Growth inhibition of *Escherichia coli* by dichloromethane in cells expressing dichloromethane dehalogenase/glutathione S-transferase

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Dichloromethane (DCM) dehalogenase converts DCM to formaldehyde via the formation of glutathione metabolites and generates 2 mol HCl per mol DCM metabolized. Growth of *Escherichia coli* expressing DCM dehalogenase was immediately and severely inhibited during conversion of 0.3 mM DCM. Intracellular pH (pHi) rapidly decreased and chloride ions were steadily released into the medium. Bacterial growth resumed after completion of DCM conversion and cell viability was unaffected. At 0.6 mM DCM there was no recovery from growth inhibition in liquid culture due to the build-up of inhibitory concentrations of formaldehyde. DCM turnover stimulated potassium efflux from cells, which was suppressed by glucose. The potassium efflux, therefore, did not contribute to growth inhibition. It was concluded that initial growth inhibition results from lowering of the cytoplasmic pH, but severity of growth inhibition was greater than expected for the change in pH. Possible contributors to growth inhibition are discussed.

Keywords: glutathione, dichloromethane, intracellular pH, chloride, formaldehyde

INTRODUCTION

Glutathione (GSH) has been proposed to have several roles in bacterial cells. It is a potential mediator of redox reactions with possible roles in signalling oxidative stress, biosynthesis of deoxyribonucleotides, methionine and cysteine and in the maintenance of proteins in the reduced state in the cytoplasm (Hibberd *et al.*, 1978; Holmgren, 1986; Prinz *et al.*, 1997; Zheng *et al.*, 1998). However, each of these functions can also be undertaken by thioredoxin and glutaredoxin (Holmgren, 1986). The function that is unique to GSH is the detoxification of toxic metabolites (Gutheil *et al.*, 1997; Ferguson & Booth, 1998; Ferguson *et al.*, 1998) and the metabolism of chlorinated hydrocarbons (Leisinger *et al.*, 1994; Leisinger, 1996; Vuilleumier *et al.*, 2000). A number of detoxification pathways are dependent on GSH, e.g. GSH oxidoreductases and GSH S-transferases (GSTs) (Gutheil *et al.*, 1992; Zablotowicz *et al.*, 1995; Vuilleumier, 1997). GST enzymes are widespread in the bacterial kingdom (Vuilleumier, 1997). Sequence analysis reveals that prokaryotic GST-like genes from more than 50 different bacteria can already be found in sequence databases and suggests that *Escherichia coli* alone contains nine GST-like genes in its genome (Vuilleumier *et al.*, 2000). In *E. coli* and related Gram-negative bacteria, and possibly throughout the purple group, GSH is also a regulator of a class of potassium efflux systems (KefB and KefC) and, thereby, a controlling influence on the cytoplasmic pH and potassium pool (Meury & Kepes, 1982; Elmore *et al.*, 1990; Douglas *et al.*, 1991; Ferguson *et al.*, 1993, 1995, 1997, 1998). There is a strong link between the detoxification of both bacterial and man-made toxic chemicals, the activity of the KefB/C systems and survival (Ferguson *et al.*, 1998).

For the majority of bacteria, the physiological functions of GSTs are unknown, although, as with their mammalian counterparts, they are likely to be instrumental...
in detoxification processes (Zablotowicz et al., 1995; Gutheil et al., 1997; Vuilleumier, 1997). For others, however, a GST enzyme is actually the central means of carbon acquisition, as in the case of methylotrophic bacteria able to assimilate carbon from dichloromethane (DCM) via a DCM dehalogenase/GST-type enzyme (Leisinger et al., 1994; Vuilleumier, 1997). Chlorinated methanes are volatile chemicals, which have been extensively used in industry and consequently are frequent environmental pollutants. Bacteria have evolved the capacity for the metabolism of these chemicals as a means of carbon acquisition, as in the case of methylophilus. Dehalogenation of chlorinated alkanes by bacteria poses several potential threats to cell viability. In the case of DCM, dehalogenation produces in the cytoplasm 2 mol HCl per mol substrate, plus 1 mol of a toxic product, formaldehyde, via two postulated GSH adducts, S-chloromethylglutathione and S-hydroxymethylglutathione (reaction 1). 

\[
\text{CH}_2\text{Cl}_2 + \text{GSH} \rightarrow \text{GS-CH}_2\text{Cl} + \text{HCl} \rightarrow \text{GS-CH}_2\text{OH} + \text{HCl} \rightarrow \text{GS} + \text{CH}_2\text{O} \tag{1}
\]

