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Survival of *Escherichia coli* Cells Exposed to Iodoacetate and Chlorodinitrobenzene Is Independent of the Glutathione-Gated K^+ Efflux Systems KefB and KefC

L. S. NESS, G. P. FERGUSON, Y. NIKOLAEV,† AND I. R. BOOTH*

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

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The KefB and KefC systems of *Escherichia coli* cells are activated by iodoacetate (IOA) and chlorodinitrobenzene (CDNB), leading to a rapid drop in the intracellular pH. However, survival of exposure to IOA or CDNB was found to be essentially independent of KefB and KefC activation. No correlation was found between the toxicity of the compound and its ability to elicit protective acidification via activation of KefB and KefC.

Adaptation to stress is essential for growth and survival of bacterial cells. In *Escherichia coli* and the other gram-negative bacteria, the tripeptide glutathione plays a central role in the defense of the cell against electrophiles (1, 2, 4). Firstly, the conjugation of glutathione to the electrophilic group inactivates the electrophile (10), and secondly, the glutathione conjugate formed activates the KefB and KefC potassium channels (2, 4). The activation of KefB and KefC by glutathione conjugates results in rapid K^+ loss from the cell, the rate and extent being dependent upon the nature of the electrophile encountered (2, 4). KefC is the kinetically dominant K^+ efflux system, but KefB shows a broader glutathione adduct specificity since it is the major system responsible for K^+ efflux caused by adducts formed by methylglyoxal (MG) and iodoacetate (IOA) (4). Activation of KefB and KefC accelerates the recovery from exposure to sublethal concentrations of MG and *N*-ethylmaleimide (NEM) and enhances survival of exposure to high concentrations of these compounds (4, 7). The KefB and KefC systems do not effect detoxification of the electrophiles; rather, it has been demonstrated that their role is to allow the lowering of the cytoplasmic pH, which has been proposed to minimize electrophilic damage to cellular macromolecules (5, 7).

Other electrophiles can interact with glutathione and form activators of the KefB and KefC systems (2). IOA forms a strong activator of KefB but is less able to activate the kinetically dominant KefC system (4). In contrast, chlorodinitrobenzene (CDNB) forms a good activator of both KefB and KefC (2). In this study, we have investigated the role of these systems in the survival of cells exposed to IOA and CDNB. Both compounds inhibit growth of *E. coli*, but only CDNB is toxic at concentrations similar to those that activate KefB and KefC. Activation of KefB and KefC systems does not significantly enhance survival of *E. coli* cells exposed to either IOA or CDNB. Rapid acidification of the cytoplasm followed activation of KefB and KefC by IOA and CDNB. However, both electrophiles also caused a rapid lowering of intracellular pH (pH_i) even in strains lacking KefB and KefC. These studies suggest that there is not a direct link between the toxicity of electrophiles and their ability to activate KefB and KefC.

All organisms used in this study were derivatives of *E. coli* K-12: Frag1 (F^- *thi rha lacZ*), Frag5 (Frag1; Δ*kdpABC5*), MJF274(Frag5; *lacI trkD1*), MJF276 (MJF274; *kefB kefC*::Tn*10*), MJF270 (MJF274; *kefB*), and MJF277 (MJF274; *kefC*::Tn*10*). All cells were grown in K_x minimal medium (where *x* is the millimolar concentration of potassium) with 0.2% (wt/vol) glucose as carbon source, as previously described (3–7). K_x minimal buffer lacked all growth supplements except 0.2% (wt/vol) glucose. Cells for K^+ and pH_i determinations were grown to late exponential phase (optical density at 650 nm [OD_{650}] = 0.7 to 0.8) in medium as stated below, and the determinations were performed in K_x minimal buffer. Potassium determinations were conducted exactly as described previously (2). For growth and viability experiments with CDNB, cells were grown to late exponential phase (OD_{650} = 0.75) in the medium described below. The cultures were then diluted 10-fold into fresh prewarmed medium. Growth and viability experiments with IOA were conducted exactly the same as with CDNB except that cells were grown to mid-exponential phase (OD_{650} = 0.4). CDNB and IOA additions were made from 100 mM stock solutions prepared in either 100% ethanol or 100 mM NaOH, respectively. Cell viability was determined exactly as described previously (4), and cells were recovered on K_{10} minimal agar plates.

