Inhibition of *Escherichia coli* growth by acetic acid: a problem with methionine biosynthesis and homocysteine toxicity

Andrew J. Roe,[†] Conor O'Byrne,[‡] Debra McLaggan and Ian R. Booth

Author for correspondence: Ian R. Booth. Tel: +44 1224 273152. Fax: +44 1224 273144.
e-mail: gen118@abdn.ac.uk

Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

The mechanism by which methionine relieves the growth inhibition of *Escherichia coli* K-12 that is caused by organic weak acid food preservatives was investigated. In the presence of 8 mM acetate the specific growth rate of *E. coli* Frag1 (in MacIlvaine's minimal medium pH 6.0) is reduced by 50%. Addition of methionine restores growth to 80% of that observed in untreated controls. Similar relief was seen with cultures treated with either benzoate or propionate. Mutants with an elevated intracellular methionine pool were almost completely resistant to the inhibitory effects of acetate, suggesting that the methionine pool becomes limiting for growth in acetate-treated cells. Measurement of the intracellular concentrations of pathway intermediates revealed that the homocysteine pool is increased dramatically in acetate-treated cells, suggesting that acetate inhibits a biosynthetic step downstream from this intermediate. Supplementation of the medium with homocysteine inhibits the growth of *E. coli* cells. Acetate inhibition of growth arises from the depletion of the intracellular methionine pool with the concomitant accumulation of the toxic intermediate homocysteine and this augments the effect of lowering cytoplasmic pH.

Keywords: enteric bacteria, amino acid synthesis, acetate, weak acids, metabolite toxicity

INTRODUCTION

The preservative properties of weak organic acids have been exploited by mankind for thousands of years. The antimicrobial activities of many different weak acid food preservatives have been well documented (Eklund, 1980; Sheu *et al.*, 1972, 1975). Their toxicity is multifactorial and includes: the ability of the undissociated acid to diffuse freely across lipid bilayers and liberate protons in the cytoplasm, lowering the cytoplasmic pH (Booth, 1985); the intercalation of the undissociated acid into the lipid bilayer at low external pH (Stratford & Anslow, 1998); and the consequences of anion accumulation (Russell & Diez-Gonzales, 1998; Roe *et*

al., 1998). Consistent with these modes of action, the inhibition provoked by these compounds is pH dependent. Several factors combine to make the inhibitory effects of weak acids more profound when the extracellular pH is acidic. The most effective compounds have acidic pK_a values and will therefore exist largely in the charged (impermeant) form at neutral pH. The transmembrane pH gradient, which is the driving force for anion accumulation and consequently dictates the magnitude of H^+ liberation in the cytoplasm, is greatest at acid pH. Finally, deviations from the cytoplasmic pH range that is compatible with growth, and survival, are greatest when the external pH is acidic. Despite a number of studies on the mode of action of organic acids (Cherrington *et al.*, 1991; Brul & Coote, 1999) their mode of growth inhibition has largely been assumed to be due to changes in cytoplasmic pH and the energetic status of the cell. A number of studies indicate that perturbation of membrane function is likely to be involved (Bakker & Mangerich, 1983; Bracey *et al.*, 1998; Freese *et al.*, 1973; Stratford & Anslow, 1998) while others indicate that effects on intracellular pH

[†] **Present address:** Zoonotic and Animal Pathogens Research Group, Department of Veterinary Pathology, Teviot Place, Edinburgh EH8 9AG, UK.

[‡] **Present address:** Department of Microbiology, National University of Ireland – Galway, Galway, Ireland.

Abbreviations: pH_i , intracellular pH; pH_o , external pH.

(pH_i) are important (Bracey *et al.*, 1998; Cole & Keenan, 1986; Salmond *et al.*, 1984). The possibility that the charged anion may contribute to inhibitory effects on growth has also been proposed (Russell & Diez-Gonzalez, 1998; Roe *et al.*, 1998).

In order to understand how cells respond to weak acid treatment a number of recent studies have looked at the global effects of weak acids on gene transcription (Arnold *et al.*, 2001; Pomposiello *et al.*, 2001) and protein expression (Blankenhorn *et al.*, 1999; Lambert *et al.*, 1997). These studies demonstrate the complexity of the cell's response to weak acid treatment. For example, treatment of cells with 5 mM sodium salicylate caused growth inhibition, and the transcription of 134 genes was significantly modulated (Pomposiello *et al.*, 2001). In the case of acetate treatment the expression of 86 genes was found to change significantly (Arnold *et al.*, 2001), and many of the changes involved genes that also show altered expression during exposure to salicylate. Proteomic studies have also led to the identification of several proteins whose expression is altered during weak acid stress (Blankenhorn *et al.*, 1999; Lambert *et al.*, 1997). Once again the response is complex, involving the induction of general stress proteins (e.g. ClpB, DnaK, GroL, UspA), transcriptional regulators (e.g. Fur, H-NS) as well as proteins involved in metabolism (e.g. MalE, MalX, AceA, PtsH). Despite all the data obtained during these studies, however, the basis for inhibition of growth by weak acids remains uncertain.