S-Chloromethylglutathione has been shown to alkylate DNA (Dechert, 1993) and this is believed to be the basis of the moderately strong mutagenicity in the Salmonella typhimurium Ames tester strain TA1535 expressing a mammalian DCM dehalogenase (Thier et al., 1993; Gisi et al., 1999). In E. coli and related bacteria, it has been shown that GSH-linked detoxification is intimately associated with potassium efflux systems and the modulation of the cytoplasmic pH (Ferguson et al., 1998). Protection against the toxic compounds methylglyoxal and N-ethylmaleimide, which are detoxified via GSH adducts, is achieved by acidification of the cytoplasm (Ferguson et al., 1993, 1995, 1997). GSH is known to be involved in acid tolerance in some bacteria (Riccillo et al., 2000) and it has been shown that protection against mutagens can be achieved by incubation with weak organic acids that also lower the cytoplasmic pH (Oktyabrsky et al., 1993). Dehalogenation reactions in E. coli, such as the conjugation of the model GST substrate 1-chloro-2,4-dinitrobenzene, are inhibitory to growth (Ness et al., 1997). However, the low expression and specific activity of GSTs may limit the impact of dehalogenation on the metabolism of E. coli cells (Zablotowicz et al., 1995; Vuilleumier, 1997). To probe the capacity of E. coli cells to cope with the multiple stresses posed by dehalogenation of chlorinated methanes, we have investigated the physiology of E. coli cells expressing DCM dehalogenase/GST of Methylophilus sp. strain DM11 (Bader & Leisinger, 1994; Vuilleumier & Leisinger, 1996; Gisi et al., 1999). We demonstrated that cells experience no long-term damage from the activity of this enzyme despite transient inhibition of growth during the metabolism of DCM. During dehalogenation cells excrete formaldehyde and chloride and the cytoplasmic pH is lowered. Growth inhibition probably arises from a combination of the stresses of internal acid production, toxicity of a reactive intermediate in the reaction and accumulation of formaldehyde. The data demonstrate that E. coli cells have the ability to cope with intracellularly generated HCl, a previously unremarked ability of this organism.

**METHODS**

**Bacterial strains and plasmids.** Three isogenic E. coli K-12 strains were used in this study; MJF274 (F-, kdpABC5 thi rha lacI lacZ), MJF276 (MJF274, kefB kefC:::Tn10) and MJF335 [MJF276, gshA:::Tn10 (kan)] (Ferguson & Booth, 1998). These strains were transformed with plasmid pME1983 (Gisi et al., 1999), a derivative of pTrc99A (Amersham) carrying the dcmA gene from Methylophilus sp. strain DM11 (Bader & Leisinger, 1994) under IPTG control, and the control plasmid pME1984 carrying the dcmA gene in antisense orientation to the P

**Potassium efflux assays.** These experiments were conducted essentially as described previously (Elmore et al., 1990; Ferguson et al., 1997). Overnight cultures of the appropriate strain were diluted into fresh medium to an OD

**Intracellular pH (pH) measurements.** The pH after DCM addition was determined using the distribution of a radio-labelled weak acid according to a previously described method (Kroll & Booth, 1983; McLaggan et al., 1994) by incubating...
Table 1. DCM dehalogenase and FDH activity in cell-free extracts of strain MJF274(pME1983)

Strain MJF274(pME1983) was grown as described and treated with 0.3 mM DCM at an OD<sub>660</sub> of 0.3. Specific activities were measured at different times during which more than 95% of dehalogenation and build-up of formaldehyde concentration occurs (see Figs 1 and 5) as described in Methods (SEM < 10%).

<table>
<thead>
<tr>
<th>Time (min)</th>
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bacteria at an OD<sub>660</sub> of 0.6 with [14C]benzoic acid (45 μM, 3.7 × 10<sup>6</sup> Bq ml<sup>−1</sup>; Sigma) and [14C]inulin (10 μM, 3.7 × 10<sup>4</sup> Bq ml<sup>−1</sup>; Sigma) as the extracellular marker. The cell pellet was separated from the supernatant by spinning the cells through bromododecane oil (McLaggan et al., 1994) and pH was calculated as described previously (Kroll & Booth, 1983). All experiments were repeated three times. For calculation of mean pH, prior to and after addition of DCM, the pH values for each condition in each experiment were averaged and the SD of the replicate experiments was calculated to be lower than 0.05 units in all cases. For samples prior to addition of DCM at least two measurements were made and after DCM addition at least four measurements were made.