Measurements of pH_i were conducted by using the distribution of radiolabelled weak acid as described previously (8) with [^{14}C]benzoic acid (5.6 μM, 0.1 mCi · ml^{-1}) and [^{3}H]inulin (0.62 mM, 1 mCi · 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. All experiments were conducted a minimum of three times. For the growth and viability experiments, variation in the actual recovery period or death phase did occur, but the appropriate controls were always performed on the same day. The patterns of growth and survival shown were always consistent for all strains or growth conditions used. For this reason, we have not taken averages from different days but have chosen to show representative data.

We have previously shown that the addition of 1 mM IOA and 0.6 mM CDNB activates KefB and KefC, resulting in loss of potassium (cells of MJF274 lost 40 and 96% of their potassium pool 8 min after the addition of IOA and CDNB, respectively) (2, 4). We sought to determine whether this activation protected *E. coli* cells during exposure to CDNB and IOA. Exponential-phase cells of MJF274 ($KefB^+$ $KefC^+$) and MJF276 ($KefB^-$ $KefC^-$) were suspended in $K_{0.2}$ medium and

* Corresponding author. Phone: 44-1224-273152. Fax: 44-1224-273144. E-mail: gen118@abdn.ac.uk.

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

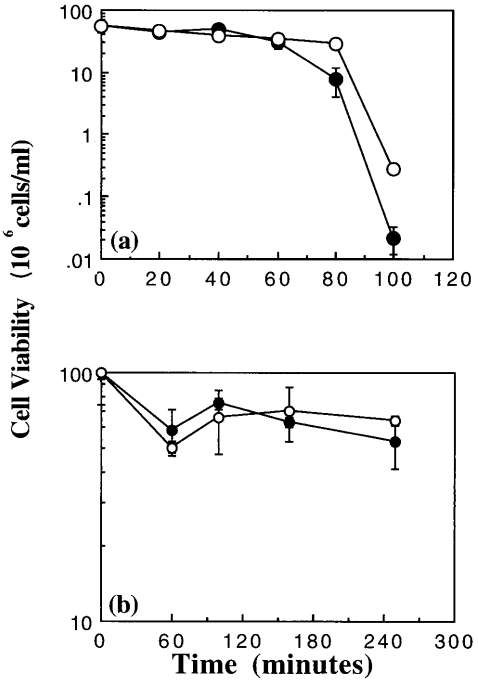

FIG. 1. The KefB and KefC systems are not essential for survival of exposure to CDNB and IOA. Exponential-phase cells were prepared, and viability experiments were conducted in $K_{0.2}$ medium exactly as described in the text. Cells of MJF274 ($KefB^+$ $KefC^+$) (●) and MJF276 ($KefB^-$ $KefC^-$) (○) were exposed to 2 mM CDNB (a) and 10 mM IOA (b) at time zero.

