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Survival during Exposure to the Electrophilic Reagent *N*-Ethylmaleimide in *Escherichia coli*: Role of KefB and KefC Potassium Channels

G. P. FERGUSON, Y. NIKOLAEV,† D. MCLAGGAN, M. MACLEAN, AND I. R. BOOTH*

Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom

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The role of the KefB and KefC potassium efflux systems in protecting *Escherichia coli* cells against the toxic effects of the electrophile *N*-ethylmaleimide has been investigated. Activation of KefB and KefC aids the survival of cells exposed to high concentrations (>100 μM) of NEM. High potassium concentrations reduce the protection afforded by activation of KefB and KefC, but the possession of these systems is still important under these conditions. The Kdp system, which confers sensitivity to the electrophile methylglyoxal, did not affect the survival of cells exposed to NEM. Survival is correlated with the reduction of the cytoplasmic pH upon activation of the channels. In particular, the kinetics of the intracellular pH (pH_i) change are crucial to the retention of viability of cells exposed to NEM; slow acidification does not protect cells as effectively as rapid lowering of pH_i. Cells treated with low levels of NEM (10 μM) recover faster if they activate KefB and KefC, and this correlates with changes in pH_i. The pH_i does not significantly alter the rate of NEM metabolism. The possible mechanisms by which protection against the electrophile is mediated are discussed.

The potassium pool of bacterial cells is subject to tight regulation through the activity of at least three uptake systems (TrkG/H, Kup, and Kdp) (2, 6, 9) and three or more efflux systems (4, 20, 22, 23). However, the addition of electrophiles such as *N*-ethylmaleimide (NEM) to *Escherichia coli* cells elicits rapid potassium efflux that is reversible by the addition of reducing agents (3, 21). These effects of NEM arise from the specific, simultaneous activation of the KefB and KefC potassium efflux systems and the nonspecific inactivation of the major potassium uptake systems TrkG and TrkH (4, 11, 24). Methylglyoxal (MG) also activates the KefB and KefC systems but does so without altering the activity of the potassium uptake systems (14, 15). Given the importance of the potassium pool for cells, deliberate perturbation of the pool must have a significant role in cell survival.

The strong activators of the KefB and KefC systems are all electrophiles. The formation of glutathione metabolites is potentially the first step in detoxification of many toxic compounds, including electrophiles (19, 25, 30). Recent studies have shown that both KefB and KefC confer protection against the toxic effects of MG (13–15). MG is an endogenous electrophile synthesized under conditions of phosphate imbalance when cells are utilizing sugars or other compounds metabolized through the upper section of glycolysis (8). The KefB system is strongly activated by glutathione metabolites of MG and confers the greatest protection against this electrophile (13). The KefC system is only weakly activated by MG, although this is sufficient to confer moderate protection in mutants that lack KefB activity. The lesser role played by the KefC system in protection against endogenous electrophiles suggested that this system was primarily involved in protecting cells against externally derived electrophiles.

Activation of KefB and KefC by NEM is dependent on the formation of a glutathione adduct, *N*-ethylsuccinimido-*S*-glutathione (ESG) (11). ESG is rapidly broken down in the presence of reducing agents, and this accounts for the reversal of the activation of potassium efflux in the presence of dithiothreitol and β-mercaptoethanol (3, 11, 21). Thus, although NEM is a man-made compound, possibly of relatively recent origin, it is a useful model substrate for investigating the survival by bacteria upon exposure to electrophiles. It has been established that the NEM activation of potassium efflux is widespread in gram-negative bacteria (10), and the genes for several KefC homologs have now been described. The widespread distribution of the KefC activity in a range of organisms strongly suggested its importance in cell physiology, and thus the possible role in survival during detoxification of environmental electrophiles has been investigated.

The data presented here show that KefB and KefC facilitate survival of cells exposed to NEM. Rapid cytoplasmic acidification achieved via activation of the KefB and KefC channels is essential for survival. The KefB and KefC systems are not essential for detoxification of NEM, and their role in protection appears to be limited to facilitating potassium efflux, leading to a rapid cytoplasmic acidification.

MATERIALS AND METHODS

Bacterial strains. All organisms used in this study were derivatives of *E. coli* K-12: Frag1 (F^- *thi rha lacZ*), Frag5 (Frag1, *kdpABC5*), MJF274 (Frag5, *lacI trkD1*), and MJF276 (MJF274, *kefB*, *kefC*::Tn*10*).