In a previous study we looked at the behaviour of *Escherichia coli* cells treated with acetic acid (Roe *et al.*, 1998). The pH_i is reduced from 7.85 to 7.48 when cells are grown in the presence of 8 mM acetate in a defined medium with a pH (pH_0) of 6.0. We also found that cells treated with inhibitory concentrations of acetate accumulate high levels of acetate anions in the cytoplasm and this accumulation is compensated for, at least in part, by a reduction in the size of the intracellular glutamate pool. Furthermore, recovery of pH_i after the removal of acetate was dependent on an ability of the cells to synthesize glutamate. These data suggested that growth inhibition could result either from the observed reduction in pH_i or from some consequence of the intracellular accumulation of the acetate anion. The question of how growth inhibition was achieved therefore remained unresolved.

Previous studies suggest that the methionine biosynthetic pathway in *E. coli* can be perturbed by environmental stresses leading to a reduced growth rate, which is caused by partial methionine auxotrophy. The growth of *E. coli* cells in minimal medium is inhibited at temperatures above 40 °C and this inhibition can be overcome by supplementation of the growth medium with methionine (Ron & Davis, 1971). This observation is explained by the heat sensitivity of MetA, the enzyme that catalyses the conversion of homoserine to O-succinylhomoserine (Fig. 1). In addition, a mutation conferring a defective heat-shock response maps to

Fig. 1. A schematic of the methionine biosynthetic pathway of *E. coli*. Pathway information derived from Greene (1996). Gene names are shown in italics. SAM, S-adenosylmethionine; THF, tetrahydrofolate; $\text{CH}_2=\text{THF}$, N^5,N^{10} -methylenetetrahydrofolate; $\text{CH}_3\text{-THF}$, N^5 -methyltetrahydrofolate.

glyA, which encodes serine hydroxymethyltransferase (Gage & Neidhardt, 1993). This enzyme is required for the biosynthesis of glycine from serine and, because of its role in regenerating the pool of N^5 -methyltetrahydrofolate, is also required for the biosynthesis of methionine (Fig. 1). It has also been observed that methionine can relieve the inhibitory effects of acetate, which accumulates as a metabolic end product during fermentations with *E. coli* growing on glucose (Han *et al.*, 1993). However, in this case no explanation for the relieving effect of methionine was offered. Since this observation had the potential to reveal more about how cells are inhibited by weak acids we investigated it further.

Here we confirm the relieving effect of methionine on the inhibition of the growth of *E. coli* by weak organic acids. We show that intermediates from the methionine biosynthetic pathway above homocysteine (Fig. 1) do not have the same relieving property. Acetate-treated cells accumulate homocysteine, the last intermediate on the methionine biosynthetic pathway, and homocysteine was found to inhibit the growth of *E. coli*. Together these observations suggest that acetate may inhibit the activity of an enzyme in the lower part of the methionine biosynthetic pathway leading to the accumulation of homocysteine. The inhibition of growth by acetate can therefore be explained by a partial auxotrophy for methionine combined with the accumulation of an inhibitory pathway intermediate.

METHODS

Materials. All chemicals were supplied by either BDH or Sigma and were Analar grade or better. Radiochemicals ($[^{14}\text{C}]$ benzoate and $[^3\text{H}]$ inulin) were supplied by NEN.

Bacterial strains. *E. coli* strains Frag1 (F^- *thi rha lac gal*) and Frag5 (Frag1, AkdpABC5) (Epstein & Kim, 1971) were from our frozen stocks. MJF556 (Frag1, *metB* *zij-2009::Tn10*) was created by transducing Frag1 to tetracycline resistance using a P1 lysate grown on strain DV62 (*metB* *zij-2009::Tn10*) (Vallari & Rock, 1987). The co-transduction of the Tn10 marker and the *metB* allele was confirmed by demonstrating that MJF556 was auxotrophic for methionine. Strains RG62 (*metK84*), RG109 (*metK85*) and their isogenic parent strain (K12 wild-type) were donated by Ron Greene (Basic Science Laboratory, Department of Biochemistry, Duke Medical Centre, Durham, NC, USA). Strain DG232 (*glyA127*) was obtained from Fred Neidhardt (University of Michigan, Medical School, Ann Arbor, MI, USA). Strains MJF378 (MJF274 *rpoS::Tn10*) and MJF358 (MJF274 *rpoS::Tn10-kan*) were used as donors in the P1 transductions to generate *rpoS* derivatives of Frag1. The *rpoS::Tn10* alleles were originally derived from *E. coli* strains RH90 (obtained from R. Hengge-Aronis) and ZK1000 (obtained from A. Martinez), respectively.