Preparation and analysis of cell-free extracts and measurements of DCM dehalogenase and formaldehyde dehydrogenase (FDH) activity. Cell-free extracts were obtained by three passages through a French press apparatus (14000 p.s.i.) followed by centrifugation at 30000 g for 30 min. Protein concentration was determined in the resulting cell-free extracts using commercial Bradford reagent (Bio-Rad) with bovine serum albumin (Sigma) as standard. Specific rates of DCM dehalogenation were determined spectrophotometrically by assaying the rate of formaldehyde production, as described previously (Vuilleumier & Leisinger, 1994). One unit is defined as the amount of enzyme catalysing the conversion of 1 μmol DCM min<sup>−1</sup>. FDH activity was calculated from the rate of NADH formation at 340 nm using ε = 6220 M<sup>−1</sup> cm<sup>−1</sup> (Gutheil et al., 1997). The reaction mixture contained 0.5 mM NAD<sup>+</sup> in a final volume of 0.5 ml 100 mM potassium phosphate (pH 8.0). To evaluate the contribution of GSH-independent FDHs, the assay was conducted either with or without 10 mM GSH in the reaction mixture. GSH in cell-free extracts was assayed using a GSH assay kit (Calbiochem) and gave values in the expected range [30–60 nmol (mg protein)<sup>−1</sup]].

The experiments were repeated with independent extracts.

Chloride assay. The concentration of chloride ions in the culture supernatant was determined as described by Bergmann & Sanik (1957). Experiments were designed as described for potassium efflux determination (above) and 0.6 ml samples were withdrawn from the sealed flasks that contained cultures metabolizing DCM. To each 600 μl aliquot of culture supernatant 200 μl 0.25 M ferric ammonium sulfate, dissolved in 9 M nitric acid, and 200 μl saturated mercuric thiocyanate in ethanol were added. After 10 min incubation at room temperature the absorbance at 460 nm was determined. A standard curve of 0.1–1 mM NaCl was used for calibration. All experiments were repeated twice.

RESULTS

Dehalogenation causes transient growth inhibition in E. coli

The expression of DCM dehalogenase from Methylophilus sp. strain DM11 in E. coli gives rise to inclusion body formation at 30 °C and higher temperatures (Bader & Leisinger, 1994; Vuilleumier & Leisinger, 1996). Therefore, all experiments in this study were conducted at 25 °C. Induction of DCM dehalogenase by IPTG caused an approximately 10–20% decrease of the growth rate compared to uninduced controls (μ = 0.31 ± 0.01 and 0.26 ± 0.01 h<sup>−1</sup>, respectively, for uninduced and induced cultures; n = 9) and MJF274(pME1984) (μ = 0.31 ± 0.006 h<sup>−1</sup>; n = 3) in which the dcmA gene is in the wrong orientation for transcription by the trc promoter (data not shown). When grown in the presence of 1 mM IPTG (Table 1), cell-free extracts of MJF274(pME1983) exhibited high levels of DCM dehalogenase activity [0.4 ± 0.05 U (mg cell protein)<sup>−1</sup>], i.e. over 5% of total soluble cell protein given a specific activity of the purified enzyme of 3.3 s<sup>−1</sup> (Vuilleumier & Leisinger, 1996), i.e. 6.4 U (mg cell protein)<sup>−1</sup>]. Activity was not significant in cells that had not been induced and was absent from MJF274(pME1984) (data not shown). Addition of 0.3–6 mM DCM caused immediate inhibition of growth of cells expressing high levels of DCM dehalogenase, but had no effect on cells that either lacked the enzyme or were not expressing it (Fig. 1a). Inhibition was transient in the presence of 0.3 mM DCM and exponential growth was restored after approximately 1 h (Fig. 1a). At 0.6 mM DCM growth inhibition was sustained for the duration of the experiment (Fig. 1a). Chloride ions released in the cytoplasm during dehalogenation could, if accumulated, affect the balance of the anion pool. The presence of a chloride channel in E. coli cells has been described (Maduke et al., 1999) and may represent a
grown in K0 expressing DCM dehalogenase. (a) Strain MJF274(pME1983) was
expressed by diminishing the chemical gradient
route for chloride excretion from the cells. The addition
of 0·1 M NaCl, which could impair the activity of an
efflux system by diminishing the chemical gradient
across the membrane, had no effect on inhibition by
DCM (Fig. 1c).