Growth media and conditions. All cells were grown as stated in the text in either K_x minimal medium (where *x* is the millimolar potassium concentration) (12) or in K_x adjusted to pH 6.9 with concentrated HCl. Unless stated otherwise, the medium was supplemented with 0.2% (wt/vol) glucose as a carbon source. K_x minimal buffer lacked all growth supplements except 0.2% (wt/vol) glucose. Cells for potassium and intracellular pH (pH_i) determinations were grown to late exponential phase (optical density at 650 nm [OD_{650}] of 0.8 to 1.0) in medium as stated in the text, and the determinations were performed in K_x minimal buffer. For the growth and viability experiments, cells were grown to late exponential phase (OD_{650} of 0.8) in the medium stated in the text and then diluted 10-fold into fresh prewarmed medium (same as before; the starting OD_{650} was approx-

* Corresponding author. Mailing address: Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, United Kingdom. Phone: 01224-273152. Fax: 01224-273144.

† Present address: Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia.

imately 0.1). NEM was added from a 50 mM stock solution prepared in 50% (vol/vol) ethanol.

Intracellular potassium determinations. The potassium content of cells was measured by a previously described method (11). For analysis of K^+ efflux into media containing high levels of K^+, the following modified method was used. Late-exponential-phase cells (OD_{650} of 0.8 to 1.0) were harvested by filtration. Before suspension to the same OD_{650} in either K_{120} or $K_{0.2}$ minimal buffer (prewarmed to 37°C), cells were washed with 5 ml of K_{120} or K_{10} minimal buffer, respectively. The suspensions were then transferred to a 37°C thermally insulated glass pot with a magnetic stirrer. A 1-ml sample was removed for the OD_{650} measurement, and then samples (0.75 ml) were removed at defined time intervals and filtered (Whatman, 0.45-μm pore size). The filtered cells were washed with 3 ml of K_0 minimal buffer supplemented with 50 mM glucose (prewarmed to 37°C). Potassium was released from the cell by drying the filters overnight at 70°C. The filters were then immersed in 1 ml of distilled water, and the potassium was measured with a Corning 400 flame photometer. NEM was added to the cell suspensions at the time indicated in the text.

Viability experiments. Cell viability was determined exactly as described previously (13). Serial dilutions were conducted in K_0 minimal buffer for cells in $K_{0.2}$ medium and in K_{120} minimal buffer for cells in K_{120} medium. Glucose was not included in these minimal buffers. Recovery of cells was always conducted on K_{10} minimal agar plates with glucose (0.2% [wt/vol]) as the sole carbon source.

pH_i measurements. pH_i was measured by using the distribution of radiolabeled weak acid, using the method of Kroll and Booth (16). This involved using ^{14}C-benzoic acid (5.6 μM, 0.1 μCi · ml^{-1}) and ^{3}H-inulin (0.62 μM, 1 μCi · ml^{-1}) as the extracellular marker. Pellet and supernatant samples were separated by centrifugation through 1-bromodecane oil in a microcentrifuge at 15,000 × *g* for 1 min. The pH_i values were then calculated as described previously (16). By this method, pH_i measurements were found to be very reproducible for cell suspensions above an OD_{650} of 0.5; cell suspensions below this OD value gave consistent results, but the data were subject to greater error.

[2-^{14}C]NEM metabolism determinations. Cells were grown in K_{10} medium to an OD_{650} of 0.8, filtered (Whatman, 4.5 cm, 0.45-μm pore size), and suspended in 10 ml of $K_{0.2}$ containing glucose (0.2% [wt/vol]) to give an OD_{650} of either 0.12 or 0.8. After incubation for approximately 10 min at 37°C in a stirred vessel, [2-^{14}C]NEM was added to give a final concentration of approximately 10 to 15 μM. At intervals, samples (0.5 ml) were taken, filtered (Whatman, 2.5 cm, 0.45-μm pore size), and washed with two 0.5-ml aliquots of $K_{0.2}$. The filter was dried, placed in a scintillation vial with 3 ml of scintillation fluid (Packard Ultima Gold), and counted for 10 min. Due to the volatility of the solvent in which the [2-^{14}C]NEM stock was dissolved, it was not possible to obtain exact equal concentrations in different experiments. Therefore, the absolute concentration of NEM present in the aqueous incubation was determined by taking a 50-μl sample and drying it without filtration onto an identical filter and determining the radioactivity.

Potassium and [2-^{14}C]NEM metabolism experiments were conducted at least twice, whereas growth, viability, and pH_i determinations were performed a minimum of three times. The data shown are representative of the results obtained on a particular day. For the growth and viability experiments, variation in the actual recovery period or death phase occurred, but the appropriate controls were always performed on the same days. Daily changes were always consistent for all strains or growth conditions used. For this reason, we have chosen to show representative data.