Growth media. The growth experiments in this study were all carried out in defined media based upon citrate/phosphate buffer at pH₆ 6.0 as used in previous studies (Roe *et al.*, 1998). Measurements of growth were made by monitoring the OD₆₅₀ of 1 ml samples. A single colony of *E. coli* was used as an inoculum for overnight growth under limiting glucose conditions (0.04%, w/v). Cells were subsequently supplemented with glucose (0.2%, w/v) and allowed one cell-doubling step prior to dilution in fresh medium to an OD₆₅₀ of 0.05. Methionine supplementation was performed using a 0.2 M (100×) methionine stock solution, prepared freshly and filter-sterilized on the day of the experiment. All specific growth rates are expressed as the mean of at least three independent growth experiments, ± SD.

pH_i measurements. The pH_i was determined by using the distribution across the cell membrane of a radiolabelled weak acid following centrifugation (Kroll & Booth, 1981). In this method, bromododecane oil was used to separate the cell pellet from the supernatant, [⁷⁻¹⁴C]benzoic acid (4.5 μM; 0.1 mCi ml⁻¹, 3.7 MBq ml⁻¹) was used as the weak acid, and [³H]inulin (1.0 mM; 1.0 μCi ml⁻¹, 1 kBq ml⁻¹) was employed as an extracellular marker.

Pathway intermediate pool measurements. Cells (OD₆₅₀ 0.6) were harvested for amino acid pool analysis by filtration of 1 ml through a Whatman membrane filter (cellulose nitrate; 0.45 μm pore size) under vacuum. Filters were washed immediately with 5 ml slightly hypertonic NaCl solution (medium + 50 mM NaCl) added drop-wise to the filter. The filters carrying the cells were then placed in Eppendorf tubes containing 1 ml ice-cold 0.1% trifluoroacetic acid with norleucine (250 pmol ml⁻¹) as an internal standard. The Eppendorf tubes were left on ice for 30 min to allow for extraction of the amino acids, the filters were removed, and the samples were then stored at -20 °C. The analysis of the amino acid pools was carried out as described previously (Amezaga *et al.*, 1995; Roe *et al.*, 1998).

Overexpression of GlyA and MetE. The *glyA* gene was PCR-amplified from *E. coli* strain Frag5 and cloned into vector pTrc99A (Amann *et al.*, 1988) using standard techniques. Since there are two potential methionine start codons for GlyA both were used for cloning to produce plasmids pGlyA1 and pGlyA2. The 5' primers PG1 (gcccatggATGCGGATGTTAAAGCGTGAAATG) and PG2 (gcccatggATGTTAAAGCGTGAAATGACCATT) were designed to amplify products with alternative methionine start codons in conjunction

with primer PGSTOP (ccctgcagGGCATGAACAACGAGCA-CATTGAC) at the 3' end of the *glyA* gene. *E. coli* Frag5 was used as a template for the PCR reaction, which followed standard conditions. Primers PG1 and PG2 incorporated *NcoI* sites and pGSTOP a *PstI* site that allowed the PCR products to be cloned into the region downstream of the inducible Trc promoter of pTrc99A. Cloned products were sequenced in both directions to ensure they were correct when compared with the published sequence of *E. coli* MG1655 (GenBank accession number NC000913).

The *metE* gene was overexpressed in Frag1 using plasmid pMetE, derived from pRSE562, which carries the *metE* and *metR* genes (Maxon *et al.*, 1989). pMetE was constructed by removing the *metR* gene from pRSE562. This was achieved by digestion of pRSE562 with *Sall* and ligation of the gel-purified plasmid backbone using standard methods. The removal of *metR* was necessary since very high levels (40% of soluble protein) of MetE are produced from pRSE562 and this leads to growth inhibition (Gonzalez *et al.*, 1992). Overexpression of MetE was confirmed by SDS-PAGE analysis of crude extracts of Frag1 (pMetE).

RESULTS

Inhibition of growth by weak organic acids is reversed by methionine

Previous workers (Han *et al.*, 1993) had reported that the accumulation of acetate in fermentation broth led to growth inhibition and that this inhibition could be reversed by the addition of methionine. To confirm these observations we grew *E. coli* Frag1 at pH 6 in minimal medium and in the presence of a range of organic acids, such that the growth rate was reduced to 50% of the uninhibited control. Addition of 8 mM acetate, 2 mM benzoate or 5 mM propionate resulted in a reduction of the specific growth rate of approximately 50% (Table 1). Methionine addition to the untreated control had no stimulatory effect on growth, but elevated the growth rate in all three acid-treated cultures to 65–78% of the control rate. Methionine stimulated the growth rate of cells treated with up to 20 mM acetate at pH 6 (data not shown). In the earlier study (Han *et al.*, 1993), glycine was also found to relieve growth inhibition, but we were unable to observe growth stimulation by this amino acid in *E. coli* Frag1 (data not shown).

