The Physiology of L-Methionine Catabolism to the Secondary Metabolite Ethylene by *Escherichia coli*

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Catabolism of L-methionine by *Escherichia coli* strain B SPAO led to the formation of ethylene as a secondary metabolite (ethylenogenesis). Methionine was initially deaminated by a transamination reaction to the 2-oxo acid 2-oxo-4-methylthiobutyric acid (KMBA) which was then converted to ethylene. The utilization of L-methionine as an additional nitrogen source was investigated by examining ethylene synthesis under different nitrogen supply conditions. Ethylene formation in batch culture was unaffected by the concentration of the precursor L-methionine in the medium although increasing concentrations of NH_4Cl resulted in progressively less ethylene formation. Cultures grown without L-methionine did not produce ethylene but were able to synthesize ethylene when L-methionine or KMBA was provided. Addition of L-tyrosine to batch cultures reduced the yield of ethylene after 42 h by 54%. Under these conditions the maximum transient level of KMBA was reduced by 32% and occurred later compared to when L-methionine was the only amino acid supplement. Continuous cultures grown under ammonia limitation produced both ethylene and KMBA. In contrast, when glucose was limiting, neither of these metabolites were produced. Cells harvested from continuous cultures grown under glucose or ammonia limitation were able to synthesize ethylene from either L-methionine or KMBA although their capacity for ethylene synthesis (ethylenogenic capacity) was optimal under ammonia limitation (C : N ratio = 20).

INTRODUCTION

Microbial secondary metabolism is one of the most poorly understood and characterized areas of biochemistry. However, it is responsible for the synthesis of many important biologically derived products such as antibiotics, and flavour and fragrance compounds. As the biochemical mechanisms underlying these systems are unknown, methods for their manipulation have few rational guidelines (Bunch & Harris, 1986). Therefore, there is a need to investigate the interaction of primary and secondary metabolic processes within cells, coupled with a detailed examination of the enzymes involved.

Ethylene formation (ethylenogenesis) by *Escherichia coli* exhibits many characteristics shared by other secondary metabolic processes (Ince & Knowles, 1985; Primrose, 1977) and its synthesis from L-methionine occurs by a different pathway to that used by plants (Adams & Yang, 1981). The first step appears to involve a soluble aminotransferase which produces 2-oxo-4-methylthiobutyrate (KMBA) from L-methionine. The enzyme can use a variety of 2-oxo acids as the co-substrate but shows highest activity with 2-oxoglutarate. Bacteria grown in a defined medium containing glucose, NH_4Cl and mineral salts supplemented with L-methionine excrete KMBA which accumulates in the culture fluid prior to ethylene production (Billington *et al.*, 1979; Ince & Knowles, 1985). The specific activity of the L-methionine aminotransferase does not change during growth of *E. coli* in batch culture (Ince & Knowles, 1986). Ethylene production is reportedly influenced by glucose and ammonia levels and ethylenogenesis may be

Abbreviations: KMBA, 2-oxo-4-methylthiobutyric acid; 2,4-DNP, 2,4-dinitrophenylhydrazine.

involved in the recovery of the amino group of L-methionine when there is a surplus of this amino acid in the medium (Ince & Knowles, 1986). It is notable that *E. coli* possesses high and low affinity systems for the uptake of exogenous L-methionine (Kadner & Watson, 1974).

The objective of this study was to examine the relationship between ethylene formation in *E. coli* and the amount and type of nitrogen source present in the culture medium.

METHODS

Organisms and growth conditions. *Escherichia coli* strain B SPAO, obtained from Dr C. S. Dow (University of Warwick), was maintained on nutrient agar slants (2.8%, w/v, Oxoid Nutrient Agar) at room temperature and subcultured at monthly intervals. The defined liquid medium used for growth and ethylene formation was described by Ince and Knowles (1985). Glucose and NH_4Cl concentrations were varied and additional amino acids added as indicated in the text. All amino acid solutions were sterilized by filtration through a 0.45 μm filter (Whatman). Growth was estimated by measuring the optical density of culture at 540 nm using an LKB 4049 spectrophotometer.