RESULTS

Protection against the electrophile NEM by the activity of KefB and KefC. Isogenic *E. coli* strains that differ only in the possession or lack of KefB and KefC were analyzed for the ability to survive exposure to NEM in $K_{0.2}$ medium. Exponential-phase cultures (see Materials and Methods) were treated with 0.1 mM NEM, and the viability of the treated cells was determined (Fig. 1a). In the absence of the electrophile, no loss of viability was observed (data not shown). Strain MJF276 incubated in the presence of NEM exhibited a slow decline in viable cell number for approximately 20 min followed by rapid loss of viability (Fig. 1a). In contrast, strain MJF274 ($KefB^+$ $KefC^+$) retained a high level of viability for the duration of the experiment. We have previously shown that MG-treated cells lose viability more rapidly if the incubation is in high-K^+ medium (K_{10}) than in low-K^+ medium ($K_{0.2}$). Although MJF274 was more sensitive to NEM in K_{120} medium than in $K_{0.2}$, the cells retained greater viability than the mutant lacking KefB and KefC (Fig. 1a).

The patterns of viability observed above were found to correlate with KefB and KefC activity. NEM-elicited efflux was

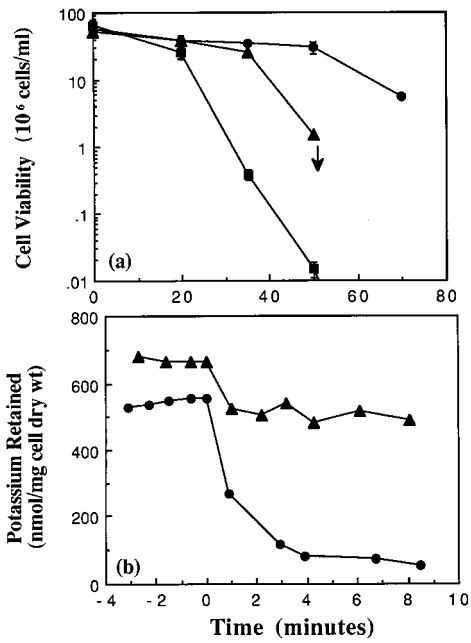

FIG. 1. The activity of the KefB and KefC systems protects against NEM. Exponential-phase cells were prepared and potassium and viability experiments were performed exactly as described in Materials and Methods. (a) Cells were grown in either K_{120} (▲) or in $K_{0.2}$ (● and ■) medium and cell viability was tested in the same medium in the presence of 0.1 mM NEM. The arrow indicates that in the next sample, no viable cells were detectable. (b) Cells of MJF274 ($KefB^+$ $KefC^+$) were grown in K_{120} medium, and potassium determinations were conducted in either K_{120} (▲) or $K_{0.2}$ (●) minimal buffer in the presence of 0.5 mM NEM. Symbols: ● and ▲, MJF274; ■, MJF276 ($KefB^-$ $KefC^-$). The K^+ content of cells grown on K_{120} medium was approximately 25% higher than that observed for cells grown on $K_{0.2}$ (15).

only partially inhibited by 120 mM K^+ (Fig. 1b): 26 and 90% K^+ lost within 4 min of addition of NEM in K_{120} and $K_{0.2}$, respectively. The K^+ pool was approximately 25% higher in K_{120}-grown cells than in $K_{0.2}$-grown cells (Fig. 1b). These data indicate that sufficient channel activity is still present in K_{120} medium (Fig. 1b) to allow protection to be observed (Fig. 1a). It is therefore clear that activation of KefB and KefC by NEM plays a significant role in the survival of *E. coli* cells during exposure to NEM.

Survival of exposure to NEM correlates with pH_i. We have previously established a direct relationship between pH_i and survival of exposure to MG (14, 15). We sought to determine whether changes in pH_i could be correlated with the observed patterns of cell death in MJF274 and MJF276. For strain MJF274, the addition of NEM elicited an immediate fall in pH_i, the rate and extent of which were determined by the K^+ concentration in the medium (Fig. 2a and b). The rate of fall of pH_i was much faster in cells incubated in $K_{0.2}$ medium than in cells incubated in K_{120} medium (pH_i fell by 1.1 ± 0.08 [n = 4] and 0.43 ± 0.06 [n = 3] units, respectively, for $K_{0.2}$ and K_{120}, within 1 min of addition of NEM). In contrast, pH_i in strain MJF276 ($KefB^-$ $KefC^-$) declined only slowly; the drop in the first minute after NEM addition was 0.025 ± 0.03 pH units (n = 4), (Fig. 2c). The steady-state pH_i reached by NEM-treated MJF276 was 7.1 ± 0.08 (n = 4), which was similar to that achieved by MJF274 in K_{120} medium, but this was achieved only after approximately 20 min of incubation with NEM. Clearly strains MJF274 and MJF276 differ significantly in the rate of the change in pH_i after exposure to NEM, and