Previous work has demonstrated that weak acids can perturb the pH_i due to their lipophilic nature and subsequent dissociation in the cytoplasm (Bracey *et al.*, 1998; Cole & Keenan, 1986; Salmond *et al.*, 1984). We have shown previously that treatment of Frag1 cells with 8 mM acetate lowers the pH_i from 7.85 to pH 7.48 (Roe *et al.*, 1998). Therefore, we tested the possibility that methionine could relieve the inhibitory effect of acetate by preventing or reducing this drop in pH_i. Cells grown in the presence of 8 mM acetate had a pH_i of 7.40 ± 0.09 and this value was not altered in the presence of 2 mM methionine despite the relieving effect of methionine on weak-acid-inhibited growth. This result demonstrates that relief from growth inhibition by methionine is independent of further changes in pH_i and that the

Table 1. Methionine relieves growth inhibition by weak organic acids

Culture conditions*	Methionine (2 mM)	Specific growth rate, μ (h ⁻¹)	Growth rate as percentage of control
Control	—	0.72 ± 0.05	100
	+	0.72 ± 0.06	100
+ Acetate (8 mM)	—	0.38 ± 0.03	52
	+	0.56 ± 0.04	78
+ Benzoate (2 mM)	—	0.37 ± 0.03	51
	+	0.48 ± 0.02	67
+ Propionate (5 mM)	—	0.3 ± 0.03	42
	+	0.47 ± 0.04	65

* Cultures were grown in MacIlvaine's minimal medium at pH 6.0.

Table 2. Methionine relief of growth inhibition is RpoS independent

Strain	Specific growth rate, μ (h ⁻¹)		
	pH 6.0	pH 6.0 + Ac*	pH 6.0 + Ac* + Met†
Frag1	0.71 ± 0.05	0.37 ± 0.05	0.55 ± 0.06
Frag1, <i>rpoS</i> ::Tn10	0.78 ± 0.07	0.45 ± 0.05	0.54 ± 0.05
Frag1, <i>rpoS</i> ::Tn10- <i>kan</i>	0.79 ± 0.04	0.43 ± 0.04	0.56 ± 0.05

* Acetate, 8 mM final concentration.

† Methionine, 2 mM final concentration.

inhibition of growth effected by acetate is not solely due to its effect on pH_i.

Methionine relief is independent of RpoS

The stress-inducible sigma factor, RpoS (σ^s), is known to play an important role in acid tolerance in *E. coli* (Castanie-Cornet *et al.*, 1999) and it accumulates under conditions of weak-acid stress (Mulvey *et al.*, 1990; Schellhorn & Stones, 1992). It was therefore possible that the relief of growth inhibition observed in the presence of methionine was dependent on this sigma factor. We examined this possibility by looking at the ability of methionine to relieve acetate-mediated growth inhibition in a strain lacking RpoS. Frag1 was transduced to either tetracycline resistance or kanamycin resistance with P1 lysates grown on strains MJF378 (MJF274 *rpoS*::Tn10) and MJF358 (MJF274 *rpoS*::Tn10-*kan*), generating Frag1 *rpoS*::Tn10 and *rpoS*::Tn10-*kan*, respectively. Western analysis confirmed that these strains did not express RpoS (data not shown). When these strains were grown at pH 6.0 in the presence of 8 mM acetate their growth was inhibited to the same extent as the wild-type parent. When methionine (2 mM) was included in the medium all three strains showed comparable relief (Table 2). These data suggest that the relieving effect of methionine on weak-acid inhibition of growth is independent of RpoS.

Intracellular accumulation of methionine leads to decreased sensitivity to acetate

The possibility that the intracellular pool of methionine could contribute to increased acetic acid tolerance was tested using mutants with altered methionine pools. Two strains (RG62 and RG109) carrying mutations in the *metK* gene have a reduced activity of MetK (S-adenosylmethionine synthetase; Fig. 1) and this leads to the intracellular accumulation of methionine (Greene *et al.*, 1973). These strains, together with the isogenic parent (K-12), were grown without acetate and the intracellular pools of methionine were measured. As expected both mutants (RG62 and RG109) had elevated methionine pools relative to the parent strain; methionine pools were approximately 1 mM and 4 mM for strains RG109 and RG62, respectively, compared with <0.1 mM for the wild-type parent strain. Other amino acid pools were similar with the exception of glycine, which was elevated approximately fourfold (data not shown). When sensitivity to weak acid treatment was investigated the parent strain exhibited normal sensitivity to acetate at pH 6 and was protected by methionine (Table 3). In contrast, the *metK* mutants displayed intrinsically slower growth rates but reduced inhibition by acetate and no restoration of growth by methionine. These data suggest that lowering the drain on the methionine pool by reducing the conversion to S-

Table 3. Acetate inhibition is suppressed by elevated methionine pools

Strain (Met pool size)*	Acetate (8 mM)	Methionine (2 mM)	Specific growth rate, μ (h ⁻¹) (n = 3)
K-12 (<i>metK</i> ⁺)	—	—	0.52 ± 0.02
(<0.1 mM)	+	—	0.25 ± 0.02
	+	+	0.42 ± 0.03
RG62 (<i>metK84</i>)	—	—	0.27 ± 0.01
(3.7 ± 1.0 mM)	+	—	0.23 ± 0.02
	+	+	0.23 ± 0.02
RG109 (<i>metK85</i>)	—	—	0.32 ± 0.01
(1.0 ± 0.2 mM)	+	—	0.26 ± 0.02
	+	+	0.25 ± 0.02

* Cells were grown to mid-exponential phase before harvesting by filtration. Intracellular methionine (Met) pools were measured as described in Methods. The values shown are the means of three triplicate experiments ± SD.