Cultures grown on orbital shakers were either in 250 ml or 2 litre Erlenmeyer flasks containing 30 ml and 500 ml of liquid growth medium respectively. Serum caps (Subaseal, Fisons) were fitted to flasks to prevent the loss of ethylene. This has not been found to result in oxygen limitation (J. E. Ince & C. J. Knowles, unpublished observations). Bacteria were grown at 36 °C and 200 r.p.m. Inocula were prepared by washing bacteria from a nutrient agar slant into 100 ml of growth medium in a 250 ml Erlenmeyer flask. This culture was then grown for 24 h under the conditions described above and a 2% (v/v) inoculum added to experimental flasks.

Continuous culture was done in a 1 litre fermenter (LH 500 Series) with a working volume of 800 ml. The defined medium described for batch cultures were used but glucose and NH_4Cl concentrations were varied in order to achieve nitrogen- or carbon-limiting growth conditions. L-Methionine was added where stated. A 2% (v/v) inoculum was used and the culture was stirred at 1000 r.p.m. Temperature was maintained at 36 °C and the pH at 6.90 by the automatic addition of 2 M-NaOH. An air flow rate of 1 litre min^{-1} was sufficient to maintain the dissolved oxygen tension at 80–95% saturation. The organism was initially cultivated under batch conditions. After 12 h a continuous nutrient feed was delivered by peristaltic pump (P-1, Pharmacia) and the culture allowed to reach steady-state at a growth rate (dilution rate) of 0.1 h^{-1} . Ethylene in the fermenter gas outlet was absorbed and concentrated using a mercuric perchlorate trapping system as described by Lynch & Harper (1974).

Analytical methods. Ethylene samples were analysed by gas chromatography using the conditions described by Ince & Knowles (1985). Glucose and ammonia were also measured as described previously (Ince & Knowles, 1985).

Amino acids were converted to their *O*-phthaldialdehyde (OPA) derivatives by the method of Gardner & Miller (1980) and identified using reversed-phase HPLC. OPA-amino acids were separated on a reverse-phase column (Spherisorb ODS-2, 5 μm ; 250 $\times$ 4.6 mm internal diameter; Jones chromatography) attached to a Milton Roy HPLC apparatus. Aqueous samples (10 μl) were filtered to 0.2 μm , injected onto the column and eluted isocratically at a flow rate of 1 ml min^{-1} using a mobile phase of methanol/0.05 M- KH_2PO_4 (adjusted to pH 4.5 with HCl)/water (55:20:25, by vol.). The OPA-amino acids were detected using a spectrofluorimetric detector (excitation maximum 355 nm; emission maximum 390 nm).

Free 2-oxo acids are unstable in aqueous environments and detection of individual 2-oxo acids on mixtures is frequently difficult. A method based on that of Hemming & Gubler (1979) was adopted whereby 2-oxo acids are converted to their 2,4-dinitrophenylhydrazones (2,4-DNPs), followed by separation and detection using paired-ion reverse-phase HPLC. The 2,4-DNPs of the 2-oxo acids were prepared at room temperature as follows: 100 μl of freshly prepared 2,4-dinitrophenylhydrazine (0.2%, w/v) in 2 M-HCl was added to 100 μl of a solution containing 2-oxo acids and allowed to react for 30 min. Insoluble hydrazones, visible as yellow precipitates, and unreacted 2,4-dinitrophenylhydrazine were then extracted into 500 μl ethyl acetate. Vigorous mixing was necessary for the complete extraction which left the aqueous phase almost colourless. Solvent was removed under a stream of air leaving a yellow residue which was stored at 4 °C until required. The residue was dissolved in methanol, samples (10 μl) injected onto the HPLC column and hydrazone derivatives eluted isocratically at a flow rate of 0.5 ml min^{-1} with methanol/0.05 M-tetrabutylammonium hydroxide (adjusted to pH 4.3 with glacial acetic acid) (80:20, v/v). Separated 2,4-DNPs were detected at 368 nm and peak data evaluated using a Milton Roy integrator/printer-plotter.

Standards for HPLC were prepared using authentic L-amino acids and 2-oxo acids without further purification.