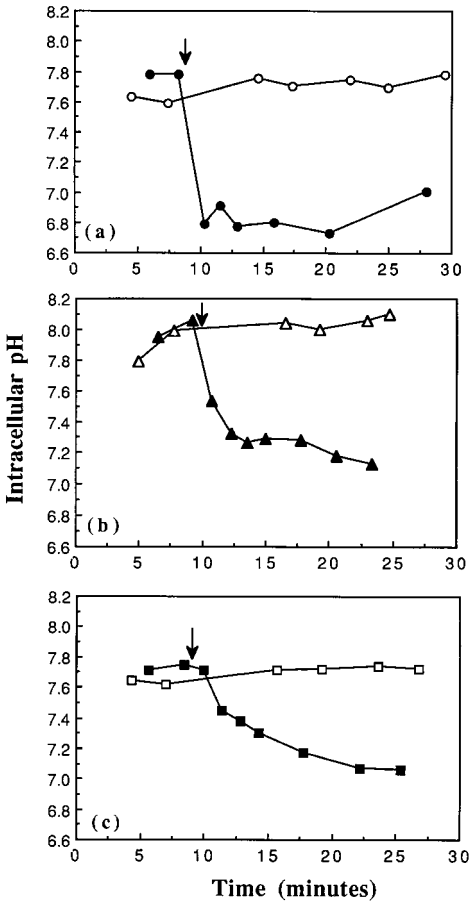

FIG. 2. Activation of KefB and KefC results in a rapid lowering of pH_i. Exponential-phase cells were prepared and pH_i measurements were conducted exactly as described in Materials and Methods. Cells of MJF274 ($KefB^+$ $KefC^+$; a and b) and MJF276 ($KefB^-$ $KefC^-$; c) were grown in K_{120} medium and then suspended in either $K_{0.2}$ (a and c) or K_{120} (b) minimal buffer. The radioactive acid and ^3H-inulin marker were added at time zero, and 0.5 mM NEM (closed symbols) was added at the time indicated by the arrow. Open symbols, control (no addition).

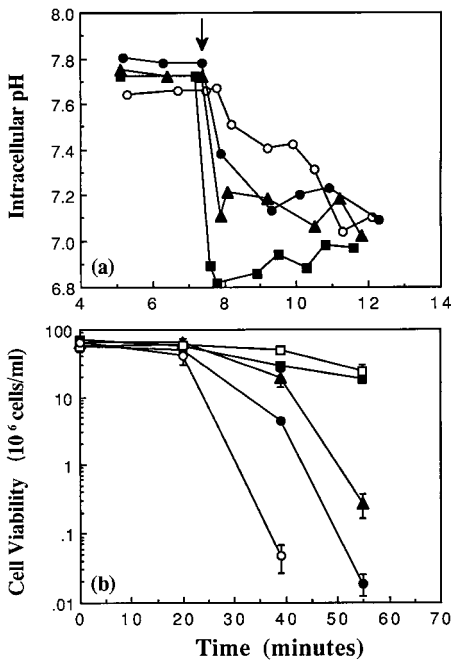

FIG. 3. The degree of cytoplasmic acidification determines the level of protection against NEM. Exponential-phase cells were prepared and pH_i and viability experiments were performed exactly as described in Materials and Methods. (a) Cells of MJF276 ($KefB^-$ $KefC^-$) were grown in K_{120} medium, and pH_i measurements were conducted in $K_{0.2}$ (pH 6.9) minimal buffer. The radioactive benzoic acid and inulin were added at time zero, and 0.5 mM NEM was added along with 0 (○), 5 (●), 15 (▲), and 25 (■) mM sodium acetate at the time represented by the arrow. (b) Cells were prepared in $K_{0.2}$ (pH 6.9) medium, and cell viability was determined in the same medium in the presence of 0.1 mM NEM added at time zero. □, MJF274 ($KefB^+$ $KefC^+$). For cells of MJF276, 0, 5, 15, and 25 mM sodium acetate was also added at time zero (symbols as panel a).

this could account for their different sensitivities to this electrophile.