Table 4. Methionine biosynthetic intermediates fail to relieve acetate inhibition

Addition (2 mM)	Specific growth rate, μ (h ⁻¹) (n = 3)		Inhibition (%)
	pH 6.0	pH 6.0 + acetate (8 mM)	
None	0.72 ± 0.02	0.35 ± 0.03	50
L-Homoserine	0.28 ± 0.03	0.23 ± 0.03	16
LL-Cystathionine	0.74 ± 0.03	0.3 ± 0.04	60
Homocysteine	0.23 ± 0.01	0.1 ± 0.03	60
L-Methionine	0.72 ± 0.03	0.63 ± 0.02	12

adenosylmethionine relieved the inhibition caused by acetate.

Supplementation with methionine precursors fails to relieve growth inhibition

The genetics of the pathways of amino acid biosynthesis were originally elucidated via cross-feeding experiments using intermediates in the pathway. Using this classical approach we tested whether intermediates of the methionine biosynthetic pathway could act to relieve acetate-mediated growth inhibition. If one of the steps in the pathway was inhibited by acetate then supplementation of the culture with intermediates downstream of the blockage should relieve the growth inhibition.

Three intermediates, homoserine, cystathionine and homocysteine (Fig. 1), were tested for their potential to relieve acetate-mediated growth inhibition. Homocysteine and homoserine were themselves found to inhibit the growth of *Frag1*, even in the absence of weak acid. The effect was most marked for homocysteine; growth of *Frag1* in the absence of acetate was inhibited by approximately 70% when 2 mM homocysteine was included in the growth medium (Table 4). Addition of acetate further increased the inhibition of growth (Table 4). These inhibitory effects made it impossible to

determine whether homoserine or homocysteine could bypass a potential acetate-mediated blockage in the methionine biosynthetic pathway. However, cystathionine, which feeds into the pathway after the MetB enzyme (cystathionine- γ -synthase), was not inhibitory to the growth of *Frag1* and we were unable to demonstrate relief from acetate inhibition when it was added to the growth medium at a concentration of 2 mM (Table 4). This result was not due to a failure of cystathionine to support the biosynthesis of methionine in *Frag1*, since a *Frag1*-derived *metB* mutant (MJF556; *Frag1*, *metB* *zlj*-2009::Tn10) could grow in methionine-deficient medium supplemented with cystathionine. Furthermore, the relief of acetate-mediated growth inhibition was only seen in MJF556 (*metB*) when methionine, but not cystathionine, was present in the growth medium (data not shown). These data suggest that inhibition by acetate might arise by blockage of a step after MetB in the methionine biosynthetic pathway.

Intracellular homocysteine levels increase following acetate addition

Inhibition of one or more steps in methionine biosynthesis should cause the build-up of pathway intermediates prior to the inhibited step(s). To test this, *Frag1* cells were grown in the presence or absence of 8 mM

Table 5. Homocysteine pools are elevated by acetate treatment at pH 6

Cells were grown to mid-exponential phase before harvesting by filtration. Intracellular pools were measured as described in Methods. The values shown are the means and standard deviations of triplicate experiments.

Intermediate	Acetate (8 mM)	Intracellular concn (mM)
Homoserine	—	0.02 ± 0.01
	+	0.03 ± 0.02
Cystathionine	—	0.01 ± 0.01
	+	0.04 ± 0.06
Homocysteine	—	0.1 ± 0.1
	+	1.6 ± 0.75

Fig. 2. Relationship between specific growth rate, μ (■), and intracellular homocysteine concentration (◆). Cultures were grown in the presence of different concentrations of homocysteine and their growth rate was determined. Samples were removed from each culture during exponential growth and the internal homocysteine pool size determined by HPLC. Error bars indicate the standard deviation from the mean. ($n=3$ for the growth rates and $n=9$ for the pool measurements).

acetate and the intracellular concentrations of homoserine, cystathionine and homocysteine were determined (Table 5). In the absence of acetate the concentrations of these intermediates were all very low (<0.1 mM). After incubation with acetate, only the concentration of homocysteine increased significantly (Table 5). This suggested that inhibition occurred at a step after conversion of cystathionine to homocysteine.