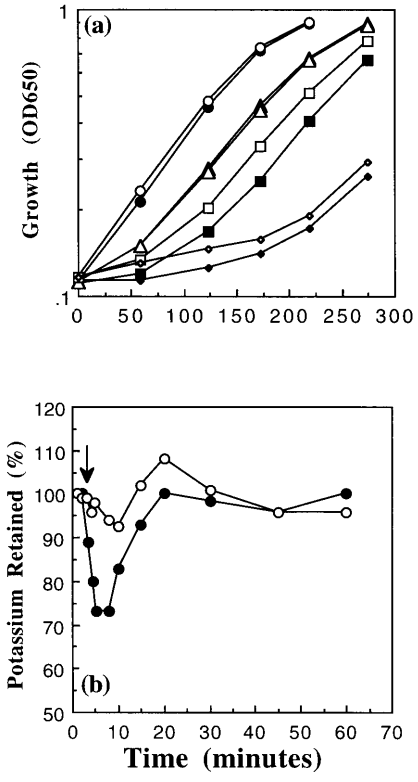

FIG. 2. Growth inhibition and K^+ efflux in the presence of CDNB. Exponential-phase cells were prepared, and growth experiments and K^+ measurements were performed exactly as described in the text in $K_{0.2}$ medium. (a) Cells of MJF274 ($KefB^+$ $KefC^+$) (closed symbols) and MJF276 ($KefB^-$ $KefC^-$) (open symbols) were exposed to 0 (● and ○), 0.15 (▲ and △), 0.2 (■ and □), and 0.25 (◆ and ◇) mM CDNB at time zero. (b) Cells of MJF274 ($KefB^+$ $KefC^+$) (●) and MJF276 ($KefB^-$ $KefC^-$) (○) were treated with 0.15 mM CDNB at the time indicated by the arrow.

treated with either CDNB or IOA (Fig. 1a and b, respectively). Treatment of cells with 2 mM CDNB resulted in loss of viability. No change in viable cell numbers was seen for the first 80 min, but thereafter cell viability declined rapidly, and this decline was essentially independent of the presence of KefB and KefC (Fig. 1a). With CDNB concentrations between 0.15 and 1 mM, no cell death was seen (data not shown). Concentrations of IOA less than 5 mM had no effect on cell viability (data not shown). However, raising the IOA concentration to 10 mM resulted in some cell death, and this was independent of KefB and KefC (Fig. 1b) (for both MJF274 and MJF276, cell viability declined by approximately 40% within an hour after the addition of 10 mM IOA). These data show that the concentrations of IOA and CDNB that activate KefB and KefC are not lethal to cells and that, although exposure to higher concentrations does lead to a loss of viability, the presence of KefB and KefC does not affect the survival at these concentrations.

Recovery from sublethal concentrations of CDNB did not depend strongly on the activation of KefB and KefC. Exponential-phase cells of MJF274 ($KefB^+$ $KefC^+$) and MJF276 ($KefB^-$ $KefC^-$) were treated with sublethal concentrations of CDNB, and the rate of recovery was observed. At the lowest concentration of CDNB tested (0.15 mM), recovery was identical in MJF274 and MJF276. At higher concentrations (0.2 to 0.25 mM), MJF276 ($KefB^-$ $KefC^-$) appeared to recover slightly faster than the parent, but the difference was small (Fig. 2a). Note that the lag prior to recovery of exponential growth is shorter than the period over which cells retained greater than 95% viability when incubated with much higher concentrations (2 mM) of CDNB (Fig. 1a), and therefore, it is unlikely that the lag corresponds to the death of a small fraction of cells. At concentrations greater than 0.25 mM CDNB, no recovery occurred over a 300-min time course, but the cells retained 100% viability (data not shown). Thus activation of KefB and KefC did not accelerate recovery of cells from exposure to CDNB and may slightly increase the time required to reestablish exponential growth. Incubation with 0.15 mM CDNB caused a transient loss of the K^+ pool from cells of strain MJF274, which was recovered after approximately 20 min (Fig. 2b). A smaller and slower decline in the K^+ pool was observed in strain MJF276, possibly due to inhibition of the Trk uptake system. However, since the magnitude of the K^+ loss was quite different in the two strains, but the growth profile was identical, inhibition does not appear to arise from the K^+ loss per se. The lag may arise from the toxicity of CDNB and/or a requirement for its detoxification prior to reinitiation of growth. It was notable that incubation with CDNB led to the accumulation of brown pigment in the growth medium, which may be equivalent to that recently reported for a range of bacteria incubated with this electrophile (12).