To determine the influence of the kinetics of pH_i change on survival, we utilized the ability of acetic acid to lower the cytoplasmic pH (5, 14, 15, 28). Strain MJF276 ($KefB^-$ $KefC^-$) was simultaneously treated with NEM and with sodium acetate solutions such that pH_i was lowered to different extents, and cell viability was determined. As noted above (Fig. 2c), even in the absence of sodium acetate, pH_i declined slowly in NEM-treated cells, but the final steady-state pH_i was similar to that achieved in the presence of 5 to 25 mM acetate (Fig. 3a). The major difference in acetate-treated cells was that pH_i fell with kinetics similar to those observed when KefB and KefC are active (compare Fig. 3a with Fig. 2a). Acetate at 5 or 15 mM gave kinetics of pH_i change intermediate between those seen with MJF274 and MJF276 in the presence of NEM. Survival of exposure to NEM correlated well with the kinetics of pH_i decline (Fig. 3b): addition of 5 mM acetate resulted in survival for longer periods of exposure to NEM, and even greater protection was observed with 15 and 25 mM acetate. With 25 mM acetate, survival was similar to that observed with MJF274 (Fig. 2b); this result is consistent with the kinetics of pH_i decline.

Further insight into the importance of the kinetics of pH_i change was obtained by varying the time at which acetate was added to cultures of MJF276 treated with NEM. Acetate (25 mM) was added to cells either simultaneously with NEM or at intervals of 1, 5, and 15 min after the addition of NEM (0.1 mM) (Fig. 4). Acetate caused an immediate drop in pH_i of 0.8 units (Fig. 3), which is equivalent to the activation of KefB and

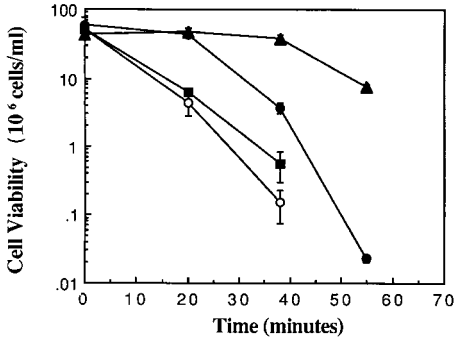

FIG. 4. A rapid cytoplasmic acidification is critical for maximum protection against NEM. Exponential-phase cells were prepared in $K_{0.2}$ (pH 6.9), and cell viability was tested in the same medium exactly as described in Materials and Methods. Cells of MJF276 ($KefB^-$ $KefC^-$) were exposed to 0.1 mM NEM at time zero, and 25 mM sodium acetate was added at 0 or 1 (▲), 5 (●), and 15 (■) min after NEM addition. No acetate was added to the control (○).

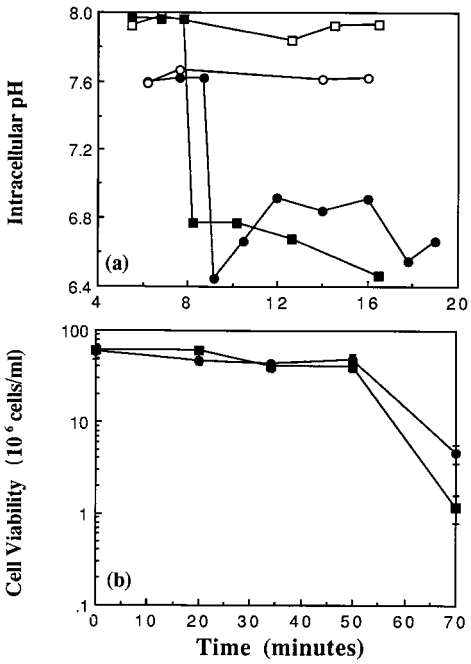

FIG. 5. The activity of the Kdp system has little effect on NEM sensitivity. Exponential-phase cells were prepared in $K_{0.2}$ medium, and pH_i and viability measurements were performed exactly as described in Materials and Methods. (a) Cells of Frag1 (Kdp^+ $KefB^+$ $KefC^+$; ■ and □) and Frag5 (Kdp^- $KefB^+$ $KefC^+$; ● and ○) were suspended in $K_{0.2}$ buffer. The radioactivity was added at time zero, and 0.5 mM NEM was added immediately after the third time point to the suspensions represented by the filled symbols. (b) Cell viability was conducted in $K_{0.2}$ medium in the presence of 0.1 mM NEM. Symbols are as in panel a.

KefC by NEM. As recorded above, the pH_i of NEM-treated cells of MJF276 fell slowly over the 20-min incubation (Fig. 2c); therefore, the pH change with acetate was additional to that caused by NEM such that pH_i was below the limit of accuracy of the pH_i determination method. Addition of acetate simultaneously with NEM and 1 min after NEM addition enhanced cell survival, but any greater delay led to a progressively faster decline in viable cell numbers (Fig. 4). If acetate was added after a 15-min delay, there was no significant protection afforded by lowering pH_i, and this accorded with the observed rate of decline of pH_i in MJF276 (Fig. 3a). These data (Fig. 3 and 4) suggest that for a very toxic electrophile, such as NEM, the rate of decline of pH_i is the most important factor determining the degree of protection obtained by activation of KefB and KefC.