The earlier observation that homocysteine was inhibitory to growth suggested that homocysteine accumulation itself might be sufficient to account for the acetate-mediated inhibition of growth. Thus, cells were incubated with a range of concentrations of homocysteine and the growth rate correlated with the observed intracellular accumulation of the metabolite (Fig. 2). The addition of 2 mM homocysteine caused almost 80% growth inhibition, but as little as 0.5 mM was sufficient to cause a severe reduction in growth rate. The intracellular pools of homocysteine mirrored this, with

an increase from approximately 0.15 mM to approximately 1.7 mM as the extracellular homocysteine concentration was increased from 0.25 mM to 0.5 mM. Since the addition of 8 mM acetate was found to increase intracellular homocysteine levels to approximately 1.6 mM in homocysteine-free medium, this could in itself be a major determinant of the growth inhibition caused by acetate.

Role of MetE and GlyA in acetate-mediated inhibition of methionine biosynthesis

The question of which step might be inhibited by inclusion of acetate in the growth medium is complicated by the fact that four different enzymes participate (directly or indirectly) in the final reaction of the pathway, the conversion of homocysteine to methionine (Fig. 1). Either of two enzymes, the transmethyases MetH and MetE, can catalyse the transfer of a methyl group from 5N -methyltetrahydrofolate to homocysteine to generate methionine in *E. coli* (Greene, 1996). Since MetH has a requirement for vitamin B₁₂ and our growth medium is not supplemented with this vitamin, MetE is the only enzyme that could catalyse this reaction in our experiments. We cloned and overexpressed *metE* (see Methods) and found no evidence to suggest that its overexpression could protect against growth inhibition by acetate (data not shown). However, two additional enzymes are required for the regeneration of 5N -methyltetrahydrofolate, without which the final step in the biosynthetic pathway could not proceed. These are GlyA (serine hydroxymethyltransferase) and MetF (N^5,N^{10} -methylene tetrahydrofolate reductase). Since GlyA had previously been shown to be involved in the heat-shock response (Gage & Neidhardt, 1993) it seemed possible that it might also be involved in the response to weak acid stress. We cloned and overexpressed *glyA* (see Methods) and showed that it could complement the heat-sensitive phenotype of a *glyA*-defective strain (DG232; *glyA127*). When this clone was transformed into Frag1 and *glyA* expression was induced with IPTG we found no protection against the inhibitory effects of acetic acid (data not shown). If either MetE or GlyA were inhibited by acetate (directly or indirectly) then overexpression of these proteins might be expected to overcome this inhibition, at least partially. It therefore seems unlikely that either of these enzymes is inhibited during acetate treatment.

DISCUSSION

Here we have confirmed the findings of an earlier study by Han *et al.* (1993), which showed that methionine could relieve the acetate-mediated inhibition of growth of an *E. coli* culture. We have shown that the effects of methionine on acetate-mediated inhibition of growth can be replicated for other organic acids. In the present study we sought an explanation for this relieving effect. Mutations that lead to elevation of the intracellular concentration of methionine (*metK* mutants) also render cells resistant to the inhibitory effects of acetate (Table

3). This result suggests that the methionine pool is limiting for growth when cells are exposed to inhibitory concentrations of acetate. This could be explained if one or more of the biosynthetic reactions leading to methionine synthesis were inhibited when cells are exposed to weak acid stress. In support of this idea is the finding that homocysteine, an intermediate in the methionine biosynthetic pathway, accumulates to a high level (1.6 mM; 16-fold higher than untreated cells) in cells treated with 8 mM acetate (Table 5). The intracellular accumulation of homocysteine suggests that an enzyme below this intermediate in the pathway is inhibited during weak acid stress, and this is consistent with the observation that cystathionine (the intermediate immediately before homocysteine; Fig. 1) fails to relieve the inhibitory effect of acetate (Table 4). Additionally, we found that the accumulation of homocysteine in the cells, as a consequence of inclusion of this pathway intermediate in the medium, is inhibitory to the growth of *E. coli* Frag1 (Table 5). There is an excellent correlation between the extent of growth inhibition and the intracellular concentration of homocysteine (Fig. 2). Indeed the intracellular concentration of homocysteine that inhibits the growth rate by 50% (approx. 1.3 mM) is similar to the concentration found in acetate-treated cells (approx. 1.6 mM). These data suggest that a second reason for growth inhibition in cells treated with acetic acid is the accumulation of the toxic intermediate homocysteine.

The data we have presented here suggest that in the presence of organic acids, at mildly acidic pH, the methionine biosynthetic pathway becomes inhibited at one of the steps below homocysteine. This has two effects on the cell: depletion of the intracellular methionine pool and accumulation of homocysteine, the immediate precursor of methionine in the biosynthetic pathway. Since methionine is essential for protein biosynthesis as well as being required for the biosynthesis of *S*-adenosylmethionine, which is itself required for several important cellular functions (Sekowska *et al.*, 2000), it is likely that this will perturb the growth rate of cells.

