decarboxylating activity was lost if the growth media contained an additional carbon source.

No evidence was obtained for the operation of the cystathionine pathway.

INTRODUCTION

Pseudomonads are known to degrade many commonly-occurring amino acids, but one exception is the sulphur amino acid, methionine (Jakoby, 1964; Stanier, Palleroni & Doudoroff, 1966). Although this plays a central role in sulphur metabolism, relatively little is known about the enzymic reactions involved in its degradative pathway.

A few strains of the genus *Pseudomonas* which can use methionine as a carbon and nitrogen source have been isolated (Kallio & Larson, 1955; Miwatani, Omukai & Nakada, 1954; Segal & Starkey, 1969). In these, the methionine sulphur was liberated as volatile methanethiol or its oxidation products in a reaction catalysed by methioninase. The resulting 2-oxobutyric acid was assumed to be decarboxylated to propionic acid (Fig. 1).

The inability of some bacteria to grow on methionine may arise because they lack certain enzymes, although the possibility cannot be ruled out that methionine itself is inhibitory, as in *Saccharomyces cerevisiae* (Yall *et al.*, 1967; Bailey & Parks, 1972). A convenient way for bacteria to eliminate excess methionine would be to liberate the sulphur as a volatile compound. Thus one might expect some bacteria to possess such an ability whether or not they could grow on methionine as carbon source.

This report compares the properties of a mutant that can use methionine for growth with its parent strain *Pseudomonas fluorescens* UK1. Using isotopically-labelled methionine, the distribution of the label amongst the supposed catabolites, 2-oxomethionine, 2-oxobutyric acid and carbon dioxide, was determined in order to identify a possible critical point in methionine catabolism.

METHODS

Chemicals. L-[^{14}C]Methionine (specific activity 62 mCi mmol $^{-1}$) and L-[^{35}S]methionine (specific activity 225 Ci mmol $^{-1}$) were purchased from New England Nuclear (Boston, Massachusetts, U.S.A.); DL-ethionine from Sigma; S-methyl-L-cysteine and S-ethyl-L-

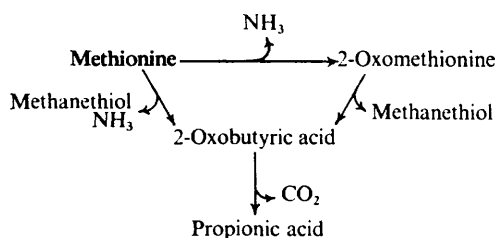


Fig. 1. Methionine degradation in *Pseudomonads*.

cysteine from California Corporation for Biochemical Research (Los Angeles, California, U.S.A.); and L-amino acid oxidase from Calbiochem. 2-Oxomethionine was prepared as described by Meister (1952) and purified by ion-exchange chromatography; DL-homocysteine thiolactone (HCl) was hydrolysed to homocysteine with 2 equiv. KOH; and L-methionine sulphoxide prepared according to the method of Greene (1957).

Bacteria. The principal organisms used were *P. fluorescens* UK1 and a mutant derived from it that could use methionine as its sole source of carbon and nitrogen (Mäntsälä, Laakso & Nurmiikko, 1974). The following strains were used as test organisms: *P. fluorescens* P2 isolated by Goodhue & Snell (1966), *Escherichia coli* K12 (W3001), and *S. cerevisiae* (ATCC7752).

Culture media. The bacteria were grown in a basal mineral medium containing (g l^{-1}): KH_2PO_4 , 1.35; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.26; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.003, adjusted to pH 7.2 with KOH. Unless otherwise stated, the concentrations of the carbon source, methionine and ammonium sulphate (added where necessary as nitrogen source) were all 10 mM.

Growth conditions. The organisms were grown in 100 ml of medium in 500 ml flasks with constant shaking at 30 °C. Exponential growth was maintained whilst keeping the turbidity below 50 Klett-readings (filter 62), by diluting if necessary with fresh medium. Before sampling, cultures were grown for several hours, with frequent measurement of turbidity to ensure that a constant growth rate had been established.

Determination of methionine catabolites. Samples containing about 4 mg dry wt bacteria were withdrawn from exponential-phase cultures, centrifuged at 5000 g for 15 min, washed twice with the basal mineral medium, and suspended in 2 ml of the same medium. Cell-free extracts were made by breaking this suspension at 0 °C in a sonic oscillator (MSE 60 W). Extracts were centrifuged at 5000 g for 15 min before use.