Ethylene assays with harvested bacteria. Bacteria were harvested from cultures by centrifugation (20000 g, 10 min, 4 °C), washed in 20 mM- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (pH 7.0) at 4 °C and resuspended in the same phosphate buffer to about 15 mg dry weight ml^{-1} . Culture samples never exceeded 10% (v/v) of the total culture volume. Bacterial suspension (1.0 ml) was added to a 25 ml Erlenmeyer flask fitted with a serum cap and

containing the following assay mixture: 20 mM- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, 0.1 mM- CaCl_2 , 1.0 mM- MgSO_4 , trace elements (as for growth), 10 mM-glucose and 10 mM-L-methionine or 10 mM-KMBA in a total volume of 1 ml. Flasks were incubated at 37°C in the dark on an orbital shaker operating at 200 r.p.m. Gaseous headspace samples (0.5 ml) were removed from the flasks at 30 min intervals for 5 h using a gas-tight syringe and the ethylene content determined by gas chromatography.

The rate of ethylene synthesis by harvested bacterial cells is referred to as the ethylenogenic capacity (Ince & Knowles, 1985).

Radioassay of L-methionine aminotransferase in the soluble fraction of cell-free extracts (high-speed supernatant). High speed supernatant was prepared as described previously (Ince & Knowles, 1986). Reaction mixtures (100 μl) contained: 50 mM-Tris/HCl (pH 8.0), 250 μM -pyridoxal 5'-phosphate, 10 mM-L-[U- ^{14}C]methionine (0.064 μCi μmol^{-1} ; 2370 Bq ml^{-1}), 24 μg bovine serum albumin, 5 mM-2-oxoglutarate and 40 μl of high speed-supernatant.

After 10 min incubation at 37 °C, the reaction was stopped with 0.3 ml 1 M-HCl followed by addition of ethyl acetate/toluene (4:1, v/v). Under these acidic conditions the 2-oxo acids are extracted into the organic solvent whilst amino acids remain in the aqueous layer. Samples (100 μl) of the organic phase were combined with 4 ml of Optiphase T liquid scintillant and radioactivity was determined by scintillation counting (Beckman LS7800).

Protein was estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

Reproducibility of results. All batch culture experiments and harvested cell ethylene assays were done in triplicate; the results given in Figs 1, 2 and 4 are means of these measurements.

Materials. L-[U- ^{14}C]methionine was obtained from Amersham. Optiphase T scintillation fluid was purchased from LKB and tetrabutylammonium hydroxide from Aldrich. All other chemicals were purchased from Sigma. Solvents were of AR grade (May & Baker or Fisons). Glass distilled water was used throughout.

RESULTS

Effect of different ammonia concentrations on ethylenogenesis in batch cultures

E. coli was grown in defined medium supplemented with 2.7 mM-L-methionine. Growth and ethylene production in cultures with various initial concentrations of NH_4Cl were determined for 30 h (Fig. 1*a, b*). Growth yields were only slightly different over a range of NH_4Cl concentrations used. Growth was slower when no NH_4Cl was present and when methionine was the sole nitrogen source. It is notable that in cultures containing more than 2 mM- NH_4Cl no residual glucose could be detected after 30 h.

After 30 h ethylene production was highest in cultures containing 2.0 mM- NH_4Cl . Above this concentration progressively less ethylene accumulated in spite of similar growth yields. After 17 h incubation all cultures were producing ethylene. Bacteria harvested from cultures at this time showed different ethylenogenic capacities (Fig. 2). Interestingly, their ethylenogenic capacity was inversely proportional to the initial concentration of NH_4Cl in the growth medium.