The role of the Kdp K^+ uptake system in determining sensitivity to NEM. Wild-type *E. coli* cells possess the Kdp, high-affinity, K^+ transport system that is expressed when the external K^+ concentration is too low for the combined activity of Trk and Kup to sustain cell turgor (17). Kdp would normally be expressed under the conditions used to analyze the sensitivity to electrophiles ($K_{0.2}$ medium), but the structural genes are deleted in strains MJF274 and MJF276. We have shown that strains possessing Kdp are more sensitive to MG (15), and thus we sought to determine whether Kdp sensitizes cells to NEM. *E. coli* Frag1 (Kdp^+) and Frag5 (Kdp^-) were grown on $K_{0.2}$ medium to induce the Kdp system as described previously (15). Kdp^+ cells maintain a higher pH_i than mutants lacking Kdp; however, both strains exhibited rapid acidification of the cytoplasm in the presence of NEM, and the steady-state pH_i values were similar (Fig. 5a). No major differences were observed in the viability of the two strains in the presence of NEM (Fig. 5b), consistent with the rapid rate of acidification seen in the two strains. In assays using K^+-depleted cells, the Kdp system was found to be completely inhibited by 0.1 mM NEM (data not shown). The failure of the Kdp system to sensitize cells to NEM is thus due to the inhibition of Kdp by NEM and to the large fall in pH_i upon activation of KefB and KefC that overcomes the higher initial pH_i value in Kdp^+ cells.

Recovery of *E. coli* cells from exposure to low concentrations of NEM. After growth to mid-exponential phase, cultures of MJF274 ($KefB^+$ $KefC^+$) and MJF276 ($KefB^-$ $KefC^-$) were diluted into fresh medium to an OD_{650} of 0.1 in the presence of 10 μM NEM, and the growth rates of the cultures were determined. Growth of both strains was inhibited by the addition 10 μM NEM, but exponential growth, at a rate close to that of the untreated cultures, was reestablished after a lag (Fig. 6a). The reestablishment of exponential growth in MJF274 was more rapid (growth lag, 130 ± 18 min; n = 3),

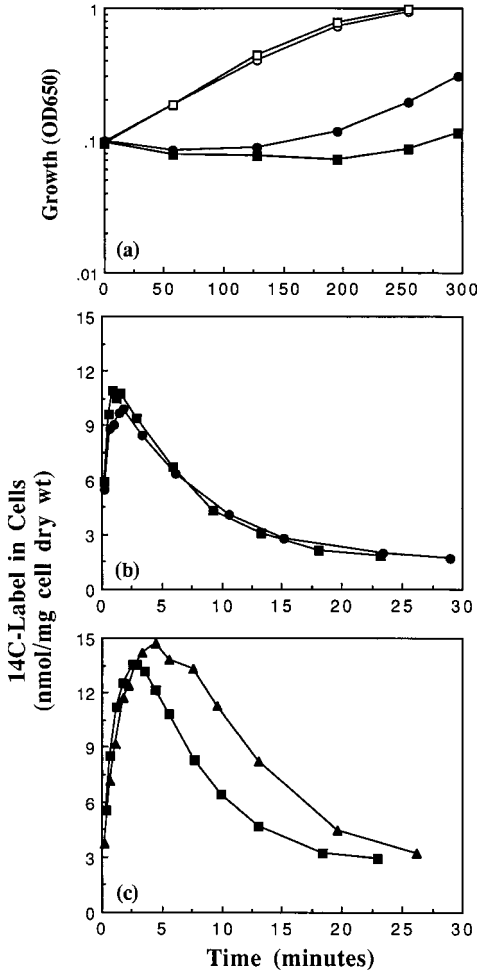

FIG. 6. KefB and KefC aid recovery from sublethal concentrations of NEM but are not required for detoxification. Exponential-phase cells were prepared for growth and for NEM metabolism as described in Materials and Methods. (a) MJF274 ($KefB^+$ $KefC^+$; ○ and ●) and MJF276 ($KefB^-$ $KefC^-$; □ and ■) cells growing in $K_{0.2}$ medium were treated with (filled symbols) and without (open symbols) 10 μM NEM at time zero. (b) MJF274 ($KefB^+$ $KefC^+$) (●) and MJF276 ($KefB^-$ $KefC^-$) (■) cells were suspended to an OD_{650} of 0.8 and treated with 13 and 14 μM [2-^{14}C]NEM, respectively, at time zero. (c) MJF276 cells suspended to an OD_{650} of 0.12 were treated with (▲) or without (■) 25 mM sodium acetate 5 s prior to the addition of 10 μM NEM at time zero.