How does the inclusion of methionine in the growth medium protect cells against inhibition by the weak acid acetic acid? First, methionine supplementation will overcome any growth inhibition arising from depletion of the intracellular methionine pool. Secondly, the presence of methionine in the medium is known to repress the methionine biosynthetic genes (Greene, 1996) and feedback-inhibit the activity of homoserine succinyltransferase (MetA), the first enzyme in the methionine biosynthetic pathway (Fig. 1; Lee *et al.*, 1966), which will prevent synthesis of homocysteine. The mechanism by which homocysteine exerts its toxic effect is unclear. However, Hahn & Brown (1967) showed that in *Sarcina lutea* homocysteine acts as a competitive inhibitor of methionyl-tRNA synthetase. When the intracellular ratio of homocysteine to methionine increases, which it is predicted to do when *E. coli* is treated with organic acids, such as acetate (Table 5), the levels

of tRNA^{Met} will decline and growth inhibition will result. Relief in the presence of methionine could then occur by decreasing the homocysteine/methionine ratio, leading to restoration of the tRNA^{Met} pools.

Previously we have considered the effects of organic acids primarily in terms of their lowering of cytoplasmic pH. At pH values close to the pK of the organic acid inhibition is multifactorial as was indicated above. It is clear that effects on cytoplasmic pH, perturbation of membrane lipids and effects of anion accumulation are likely to be maximal at these low pH values. However, at mildly acidic pH (pH 6) it is clear that the reduction in cytoplasmic pH by acetate is insufficient to cause profound growth inhibition, since in the presence of methionine the growth rate is restored to around 80% of the untreated control. Whether the enzymes of methionine synthesis are themselves sensitive to the lowered pH, organic anions or a combination of both is not clear. Incubation with acetate at pH 6 causes the accumulation of approximately 240 mM acetate anions in the cytoplasm (Roe *et al.*, 1998). This accumulation is partially compensated for by the depletion of the glutamate pools. However 75% of the acetate anion pool must be compensated for by lowering the intracellular concentration of other anions, whose identity remains unknown (Roe *et al.*, 1998). It is conceivable that it is the depletion of these anions, which may represent important metabolic intermediates, that results in inhibition of methionine biosynthesis.

It is clear that methionine biosynthesis is unlikely to be the only pathway affected by incubation of cells with organic acids, since restoration of growth by methionine is not complete. Thus, we need to keep in mind that organic acids may exert their effects via changes in the activity of particularly sensitive pathways, such as that for methionine biosynthesis, and the more general inhibition arising from the change in cytoplasmic pH and anion composition. Clearly, in *E. coli* K-12 the inhibition arising from inclusion of acetate in the growth medium at mildly acidic external pH is more complex than simply altered cytoplasmic pH.

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REFERENCES

- Amann, E., Ochs, B. & Abel, K. J. (1988). Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. *Gene* **69**, 301–315.
- Amezaga, M. R., Davidson, I., McLaggan, D., Verheul, A., Abee, T. & Booth, I. R. (1995). The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity. *Microbiology* **141**, 41–49.
- Arnold, C. N., McElhanon, J., Lee, A., Leonhart, R. & Siegele, D. A. (2001). Global analysis of *Escherichia coli* gene expression during