Effect of different L-methionine concentrations on ethylenogenesis in batch cultures

E. coli was grown for 15.5 h in defined liquid medium initially containing 0.5, 1.0, 2.0, 2.5 or 3.0 mM-L-methionine. The glucose concentration was initially 10 mM and that of NH_4Cl 3.0 mM. There was no difference in the growth rate (doubling time 2 h) and the final cell yield was the same in each culture (OD_{540} after 15.5 h was 1.02 ± 0.056). Ethylene was present in all flasks after 10 h and all cultures produced ethylene at a similar rate (0.107 ± 0.0176 μmol per flask) to the culture grown at 3.0 mM- NH_4Cl (Fig. 2) except that no ethylene production was seen in the absence of methionine. After 15.5 h, the ethylene yield of the cultures was 0.029 ± 0.0063 μmol (mg cell dry wt) $^{-1}$. Harvested, washed cells, including those grown without L-methionine, all had similar ethylenogenic capacities [4.73 ± 1.17 nmol h $^{-1}$ (mg cell dry wt) $^{-1}$].

Ethylenogenesis in ammonia- and glucose-limited continuous culture in the presence and absence of L-methionine

Fig. 3 shows the residual concentrations of glucose and NH_4Cl and ethylenogenic capacities of bacteria harvested from continuous cultures grown over a range of carbon : nitrogen ratios (C : N) from 2 to 60. Cultures were ammonia limited above a C : N of 12. Harvested bacteria exhibited the highest ethylenogenic capacity with L-methionine or KMBA as a substrate when

Fig. 1. Growth (a) and ethylene formation (b) by *E. coli* B SPAO in medium containing 10 mM-glucose, 2.7 mM-L-methionine and concentrations of NH_4Cl as follows: 0 (●), 0.5 (■), 3.0 (▲), 6.0 (○), 10 (□) and 20 mM (△). Bacteria were grown in 250 ml Erlenmeyer flasks (culture volume 30 ml). For comparison, ethylene production is expressed in $\mu\text{mol (mg cell dry wt)}^{-1}$.

Fig. 2. Ethylenogenic capacity of *E. coli* B SPAO grown in medium containing 10 mM-glucose, 2.7 M-L-methionine and concentrations of NH_4Cl as follows: 0.5 (●), 3.0 (▲), 10 (■) and 20 mM (○). Bacteria were grown in 2 litre Erlenmeyer flasks (culture volume 500 ml) and harvested after 17 h, corresponding to the end of exponential growth. Washed cell suspensions were assayed for the ability to form ethylene from L-methionine as indicated in the text.

cultures were grown under conditions of ammonia limitation at a C : N of 20 (glucose 10 mM, NH_4Cl 3 mM, no L-methionine). The rate of ethylene synthesis by non-growing cell suspensions was at least 10-fold higher when KMBA was the assay substrate rather than L-methionine over the range of carbon : nitrogen ratios tested.

Fig. 3. Ethylenogenic capacity of *E. coli* B SPAO growing in continuous culture as a function of the C:N ratio of the inflowing medium. Bacteria were grown in a fermenter (800 ml working volume), stirred continuously at 1000 r.p.m. The temperature was 36 °C and a pH of 6.9 was maintained by the automatic addition of 2 M-NaOH. An air flow rate of 1 litre min⁻¹ was sufficient to aerate the cultures. The organism was initially cultivated under batch conditions. After 12 h a continuous nutrient feed (80 ml h⁻¹) was delivered by a peristaltic pump and the culture allowed to reach steady-state at a growth rate (dilution rate) of 0.1 h⁻¹. Essentially, the defined medium described by Ince & Knowles (1985) was used and different C:N ratios were obtained by varying the concentration of NH₄Cl at a fixed concentration of 10 mM-glucose. Samples (50 ml) of culture were removed at intervals from the fermenter and bacteria were harvested, washed and assayed for their ability to form ethylene from L-methionine (○) and KMBA (●). Concentrations of glucose (▲) and NH₄Cl (□) in samples of culture supernatant were recorded.

When L-methionine was added at a final concentration of 2.7 mM to the continuous culture at steady-state under ammonia limitation (C:N = 30), ethylene was present in the effluent gas from the fermenter and KMBA and 2-oxoglutarate were detected in the culture medium. In glucose-limited cultures (C:N = 2) containing L-methionine no ethylene could be detected and neither KMBA nor 2-oxoglutarate accumulated in the culture medium. Although growth was not significantly affected by the presence of L-methionine ethylene was not produced when L-methionine was absent from the growth medium.