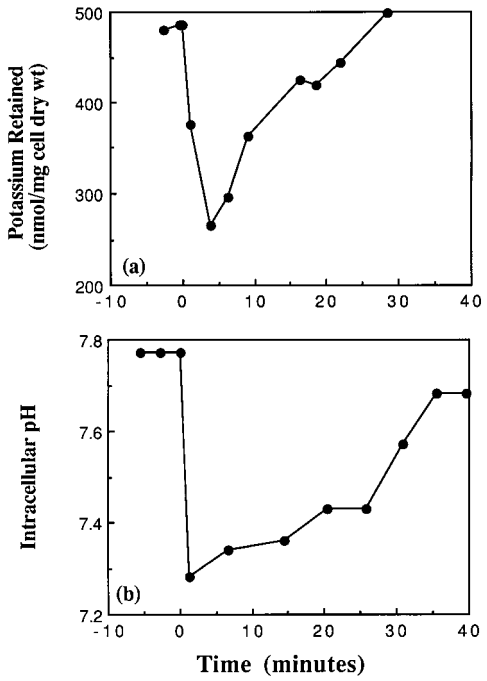

FIG. 7. Rapid recovery of the K^+ pool and pH_i after treatment with sublethal concentrations of NEM. Exponential-phase cells of MJF274 ($KefB^+$ $KefC^+$) were prepared and suspended in $K_{0.2}$ minimal buffer containing glucose (0.2% [wt/vol]). (a) K^+ measurements were conducted as described previously (11). (b) pH_i determinations were performed as described in Materials and Methods. The radioactive benzoic acid and inulin markers were added 6 min prior to the first sample. NEM (30 μM) was added to both experiments at time zero.

whereas recovery of strain MJF276 ($KefB^-$ $KefC^-$) showed an extended lag (growth lag, 195 ± 15 min; $n = 3$). The cytoplasmic pH was observed to decline in these cells by 0.37 and 0.16 units, respectively, for MJF274 and MJF276. Recovery of strain MJF274 from exposure to 10 μM NEM was slower in K_{120} than in $K_{0.2}$ medium (data not shown) but was faster than for strain MJF276. Inhibition by NEM was cell density dependent; at a higher cell density (OD_{650} of 0.3), there was no observed growth inhibition by 10 μM NEM.

The data presented above are consistent with the ability of *E. coli* cells to detoxify NEM. We have previously shown the formation of the glutathione adduct ESG (11), which by analogy with other electrophiles is the first step in detoxification (14, 19). Cells were incubated with [2-^{14}C]NEM to determine whether acidification influenced the fate of the electrophile. The intracellular level of radioactivity was not affected by the cell mass present in the incubation, and thus it was possible to carry out experiments both under growth conditions (OD_{650} of 0.1) and in those used for assay of K^+ efflux (OD_{650} of 0.8). Accumulation of the radiolabel was transient (Fig. 6b), and after 20 min most of the cellular radiolabel was associated with cellular macromolecules (data not shown). This accumulation profile was not affected by the activity of KefB and KefC (Fig. 6b). Further, when cells of MJF276 ($KefB^-$ $KefC^-$) were acidified with 25 mM acetate, the fate of NEM was qualitatively similar although the release of counts from the cell was slightly slowed (Fig. 6c). Therefore, it does not appear that the protective effect of intracellular acidification, provoked by activation of KefB and KefC, can be mediated by enhanced metabolism of NEM.

Treatment of MJF274 with 30 μM NEM resulted in K^+ efflux that persisted for approximately 5 min and was followed by recovery of the K^+ pool (Fig. 7a). The recovery of the K^+ pool was spontaneous and did not require the addition of dithiothreitol, which has previously been shown to accelerate the breakdown of ESG (11). During this time, pH_i declined from 7.8 to 7.3 and then recovered to a value close to the pre-NEM value (Fig. 7b). These data demonstrate the essentially transient nature of the activation of K^+ efflux in the presence of low concentrations of NEM that can readily be detoxified.

DISCUSSION

The data presented here demonstrate that the KefB and KefC systems, which are activated by the formation of adducts between glutathione and electrophiles, play an important role in the survival of exposure to NEM. Although NEM is a chemical to which bacteria are not exposed frequently, *E. coli* cells have evolved the capacity to detoxify this electrophile. This is illustrated by the ability of cells to recover from exposure to low concentrations of NEM (Fig. 6a), by the glutathione-dependent metabolism of NEM (11) (Fig. 6b) (19a), and by the time-dependent reversal both of NEM-elicited K^+ efflux and the associated fall in pH_i (Fig. 7). Similar detoxification and recovery from exposure to NEM have been demonstrated for *Pseudomonas putida* (31). Thus, although NEM may not be a natural antagonist of growth, it is clearly related in structure to other compounds that are sufficiently frequently encountered to have favored the acquisition of metabolic systems for their detoxification.