The basis of the growth inhibition was investigated.
First, neither 0·6 mM DCM nor up to 0·6 mM for-
maldehyde, which is the product of DCM dehalo-
genation, affected cell viability (cells retained greater
than 98% viability; data not shown). Formaldehyde,
the end-product of dehalogenation, is toxic to E. coli
(O’Donovan & Mee, 1993). The accumulation of
formaldehyde in the growth medium, when cells were
incubated with 0·3 mM DCM, was determined and was
found to be transient (Fig. 1b). The initial rate of
accumulation was similar for 0·3 and 0·6 mM DCM,
indicating that the DCM dehalogenase was probably
saturated by substrate. However, after 90 min incu-
bation with 0·3 mM DCM, the formaldehyde pool
decayed (Fig. 1b) and this paralleled the induction
of GSH-dependent FDH activity (Table 1). No
detoxification of formaldehyde was observed when
0·3 mM formaldehyde was added to cells of
MJF335(gshA::Tn10; GshA'), which is unable to
synthesize GSH (Ferguson & Booth, 1998). Cells of
MJF335 were more sensitive to formaldehyde than their
isogenic parent strain (0·3 mM formaldehyde caused
approximately 50% inhibition of growth in the mutant,
compared with 10% inhibition of the GshA' parent
strain MJF274; data not shown). This confirmed the
proposed requirement for GSH in this process
(Kummerle et al., 1996; Gutheil et al., 1997) and
suggested that the initial accumulation of formaldehyde
observed in strain MJF274 expressing DCM dehalo-
genase (Fig. 1b) is followed by GSH-dependent enzymic
conversion to formate (Gutheil et al., 1997).

The toxicity of formaldehyde was investigated in strain
MJF274(pME1983) (DCM+) (Fig. 2a). Addition of
formaldehyde reduced the growth rate, but was only
strongly inhibitory above 0·6 mM (Fig. 2a). Therefore,
since growth inhibition by 0·3 mM DCM was immediate
(Fig. 1a), but strongly inhibitory concentrations of the
formaldehyde product were not achieved (Fig. 1b), we
conclude that this product of dehalogenation is not the
cause of growth inhibition. The growth rate sustained
by MJF274(pME1983) after completion of DCM dehalo-
genation (µ = 0·22 ± 0·01 h⁻¹; n = 3) was similar to
that achieved in the presence of the equivalent concentra-
tion of formaldehyde (µ = 0·18 ± 0·01 h⁻¹; n = 3). Cells
washed free of DCM and the products of dehalogenation
soon after addition of DCM re-established growth rates
identical to those of cultures not treated with DCM (µ =
0·28 ± 0·01 h⁻¹; n = 3; Fig. 2b). Similarly, cells incubated
in the presence of 0·3 mM formaldehyde recovered
normal exponential growth after washing and
resuspension in fresh growth medium (Fig. 2b). These
data suggest that cells do not sustain significant damage
when dehalogenating DCM under the chosen con-
ditions.

Lowering of cytoplasmic pH during DCM metabolism

Dehalogenation of DCM releases 2 mol HCl per mol
DCM metabolized. The buffering capacity of the cyto-
plasm is quite limited in the neutral pH range (Booth,
DCM (Fig. 3). For accurate pH growth was followed at OD650. (b) Strain MJF274(pME1983) was but the time course of DCM metabolism was shorter 7 rapid lowering of pH 1985) and lowering of pHi is known to inhibit growth of E. coli cells (Roe et al., 1998). Therefore, we measured the cytoplasmic pH during metabolism of DCM. Addition of DCM to MJF274(pME1983) (DcmA+) led to a rapid lowering of pHi. Prior to DCM addition pHi was 7.75 ± 0.05 and fell to pH 7.45 ± 0.05 upon addition of DCM (Fig. 3). For accurate pHi measurements one had to double the cell number in the incubation (cells grown to OD650 = 0.3 provided too little biomass). Since this also doubled the amount of DCM dehalogenation, we did most of the experiments on pHi with 0.6 mM DCM to retain the same ratio of biomass to DCM, which would give a more accurate representation of what was happening in the growth experiments with 0.3 mM DCM at an OD650 of 0.3 at which DCM was added. The Km of the enzyme being 59 µM (Vuilleumier & Leisinger, 1996), the enzyme was saturated at both 0.3 and 0.6 mM, but the time course of DCM metabolism was shorter with 0.3 mM than with 0.6 mM DCM. Control cells, either not expressing DCM dehalogenase [MJF274(pME1984)] or not incubated with DCM, exhibited no change in pHi over the time course of the experiment (Fig. 3).