Cell suspension (0.5 ml; 1 mg dry wt/ml) or cell-free extract was pipetted into the main chamber of a double side arm Warburg flask. One side arm contained 0.5 ml of 10 μM -[1- ^{14}C]methionine (specific activity 7.9 mCi mmol $^{-1}$) and the other 0.5 ml of 2 M-HClO $_4$. The centre well contained a filter paper moistened with 0.2 ml of 5 M-KOH to absorb the radioactive carbon dioxide. After equilibration, the methionine was tipped into the main chamber and incubation at 30 °C was continued with constant shaking for 30 min. At intervals, HClO $_4$ from the side arm was poured into the main chamber and incubation continued for an additional 15 min so that all the carbon dioxide liberated from the acidic reaction mixture would be absorbed. The filter papers were air dried and counted for radioactivity in a scintillation spectrometer (Wallac, Decem-NTL 314).

Separation of the 2-oxo acids. The contents of the Warburg vessels were transferred to test tubes and neutralized with KOH. The neutralized supernatant fluids were incubated with 0.5 ml of 2.5 mM-2,4-dinitrophenylhydrazine (in 1.2 M-HCl) for 30 min at 30 °C

before extracting the 2-oxo acids as their dinitrophenylhydrazones with ethyl acetate (Katsuki *et al.*, 1971). The latter were separated by kieselgel thin-layer chromatography (kieselgel G in 0.1 M-NaHCO₃, 1:2, w/v) (Ariga, 1972), extracted from the plates with 1% (v/v) ammonia (in methanol) and counted for radioactivity.

Thin-layer chromatography and autoradiography. Two-dimensional chromatography followed by autoradiography was used to identify components derived from [1-¹⁴C]methionine and [³⁵S]methionine.

Mutant bacteria growing exponentially on 10 mM-methionine were transferred into fresh medium containing 0.6 mM-methionine. This suspension (10 ml; approx. 0.6 mg dry wt/ml) was incubated for 30 min and then supplemented with 1.5 μ Ci [1-¹⁴C]methionine (specific activity 62 mCi mmol⁻¹) or with 1.5 μ Ci [³⁵S]methionine (specific activity 225 Ci mmol⁻¹). After an additional 10 min incubation, a 5 ml sample was pipetted into 10 ml of 50% (v/v) aq. ethanol at 80 °C. The ethanol-insoluble material was centrifuged (5000 g, 15 min) and the precipitate washed twice with 50% (v/v) aq. ethanol and twice with water before hydrolysing with 0.5 ml of 6 M-HCl (110 °C, 20 h), evaporating to dryness and dissolving in 0.2 ml of water. The supernatant fraction was also evaporated to dryness and dissolved in water (1 ml). Standard compounds were treated with ethanol in a similar manner. Samples were spotted on to t.l.c. plates covered with a mixture of cellulose and kieselgel (cellulose MN 300, silica gel H, 5:1, w/w). Chromatograms were developed first with phenol-water (78:28, w/v) and then with n-propanol-water-n-propyl acetate-acetic acid-pyridine (120:60:20:4:1, v/v) (Bielecki & Turner, 1966). The radioactive spots were identified according to their R_F values and rechromatographed with appropriate standards.

Determination of demethiolation. The methods described by Challenger (1959) and Segal & Starkey (1953) were used to detect volatile sulphur products. Demethiolating activity (methioninase) was determined by a procedure based on spectrophotometric determination of a coloured aryl mercaptan produced by a quantitative reaction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) with liberated methanethiol. The standard assay mixture contained 3 μ mol L-methionine, 0.2 μ mol DTNB and 0.25 mg dry wt of washed organisms, resuspended in 1.0 ml basal mineral medium. The increase in absorbance at 412 nm was followed with a Unicam SP 800 spectrophotometer.

RESULTS

Occurrence of demethiolating activity in micro-organisms

A qualitative test to detect volatile sulphur products derived from methionine was made by drawing sterile air through a culture of the mutant *P. fluorescens*, growing exponentially on methionine, into two serial washing bottles, one containing aq. Hg(CN)₂ (3%, w/v) and the other HgCl₂ (3%, w/v). Methanethiol and trace amounts of dimethyl disulphide were the only sulphur compounds identified. For quantitative methanethiol determinations, the DTNB method was used: in this DTNB functions as the primary oxidant and dimethyl disulphide, presumably a product of autoxidation, is determined as methanethiol.