KefB and KefC are components of the defensive system against electrophiles. It is clear that both KefB and KefC, which are strongly activated by exogenous electrophiles (4, 11), play a major role in protection of cells against the toxic effects of NEM. Cells that lack KefB and KefC recover more slowly from exposure to low concentrations of NEM and die more rapidly when exposed to high concentrations of this electrophile. The fate of radiolabeled NEM is unaffected by the loss of the KefB and KefC systems, and this implies that the two efflux systems are not directly involved in detoxification of NEM but potentiate survival by another route. Once detoxification of the NEM is complete, the K^+ pool is recovered and pH_i is restored. Recovery was found to be glucose dependent (data not shown) and has previously been shown to be dependent on the activity of the major K^+ uptake systems (3).

We have now established the central importance of the pH_i changes, wrought by the activation of the K^+ efflux systems, in survival of exposure to electrophiles (this work and references 14 and 15). The much larger pH_i changes in the presence of NEM, caused by activation of KefB and KefC and inhibition of the TrkG/H and Kdp uptake systems (11), render protection much less sensitive to environmental variables than is the case for MG (13–15). The rate of acidification after addition of NEM to cells has a major effect on survival. Strain MJF276 ($KefB^-$ $KefC^-$) experiences acidification of the cytoplasm upon treatment with NEM, and the steady-state pH_i is similar to that observed in MJF274 ($KefB^+$ $KefC^+$) in K_{120} medium. However, the survival of MJF276 is much poorer due to the difference in the rate at which the low value of pH_i is achieved (Fig. 1a and 2b and c). Acidification of the cytoplasm in MJF276 is not brought about by activation of KefB and KefC since these systems are missing. Rather, the acidification may be due to the inhibition of K^+ uptake, which we have previously established (16). K^+ uptake capacity is known to correlate with maintenance of an alkaline cytoplasmic pH (5). The importance of pH_i for survival of exposure to NEM is supported by protection afforded cells by acetate (Fig. 3 and 4).

When acetate was added to MJF276 cells to provoke an initial drop in pH_i, survival was dependent on the size of the pH_i change and on the time at which it was imposed. Thus, survival of exposure to NEM requires rapid, large-scale changes in pH_i.

The mechanism(s) by which acidification of the cytoplasm protects against electrophiles is not established. We have shown that acidification does not accelerate detoxification of either NEM (Fig. 6b and c) or MG (14). Chloramphenicol added at the same time as NEM does not reduce survival, suggesting that new protein synthesis is not required (19b). Similarly we have shown that inhibition of protein synthesis does not impair acidification-based protection against the electrophile MG (14). We have previously suggested that acidification may reduce the rate of damage to macromolecules in the cell (13, 14). The requirement for essential repairs to macromolecules could explain why recovery of K^+ and pH_i and NEM detoxification are relatively rapid (Fig. 7a, 7b, and 6b, respectively) but restoration of growth is slow (Fig. 6a) (16 to 30 min for K^+, pH_i, and detoxification, compared with 125 min for growth). These data suggest that detoxification is not the rate-determining factor for recovery.

Changes in pH_i have been implicated in resistance to methylating mutagens. Preincubation of cells with 25 mM acetate decreased the frequency of Trp^+ revertants in cells surviving exposure to *N*-methyl-*N*′-nitro-*N*-nitosoguanidine (26). These workers have also suggested that the induction of the *aidB* locus, which is involved in some forms of DNA repair, is controlled both by NEM and by cytoplasmic pH (18, 29). These data are consistent with a generalized effect of changes in pH_i in protecting DNA against covalent modification. The importance of protection of DNA against damage and of a high capacity for DNA repair are suggested by the induction of the Dps protein and exonuclease III (1, 27) in cells entering the stationary phase. Preliminary data show that *rpoS* mutants and strains lacking exonuclease III and Dps are more sensitive to NEM. Although these systems are generally considered to be inducible, we have found that they are all expressed at a significant level in exponentially growing cells, and therefore their activities contribute to setting the level of sensitivity to NEM. Thus, DNA damage is likely to be a significant component of the mechanism of killing by NEM, and acidification may act by reducing the rate at which this damage occurs.

In conclusion, we have demonstrated that activation of KefB and KefC is a major determinant of the protection of *E. coli* cells against the toxic effects of NEM. This protection is largely mediated by changes in the cytoplasmic pH. KefB and KefC activity is widespread in gram-negative bacteria, which suggests that this mechanism of protection against electrophiles is also of general importance. In contrast, gram-positive bacteria generally lack both glutathione and KefB and KefC activity and either must be exquisitely sensitive to electrophiles or must have evolved alternative mechanisms for protection.

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