- the acetate-induced acid tolerance response. *J Bacteriol* **183**, 2178–2186.
- Bakker, E. P. & Mangerich, W. E. (1983).** The effects of weak acids on potassium uptake by *Escherichia coli* K-12 inhibition by low cytoplasmic pH. *Biochim Biophys Acta* **730**, 379–386.
- Blankenhorn, D., Phillips, J. & Slonczewski, J. L. (1999).** Acid- and base-induced proteins during aerobic and anaerobic growth of *Escherichia coli* revealed by two-dimensional gel electrophoresis. *J Bacteriol* **181**, 2209–2216.
- Booth, I. R. (1985).** Regulation of cytoplasmic pH in bacteria. *Microbiol Rev* **49**, 359–378.
- Bracey, D., Holyoak, C. D. & Coote, P. J. (1998).** Comparison of the inhibitory effect of sorbic acid and amphotericin B on *Saccharomyces cerevisiae*: is growth inhibition dependent on reduced intracellular pH? *J Appl Microbiol* **85**, 1056–1066.
- Brul, S. & Coote, P. (1999).** Preservative agents in foods. Mode of action and microbial resistance mechanisms. *Int J Food Microbiol* **50**, 1–17.
- Castanie-Cornet, M. P., Penfound, T. A., Smith, D., Elliott, J. F. & Foster, J. W. (1999).** Control of acid resistance in *Escherichia coli*. *J Bacteriol* **181**, 3525–3535.
- Cherrington, C. A., Hinton, M., Mead, G. C. & Chopra, I. (1991).** Organic acids: chemistry, antibacterial activity and practical applications. *Adv Microb Physiol* **32**, 87–108.
- Cole, M. B. & Keenan, M. H. (1986).** Synergistic effects of weak-acid preservatives and pH on the growth of *Zygosaccharomyces bailii*. *Yeast* **2**, 93–100.
- Eklund, T. (1980).** Inhibition of growth and uptake processes in bacteria by some chemical food preservatives. *J Appl Bacteriol* **48**, 423–432.
- Epstein, W. & Kim, B. S. (1971).** Potassium transport loci in *Escherichia coli*. *J Bacteriol* **108**, 639–644.
- Freese, E., Sheu, C. W. & Galliers, E. (1973).** Function of lipophilic acids as antimicrobial food additives. *Nature* **241**, 321–325.
- Gage, D. J. & Neidhardt, F. C. (1993).** Modulation of the heat shock response by one-carbon metabolism in *Escherichia coli*. *J Bacteriol* **175**, 1961–1970.
- Gonzalez, J. C., Banerjee, R. V., Huang, S., Sumner, J. S. & Matthews, R. G. (1992).** Comparison of cobalamin-independent and cobalamin-dependent methionine synthases from *Escherichia coli*: two solutions to the same chemical problem. *Biochemistry* **31**, 6045–6056.
- Greene, R. C. (1996).** Biosynthesis of methionine. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 542–560. Edited by F. C. Neidhardt. Washington, DC: American Society for Microbiology.
- Greene, R. C., Hunter, J. S. & Coch, E. H. (1973).** Properties of *metK* mutants of *Escherichia coli* K-12. *J Bacteriol* **115**, 57–67.
- Hahn, G. A. & Brown, J. W. (1967).** Properties of a methionyl-tRNA synthetase from *Sarcina lutea*. *Biochim Biophys Acta* **146**, 264–271.
- Han, K., Hong, J. & Lim, H. C. (1993).** Relieving effects of glycine and methionine from acetic acid inhibition in *Escherichia coli* fermentation. *Biotechnol Bioeng* **41**, 316–324.
- Kroll, R. G. & Booth, I. R. (1981).** The role of potassium transport in the generation of a pH gradient in *Escherichia coli*. *Biochem J* **198**, 691–698.
- Lambert, L. A., Abshire, K., Blankenhorn, D. & Slonczewski, J. L. (1997).** Proteins induced in *Escherichia coli* by benzoic acid. *J Bacteriol* **179**, 7595–7599.
- Lee, L. W., Ravel, J. M. & Shive, W. (1966).** Multimetabolite control of a biosynthetic pathway by sequential metabolites. *J Biol Chem* **241**, 5479–5480.
- Maxon, M. E., Redfield, B., Cai, X. Y., Shoeman, R., Fujita, K., Fisher, W., Stauffer, G., Weissbach, H. & Brot, N. (1989).** Regulation of methionine synthesis in *Escherichia coli*: effect of the MetR protein on the expression of the *metE* and *metR* genes. *Proc Natl Acad Sci U S A* **86**, 85–89.
- Mulvey, M. R., Switala, J., Borys, A. & Loewen, P. C. (1990).** Regulation of transcription of *katE* and *katF* in *Escherichia coli*. *J Bacteriol* **172**, 6713–6720.
- Pomposiello, P. J., Bennik, M. H. & Demple, B. (2001).** Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. *J Bacteriol* **183**, 3890–3902.
- Roe, A. J., McLaggan, D., Davidson, I., O'Byrne, C. & Booth, I. R. (1998).** Perturbation of anion balance during inhibition of growth of *Escherichia coli* by weak acids. *J Bacteriol* **180**, 767–772.
- Ron, E. Z. & Davis, B. D. (1971).** Growth rate of *Escherichia coli* at elevated temperatures: limitation by methionine. *J Bacteriol* **107**, 391–396.
- Russell, J. B. & Diez-Gonzalez, F. (1998).** The effects of fermentation acids on bacterial growth. *Adv Microb Physiol* **39**, 205–234.
- Salmond, C. V., Kroll, R. G. & Booth, I. R. (1984).** The effect of food preservatives on pH homeostasis in *Escherichia coli*. *J Gen Microbiol* **130**, 2845–2850.
- Schellhorn, H. E. & Stones, V. L. (1992).** Regulation of *katF* and *katE* in *Escherichia coli* K-12 by weak acids. *J Bacteriol* **174**, 4769–4776.
- Sekowska, A., Kung, H. F. & Danchin, A. (2000).** Sulfur metabolism in *Escherichia coli* and related bacteria: facts and fiction. *J Mol Microbiol Biotechnol* **2**, 145–177.
- Sheu, C. W., Konings, W. N. & Freese, E. (1972).** Effects of acetate and other short-chain fatty acids on sugar and amino acid uptake of *Bacillus subtilis*. *J Bacteriol* **111**, 525–530.
- Sheu, C. W., Salomon, D., Simmons, J. L., Sreevalsan, T. & Freese, E. (1975).** Inhibitory effects of lipophilic acids and related compounds on bacteria and mammalian cells. *Antimicrob Agents Chemother* **7**, 349–363.
- Stratford, M. & Anslow, P. A. (1998).** Evidence that sorbic acid does not inhibit yeast as a classic 'weak acid preservative'. *Lett Appl Microbiol* **27**, 203–206.
- Vallari, D. S. & Rock, C. O. (1987).** Isolation and characterization of temperature-sensitive pantothenate kinase (*coaA*) mutants of *Escherichia coli*. *J Bacteriol* **169**, 5795–5800.

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