We have previously shown that some GSH adducts activate the KefB and KefC potassium efflux systems of E. coli leading to acidification of the cytoplasm (Ferguson et al., 1995, 1997, 2000; Ferguson & Booth, 1998). We determined the rate of potassium efflux when strain MJF274(pME1983) (DcmA+) was incubated with DCM. The presence of glucose can mask efflux activity due to the rapid energy-dependent recovery of potassium that is lost from cells. Thus experiments were initially conducted in the absence of glucose. After addition of DCM the potassium pool was initially constant for approximately 3 min, but rapid potassium efflux then ensued, leading to loss of 50% of the potassium pool in 15 min (Fig. 4a). No efflux was seen from MJF274(pME1984) cells that did not express DCM dehalogenase (Fig. 4a) and therefore the potassium efflux is a consequence of DCM metabolism. However, efflux does not require the GSH adduct-regulated KefB and KefC systems since addition of 0.3 mM DCM to MJF276(pME1983) (DcmA+) caused similar potassium efflux to that seen in MJF274(pME1983) (Fig. 4a). In both MJF274(pME1983) and MJF276(pME1983), DCM-induced potassium efflux was not seen in the presence of glucose (Fig. 4b), suggesting that the rate was sufficiently slow to be counteracted by the activity of the Trk uptake system (Elmore et al., 1990). However, growth inhibition by DCM took place in complete growth medium containing glucose as sole carbon and energy source. Thus,

![Fig. 2. Effect of formaldehyde and DCM on the growth of E. coli expressing DCM dehalogenase. (a) Strain MJF274(pME1983) was grown to mid-exponential phase in K02 minimal medium as described in the legend to Fig. 1, 0 (○), 0.3 (●), 0.6 (▲) or 0.9 mM (■) formaldehyde was added at 3.5 h (arrow) and growth was followed at OD650. (b) Strain MJF274(pME1983) was grown to mid-exponential phase (OD650 = 0.3) and at t = 4 h (arrow) exposed to 0.3 mM DCM (○, ●), 0.3 mM formaldehyde (▲, ▲) or left untreated (□, □). After 10 min incubation approximately half of the culture was filtered onto paper disks using a gentle vacuum and washed free of the toxic chemical, resuspended in fresh, pre-warmed growth medium and growth was observed. Open symbols, cultures filtered and resuspended in fresh growth medium after 10 min exposure to chemicals; filled symbols, cultures incubated without filtration and washing steps.](image1)

![Fig. 3. Effect of DCM treatment on pHi. After growth of strain MJF274(pME1983) or MJF274(pME1984) to an OD650 of 0.3 in K02 medium, the cells were harvested by filtration and resuspended to an OD650 of 0.6 in K02 medium containing glucose. The pHi of the cells was determined as described previously (Kroll & Booth, 1983; McLaggan et al., 1994). Symbols: strain MJF274(pME1983) to which 0.3 (●) or 0.6 mM DCM (▲) was added (arrow); control strain MJF274(pME1984) to which 0.3 mM DCM was added (■); strain MJF274(pME1983) to which no DCM was added (◇).](image2)
although DCM-derived metabolites can cause potassium efflux, this is not a component of the observed growth inhibition.

**Fate of chloride ions generated during dehalogenation**

Anion homeostasis is central to the regulation of turgor and of the potassium pool (McLaggan et al., 1994). Therefore, it is likely that cells must expel the chloride generated by dehalogenation into the medium. DCM dehalogenation is accomplished in approximately 60 min under the chosen conditions (0.3 mM DCM at OD<sub>650</sub> = 0.3). If all chloride ions were retained in the cytoplasm, approximately 2.5 M chloride ions would accumulate [assuming an intracellular volume of 1.6 µL (mg dry wt)<sup>-1</sup> and 2 OD<sub>650</sub> units ml<sup>-1</sup> (mg dry wt)<sup>-1</sup>]. Clearly this would pose an unacceptable stress for the cell. The rate of chloride ion efflux was determined by assaying culture supernatants of cells detoxifying DCM.