Three strains of *P. fluorescens* (UK1 and its mutant, and P2), *E. coli* K12 and *S. cerevisiae* were tested for demethiolating activity. Only the mutant of *P. fluorescens* UK1 could utilize methionine as sole source of carbon and nitrogen. Therefore, demethiolating activity was measured in organisms growing exponentially on glucose with 10 mM DL-methionine. The rates of demethiolation (per mg dry wt of organisms) were constant and independent of the time of sampling, indicating steady-state conditions of growth. Methionine was demethiolated by all three cultures of *P. fluorescens* at about the same rate. *Escherichia coli* and *S. cerevisiae* showed only about 1% of this activity.

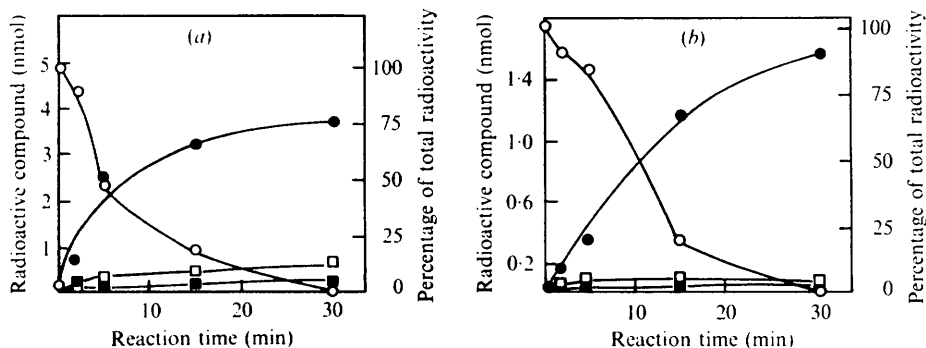


Fig. 2. Distribution of radioactivity derived from [1-¹⁴C]methionine amongst 2-oxomethionine, 2-oxobutyric acid and carbon dioxide. Cell-free extracts (a) or washed cell suspensions (b) were prepared from the mutant grown on methionine (10 mM) as carbon and nitrogen source. The experimental conditions were as described in Methods. ○, [¹⁴C]Methionine; ●, ¹⁴CO₂; □, oxo[¹⁴C]methionine; ■, oxo[¹⁴C]butyric acid.

Stoichiometry of methionine degradation

Although 95% of the catabolic activity was lost during cell disruption, most of the radioactivity of [1-¹⁴C]methionine was released as ¹⁴CO₂ by both whole cell suspensions (90%) and cell-free extracts (80%) of the mutant grown on methionine as carbon and nitrogen source.

Radioactive oxo acids which accumulated were separated as their dinitrophenylhydrazine derivatives on kieselgel thin-layer plates with ethyl acetate-methanol (5:1, v/v) as solvent. The only labelled oxo acids found were 2-oxomethionine and 2-oxobutyric acid. Of the labelled methionine consumed by whole cells, 95% was recovered as ¹⁴CO₂, 2-oxo[1-¹⁴C]-methionine and 2-oxo[1-¹⁴C]butyric acid (Fig. 2a). The corresponding value for cell-free extracts was 88% (averages of four determinations) (Fig. 2b).

The possible role of the identified oxo acids as methionine catabolites was tested by determining the distribution of ¹⁴C derived from labelled methionine in cell-free extracts supplemented with either 2-oxomethionine or 2-oxobutyric acid (Table 1). The latter caused a significant decrease in the rate of labelled CO₂ evolution and a nine-fold increase in production of labelled 2-oxobutyric acid. This suggests that decarboxylation of 2-oxobutyric acid rather than 2-oxomethionine is involved in methionine degradation. Neither pyridoxal phosphate nor pyridoxamine had a significant effect on CO₂ production from methionine in the presence of 2-oxomethionine, whereas pyridoxamine greatly reduced the radioactivity found in both oxo acids.

[1-¹⁴C]Methionine sulphoxide, a poor growth substrate for both the parent and mutant of *P. fluorescens* UKI, was converted into ¹⁴CO₂ at only 5.5% of the rate of methionine.