Supplementing the culture medium with selected amino acids and its effect on ethylene production

L-Methionine uptake by batch cultures of *E. coli* is highest after ammonia present in the growth medium has been completely utilized (Ince & Knowles, 1985). The aminotransferases present in *E. coli* enable it to utilize many amino acids as carbon and nitrogen sources. The enzymes have broad and overlapping substrate specificities (Jensen & Calhoun, 1981) and the possibility that other amino acids might compete with or regulate 'methionine aminotransferase' activity, and hence ethylenogenesis, was investigated.

Amino acids tested were selected on the basis that they are known substrates for the aminotransferases of *E. coli* and were added individually with L-methionine to the defined medium at the concentrations specified.

Growth and ethylene production were similar in batch cultures grown on 10 mM-glucose and 3 mM-NH₄Cl containing 2.7 mM-L-methionine or 2.7 mM-L-methionine supplemented with 3.0 mM-L-alanine, L-aspartate, L-glutamate, L-glutamine, L-isoleucine, L-leucine or L-valine. L-Tyrosine (2.0 mM), although not affecting growth (Fig. 4*a, b*) reduced the yield of ethylene after 42 h by 54%. The maximum transient level of KMBA was also reduced by 32% in the presence of L-tyrosine and occurred later compared to when tyrosine was absent.

The 2-oxo acid *p*-hydroxyphenylpyruvate was observed to accumulate in the medium and the time of its maximum appearance coincided with that for KMBA. High levels of *p*-hydroxyphenylpyruvate accumulated in medium containing both L-methionine and L-tyrosine (Fig. 4*b*). No ethylene accumulated in flasks containing only L-tyrosine.

Fig. 4(a)

Fig. 4(b)

Fig. 4. Growth (◆) and ethylene production (■) by *E. coli* B SPAO in medium containing 10 mM-glucose, 3 mM-NH₄Cl; (a) 2.7 mM-L-methionine (b) 2.7 mM-L-methionine and 2.0 mM-L-tyrosine. Bacteria were grown in 250 ml Erlenmeyer flasks (culture volume 30 ml). Concentrations of glucose (□), L-methionine (●), L-tyrosine (▲), K MBA (△) and *p*-hydroxyphenylpyruvate (○) were recorded.

Repression of methionine aminotransferase activity by L-methionine and L-tyrosine

High-speed supernatants were prepared from cultures (500 ml) harvested after exponential growth (14 h) in defined medium containing glucose (10 mM), NH_4Cl (3 mM) and L-methionine (2.7 mM), tyrosine (2.0 mM) or L-methionine (2.7 mM) and L-tyrosine (2.0 mM). Methionine and tyrosine reduced the specific activity of methionine aminotransferase by 32% and 44% respectively compared to a specific activity of $3.17 \mu\text{mol h}^{-1} (\text{mg protein})^{-1}$ which was obtained when no amino acid supplement was made. In combination, the two amino acids repressed aminotransferase activity by 65%.

DISCUSSION

Many bacteria and fungi are able to synthesize ethylene (Primose, 1979). The most prolific ethylene producer so far identified, *E. coli* strain B SPAO, was the subject of this study. Ethylene, CO_2 and methanethiol are produced from L-methionine by *E. coli* via the transamination of L-methionine to KMBA (Ince & Knowles, 1986). It was suggested by Ince & Knowles (1986) that ethylenogenesis by *E. coli* might enable scavenging of the amino group of methionine as a source of nitrogen when other sources are unavailable.

The assimilation of ammonia by *E. coli* is well-documented (Brown *et al.*, 1974; Brown, 1976) but little information is available concerning growth on mixed nitrogen sources (Harder & Dijkhuizen, 1976). In batch culture ethylene synthesis from the precursor L-methionine was influenced by the initial concentration of ammonia in the growth medium (Fig. 1). Further metabolism of ethylene by this organism has not been reported (S. Mansouri & A. W. Bunch, unpublished results). Thus these differences must reflect the ability of the bacterium to synthesize ethylene. The ethylenogenic capacity of intact bacteria harvested at the end of the exponential growth phase (17 h) was inversely proportional to the initial concentration of NH_4Cl in the growth medium.