After addition of DCM to MJF274(pME1983) there was a linear increase in the chloride concentration in the medium with time (Fig. 5). A short lag may precede the appearance of chloride ions in the medium (Fig. 5). The rate of accumulation of chloride in the medium was approximately double that of formaldehyde. The rate of chloride release was calculated to be 0.59 ± 0.07 mM h<sup>-1</sup> compared to 0.26 ± 0.06 mM h<sup>-1</sup> for formaldehyde production (Fig. 5), which gives a stoichiometry of 2:3. This accords with the predicted stoichiometry of the dehalogenation reaction and suggests close coupling between DCM metabolism and chloride release from the cell. Indeed, the in vivo rate of DCM consumption, estimated from the specific activity of DCM dehalogenase in cell-free extracts [approx. 0.4 U (mg protein)<sup>-1</sup> h<sup>-1</sup>] and assuming that approx. 50% of the cell dry weight is protein, yields a value of about 0.2 mM h<sup>-1</sup> which also accords well with the observed formaldehyde production. Thus, E. coli cells can rapidly expel chloride that is generated intracellularly through metabolism of chlorinated compounds.

**DISCUSSION**

Cells that are metabolizing chlorinated hydrocarbons face a number of potential problems: the metabolism is acidogenic, chloride ions are produced in the cytoplasm, the intermediates may be reactive against DNA and proteins and the products of dehalogenation may themselves be toxic. We have previously shown that E. coli cells can survive the dehalogenation of 1-chloro-2,4-dinitrobenzene (CDNB) (Ness et al., 1997), which is a substrate for at least one of the GSTs of E. coli.
(Vuilleumier et al., 2000). However, the very slow rate of metabolism of CDNB that was observed in these experiments raises the possibility that none of the above stresses associated with dehalogenation provides a significant challenge to the cell. The metabolism of DCM by E. coli cells expressing DCM dehalogenase presents a more significant, albeit artificial, problem due to the high rates of metabolism that are achieved (Fig. 1).

The data presented here show that E. coli cells are very robust during the dehalogenation of low concentrations of DCM. At 0.6 mM DCM, cells do not recover exponential growth within 4 h. However, this is not due to the intrinsic rate of dehalogenation, which is similar to that observed at 0.3 mM DCM (see Fig. 3) due to saturation of DCM dehalogenase (K_{m} = 59 µM; Vuilleumier & Leisinger, 1996). Hence, it may arise from the accumulation of formaldehyde to inhibitory levels (see Fig. 2a).

At 0.3 mM DCM neither the formaldehyde produced, the lowering of cytoplasmic pH nor the chloride ions generated in the cytoplasm appear to cause significant long-lasting damage to the cells. Transfer of cells incubated with either DCM or formaldehyde to fresh growth medium resulted in immediate exponential growth at the same rate (Fig. 2b). The cells respond to the intracellular generation of formaldehyde by the induction of the E. coli GSH-dependent FDH (Table 1). GSH-dependent FDH has been shown previously to be a class III alcohol dehydrogenase that also acts on hydroxymethylglutathione (Gutheil et al., 1992), which is readily formed from formaldehyde and GSH in aqueous solution (Uotila & Koivusalo, 1974). Induction of GSH-dependent FDH by the addition of formaldehyde to the growth medium has also been demonstrated (Gutheil et al., 1997) and has been suggested to represent a significant component of the detoxification machinery for this reactive compound (Uotila & Koivusalo, 1989; Kummerle et al., 1996). Our data are quite consistent with these observations. In addition, our inability to detect significant levels of GSH-independent FDH (Table 1) also explains the observed lack of metabolism of this compound by the GSH-deficient strain MJF335.