Chromatography of methionine catabolites

Washed suspensions of the mutant and its parent strain were incubated with [³⁵S]methionine or [1-¹⁴C]methionine and radioactive products were separated by two-dimensional cellulose-kieselgel thin-layer chromatography. Parent and mutant cultures gave identical autoradiograms with both isotopes. Spots 1 and 2 (Table 2) were identified as methionine and its sulphoxide, methionine sulphoxide being formed spontaneously during ethanol extraction and chromatography. Only two other spots were detectable when [¹⁴C]methionine was used as substrate. With [³⁵S]methionine the same four spots appeared and, in addition,

Table 1. Effect of supplements on the rate of production of $^{14}\text{CO}_2$ and $[\text{I-}^{14}\text{C}]$ oxo acids derived from $[\text{I-}^{14}\text{C}]$ methionine by extracts of the mutant

The reaction mixture contained 0.075 μCi $[\text{I-}^{14}\text{C}]$ methionine (specific activity 7.9 mCi mmol $^{-1}$), and each supplement was added to give a concentration of 1.5 $\mu\text{mol ml}^{-1}$.

Supplement	Rate of $^{14}\text{CO}_2$ production (% of control)	Rate of oxo- $[\text{I-}^{14}\text{C}]$ butyrate production (% of control)	Rate of oxo- $[\text{I-}^{14}\text{C}]$ methionine production (% of control)
None (control)	100	100	100
Oxomethionine	79.4	270	880
Oxomethionine + pyridoxal phosphate	65.0	550	350
Oxomethionine + pyridoxamine	77.0	85	80
Oxobutyrate	28.3	930	300

Table 2. Radioactive materials produced by washed suspensions of the mutant incubated with $[\text{I-}^{14}\text{C}]$ or $[\text{S-}^{35}\text{S}]$ methionine

The compounds were extracted as described in Methods and separated on cellulose-silica gel thin-layer plates (20 \times 20 cm) by two-dimensional chromatography. Solvent I, phenol-water (78:28, w/v); solvent II, n-propanol-water-n-propyl acetate-acetic acid-pyridine (120:60:20:4:1, v/v).

Spot no.	Radioactive spots detected from:				R_F values	
	$[\text{I-}^{14}\text{C}]$ Methionine		$[\text{S-}^{35}\text{S}]$ Methionine		Solvent I	Solvent II
	Ethanol soluble	Ethanol insoluble	Ethanol soluble	Ethanol insoluble		
1	+	+	+	+	0.70	0.61
2	+	+	+	+	0.70	0.37
3	+	○	+	+	0.55	0.78
4	+	○	+	○	0.55	0.42
5	○	○	+	○	0.76	0.80
6	○	○	+	○	0.41	0.65
7	○	○	+	○	0.43	0.53
8	○	○	+	○	0.34	0.55

+, Detected; ○, not detected.

there were spots corresponding to four other labelled sulphur compounds (spots 5, 6, 7 and 8). Spots 3 and 4 were present in both ^{35}S and ^{14}C chromatograms, indicating that these compounds probably had a molecular structure similar to methionine. Spot 4 was also present in the ethanol-insoluble fraction chromatographed after acid hydrolysis, suggesting it was an oxidation product of methionine. The R_F values of spots 7 and 8 were similar to those of reduced glutathione, cysteine or taurine. However, rechromatography with internal standards ruled out these compounds. It is remarkable that the exponential culture did not accumulate detectable intermediates of the cystathionine pathway even when the incubation time was prolonged from 10 to 30 min or when the concentrations of radioactive methionine were four times higher (6 μCi , 225 mCi mmol $^{-1}$). Some of the radioactive compounds have not been identified. After acidic or alkaline treatment, for instance, cysteine, cystine and cystathionine gave several spots on the chromatograms, but none of them corresponded to those of the radioactive samples.

Since trans-sulphuration from methionine to cysteine could not be detected, either the amounts of cysteine and the components of the cystathionine pathway must be low, or the ultimate source of cysteine sulphur is inorganic sulphate. The effect of inorganic sulphate

Table 3. *Effect of growth substrates on demethiolating activity and on $^{14}\text{CO}_2$ production from methionine in cell suspensions of *P. fluorescens* UK1 mutant and parent strains*

Bacteria were grown in a basal mineral medium supplemented with growth substrates as indicated. Cell suspensions were prepared from exponentially-growing cultures and the activities were determined as described in Methods. Results are expressed as a percentage of the activities found with mutant cells grown on methionine.