When L-methionine was added to a continuous culture of *E. coli* maintained at a growth rate of 0.1 h^{-1} under ammonia limitation, ethylene was synthesized and KMBA and 2-oxoglutarate were excreted into the culture fluid. The rate-limiting step in the dissimilation of L-methionine to ethylene appears to be the conversion of KMBA to ethylene, which would account for the accumulation of the KMBA in the culture fluid (see also Fig. 4).

Methionine is utilized as a sole nitrogen source which results in a lower growth rate (Fig. 1). It is apparent that methionine transamination occurs towards the end of the exponential growth phase and is followed by the slower conversion of KMBA to ethylene during the stationary phase (Fig. 4). L-Methionine aminotransferase activity is present throughout growth even in the absence of L-methionine (Ince & Knowles, 1986).

In the presence of excess ammonia the principal mechanism for ammonia assimilation is via the reductive amination of 2-oxoglutarate catalysed by glutamate dehydrogenase (Kavanagh & Cole, 1976). This may result in a lower availability of 2-oxoglutarate which is a cosubstrate for methionine dissimilation by the transamination reaction. At intracellular ammonia concentrations below 1 mM (Sakamoto *et al.*, 1975) the glutamine synthetase/glutamate synthase assimilatory mechanism is stimulated. Provided the carbon source is abundant, a high intracellular concentration of 2-oxoglutarate is maintained (Neijssel & Tempest, 1979) in order to saturate glutamate synthase for optimum activity (Huetting & Tempest, 1979). These conditions may also favour the transamination reaction as an additional nitrogen salvage reaction and would explain the absence of ethylene, KMBA and 2-oxoglutarate as metabolic products when L-methionine is added to a glucose-limited continuous culture.

Cells harvested from continuous cultures grown in the absence of methionine were able to synthesize ethylene when methionine was added to harvested cells. The highest ethylenogenic capacity was shown by cells grown under ammonium limitation (C:N = 20, Fig. 3). Ethylene formation in batch culture was independent of the L-methionine concentration over the range 0.5–3.0 mM, although L-methionine is reported to inhibit the activity of the methionine transport systems (Kadner, 1977). These observations support the suggestion that the primary function of the ethylene-forming enzymes may not be for ethylene synthesis.

An alternative route of L-methionine dissimilation, which does not yield ethylene, may exist. For example, methionine γ -lyase, which is induced by L-methionine has been characterized in *Pseudomonas* spp. (Tanaka *et al.*, 1976; Ito *et al.*, 1976). The possibility that this activity is also present in *E. coli* is currently under investigation.

Thus physiological circumstances, particularly the nitrogen supply, may favour initial dissimilation of methionine by the aminotransferase route and subsequent conversion of KMBA to ethylene, either by influencing the levels of a precursor necessary for ethylene synthesis (e.g. 2-oxoglutarate) or by favouring an alternative route of L-methionine dissimilation.

The presence of ammonia can also affect uptake of amino acids by repressing the synthesis of specific permeases although this has not been reported for L-methionine (Kustu *et al.*, 1979).

Supplementing the batch culture medium with other amino acids in addition to L-methionine showed that L-tyrosine lowered ethylene production, an effect which could not be attributed to differences in biomass (Fig. 4). This suggests that L-tyrosine either represses the synthesis of the ethylene-forming enzyme(s) or can compete as a substrate, probably for the transamination reaction, since *p*-hydroxyphenylpyruvate is observed to accumulate in batch culture (Fig. 4). The presence of two multispecific tyrosine aminotransferases in *E. coli*, one of which is tyrosine repressible, is well-documented (Collier & Kohlaw, 1972; Gelfand & Steinberg, 1977; Powell & Morrison, 1978).

The results presented in this paper have shown that the cellular activity of the ethylenogenic enzymes is influenced by the availability of ammonia in the growth medium. However, this may not represent a direct involvement of the ethylenogenic system with nitrogen recovery from methionine. Indeed, it is possible that ethylenogenesis has no metabolic role but arises as a fortuitous combination of reactions brought about by non-specific enzymes whose main metabolic functions lie elsewhere. We are currently investigating this possibility.

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