Chloride ion transport is poorly characterized in bacterial cells. In our experiments the rate of conversion of DCM was approximately 35–40 nmol DCM min^{-1} (mg cell dry wt)^{-1}, which corresponds to 70–80 nmol chloride ion expelled min^{-1}. This rate is essentially set by the metabolism of DCM and thus may not represent the fastest rate of chloride efflux that E. coli can sustain. Inverted membrane vesicles from E. coli are known to be permeable to chloride ions, but the mechanism underlying this observation is not known (Reenstra et al., 1980). A chloride channel has been detected in E. coli (Maduke et al., 1999), but whether such a channel can participate in the efflux of this anion is unknown. Such a system would be subject to influence by the external chloride concentration. Based on data from Figs 1 and 5 and an intracellular volume of 1.6 µl (mg dry wt)^{-1}, the intracellular concentration of chloride ions would be approximately 40 mM after 1 min of dehalogenation. Allowing for a delay of 2–3 min (Fig. 5) before the onset of chloride excretion, the intracellular chloride concentration would be approximately 120–160 mM. We have previously shown that a rise in anion concentration of this magnitude can be accommodated in E. coli by the release of glutamate and other physiological anions (Roe et al., 1998). The external concentration of chloride in the experiments of Roe et al. (1998) was approximately 0.25 mM. Thus, the considerable transmembrane gradient generated under these conditions would allow a channel to operate to release chloride. Given a membrane potential (Δφ) of greater than −120 mV however, the chloride concentration required to block channel activity can be estimated to be at least 10 M (−120 mV is equivalent to a 100-fold gradient and the estimated internal concentration is 100 mM), which is beyond the upper limit of that tolerable for growth by E. coli. In this work, the presence of 0.1 M NaCl during dehalogenation did not significantly alter growth inhibition. Alternatively, the presence in E. coli of a uniporter energized only by the Δφ would provide another mechanism for chloride ion egress. This would have the potential benefit of chloride efflux becoming independent of any channel gating mechanism and allow the cell to sustain gradients of 100-fold or more (Δφ ≥ −120 mV; Booth, 1985).

Lowering of the cytoplasmic pH is expected to cause growth inhibition. We have previously shown that when the cytoplasmic pH falls below pH 7.4, which is a similar value to that observed in the presence of DCM (Fig. 3), the growth rate falls by 50% (Roe et al., 1998). During DCM dehalogenation growth was completely inhibited and this suggests that the change in the cytoplasmic pH is not the sole cause of inhibition. The levels of formaldehyde produced by DCM dehalogenation are insufficient to cause the growth inhibition, the major concentration of formaldehyde reached in the presence of 0.3 mM DCM, does not significantly inhibit growth (Fig. 2a). The cause of growth inhibition can be rapidly removed, since washing cells restored normal exponential growth almost immediately (Fig. 2b). One possible explanation for the greater growth inhibition observed during DCM dehalogenation is toxicity of GSH adducts generated during DCM metabolism. The observed mutagenicity of GST-mediated DCM conversion (Thier et al., 1993; Gisi et al., 1999) is possibly due to the modification of DNA bases by S-chloromethylglutathione, which was demonstrated in vitro (Deichert, 1995). Insertion mutants of the DCM-degrading bacterium Methylobacterium dichloromethanum DM4 generated by mini-Tn5 mutagenesis were isolated that are unable to grow with DCM, but which still possess GSH and active DCM dehalogenase. These mutants grow well with methanol, which is oxidized during growth to formaldehyde, a central metabolic intermediate in methylo trophic bacteria. In one of these mutants, the transposon insertion is located in the gene encoding DNA polymerase I, a key enzyme in DNA...
repair (Kayser et al., 2000). Thus, it seems possible that the unexpected severity of the growth inhibition observed during exposure to DCM of E. coli cells expressing DCM dehalogenase is due in part to genotoxic GSH adducts. In this context the fall in the cytoplasmic pH, caused by the production of acid during dehalogenation, may even protect the cells from further damage (Ferguson et al., 1995, 1997, 2000).

This study demonstrates that E. coli experiences growth inhibition during dehalogenation of DCM. In contrast, some DCM-degrading methylotrophic bacteria grow whilst actively dehalogenating DCM (Leisinger et al., 1994). It is possible that they are more tolerant of the perturbations of pH and chloride ion concentration arising from dehalogenation and conceivably they have developed tolerance of the DCM adducts formed with GSH. Growth of these organisms with DCM is associated with rapid acidification of the medium, which is consistent with high rates of DCM metabolism. Nevertheless, the slow maximal growth rates of these organisms compared to that of E. coli (approx. 0.08 h⁻¹) may reflect the burden faced by methylotrophic bacteria growing with DCM due to constant acidification of the cytoplasm, rather than slow rates of carbon and energy acquisition. Whatever the case may be, our study suggests that significant physiological adaptations may have been required by methylotrophic bacteria to utilize chlorinated methanes as a means of generating carbon and energy.

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