Carbon source for growth (10 mM)	Mutant strain		Wild type	
	Demethiolating activity (%)	Rate of $^{14}\text{CO}_2$ production (%)	Demethiolating activity (%)	Rate of $^{14}\text{CO}_2$ production (%)
Methionine	100	100	—	—
Methionine + glutamate	84.2	25.0	86.8	24.0
Glutamate	10.5	28.0	10.8	26.7
Glucose	5.3	6.6	8.0	7.0
Oxobutyrate	10.5	43.9	—	—

on the fate of methionine sulphur was studied by cultivating the mutant on methionine as carbon and nitrogen source in a medium deficient in exogenous sulphate. A decrease of 15% was observed in the ability of the washed organisms to liberate $^{14}\text{CO}_2$ from [$1\text{-}^{14}\text{C}$]-methionine; demethiolating activity remained unchanged. The products detected by autoradiography and chromatography were also unchanged.

Effect of carbon source on methionine degradation

Highest demethiolating activities were found in organisms grown in the presence of methionine. When the growth medium contained glutamate in addition to methionine, carbon dioxide production was much lower. On the other hand, the ability to demethylate methionine was only slightly decreased by the presence of glutamate or glucose in the culture medium (Table 3). When the sole carbon source was glutamate, glucose or 2-oxobutyrate, demethiolating activity was diminished to 10% of that of bacteria grown on methionine.

DISCUSSION

In *P. fluorescens* UK1 and its methionine-utilizing mutant the catabolic reactions of methionine were rather labile. Similar observations were made by Kreis & Hession (1973) during purification of methioninase from *Clostridium sporogenes*. In spite of 95% inactivation, the crude extracts of *P. fluorescens* UK1 attacked methionine with almost the same stoichiometry as whole cells. This suggests that the labile point lies at the initial stages of breakdown. 2-Oxobutyric acid may be regarded as a more immediate substrate for decarboxylation than 2-oxomethionine or methionine. However, Ruiz-Herrera & Starkey (1970) showed that *Achromobacter starkeyi*, which is able to use methionine for growth, decarboxylates 2-aminobutyric acid more effectively than 2-oxobutyric acid. This may also occur in our *Pseudomonas* strains.

Artificial enhancement of the 2-oxo acid pools resulted in accumulation of label in these pools. This might indicate that decarboxylation of 2-oxobutyric acid is a rate-limiting step in the degradative pathway so that unlabelled 2-oxobutyric acid at concentrations 150 times higher than methionine saturated the decarboxylative system. The increased label in the 2-oxo acid fractions also suggests that demethiolation is irreversible.

In mammalian cells the conversion of methionine sulphur to cysteine via the cystathionine pathway is important, and this pathway is operative in *Neurospora* and *Saccharomyces*

(Flavin, 1962; Delavier-Klutcko & Flavin, 1965). If this were the catabolic route in *P. fluorescens* UK1, the ultimate catabolites might be 2-oxobutyric acid and carbon dioxide, and the component determined by the DTNB method might be hydrogen sulphide. However, the operation of the cystathionine pathway as a catabolic route for methionine seems unlikely. Chromatographic analysis of reaction mixtures containing [³⁵S]methionine or [1-¹⁴C]methionine did not reveal any traces of members of the cystathionine pathway. If homocysteine, cystathionine, cysteine or homoserine were catabolites of methionine, it is difficult to believe that they would not have accumulated in detectable amounts, especially when the relatively unstable 2-oxo acids were found.

In most studies of methionine degradation the organisms were cultivated in the presence of methionine. One exception was the study of Kreis & Hession (1973) on *Clostridium sporogenes*, from which they extracted methioninase. Further evidence of the constitutive nature of methionine degradation has been presented for *Aspergillus* sp. by Ruiz-Herrera & Starkey (1969). In *P. fluorescens* UK1, demethiolating activity was influenced by the presence of methionine in the medium, both organisms (wild type and mutant) exhibiting their highest demethiolating activities when grown on methionine. These results suggest that the difference between the strains is not in the demethiolation step.

The liberation of carbon dioxide was strongly influenced by the presence of a carbon source in addition to methionine. This, in conjunction with the inability of the wild type to grow at the expense of 2-oxobutyric acid, suggests that 2-oxobutyric acid is one of the components of the methionine catabolic pathway and that the ability to grow on methionine depends on the ability to decarboxylate it.

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