Activation of KefB and KefC led to growth inhibition in the presence of sublethal concentrations of IOA (Fig. 3a). Strain MJF274 ($KefB^+$ $KefC^+$) did not grow in the presence of 0.05 to 0.08 mM IOA, whereas the mutant strain, MJF276 ($KefB^-$ $KefC^-$), achieved growth rates of between 40 and 80% of those of the uninhibited control cells (Fig. 3a). KefB is the dominant system in this phenomenon, since mutants that possess only KefB (strain MJF277) were similarly inhibited whereas those that possessed only KefC (strain MJF270) were unaffected (data not shown). These data suggested that loss of K^+ due to

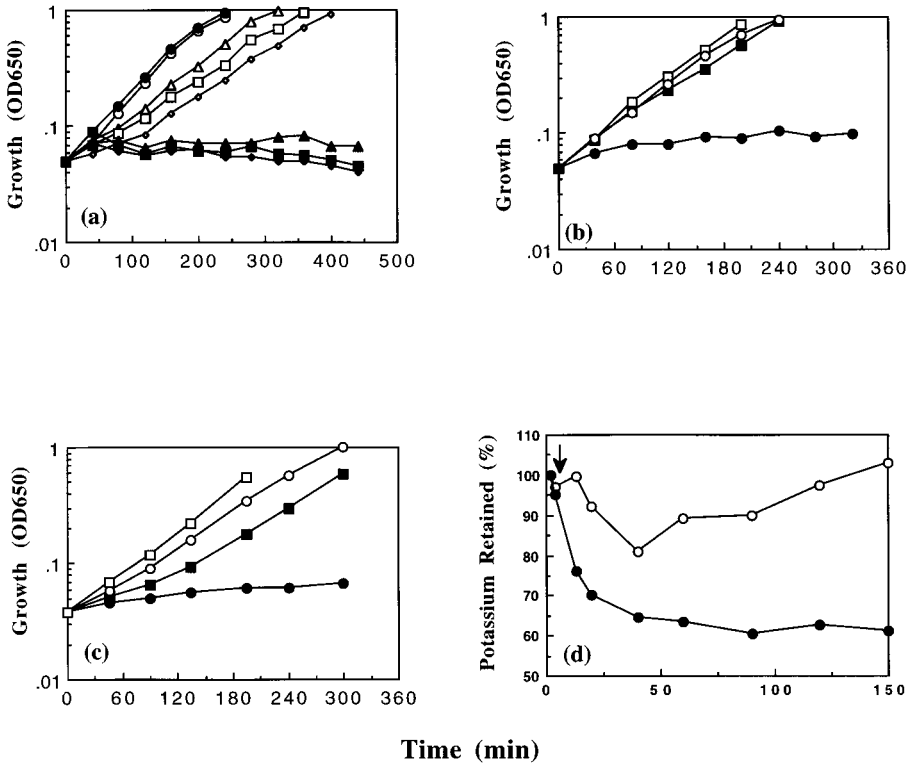

FIG. 3. Growth inhibition by IOA is reversed either by inactivation of KefB or by expression of the Kdp system. Exponential-phase cells were prepared as described for Fig. 2. (a) Cells of MJF274 (closed symbols) and MJF276 (open symbols) were exposed to 0 (● and ○), 0.05 (▲ and △), 0.06 (■ and □), and 0.08 (◆ and ◇) mM IOA at time zero. (b) Cells of MJF274 ($KefB^+$ $KefC^+$) were suspended either in $K_{0.2}$ (● and ○) or in K_{10} (■ and □) medium and treated with (closed symbols) or without (open symbols) 0.05 mM IOA at time zero. (c) Cells of Frag1 (Kdp^+ $KefB^+$ $KefC^+$) (■ and □) and Frag5 (Kdp^- $KefB^+$ $KefC^+$) (● and ○) were suspended in $K_{0.2}$ medium and treated with (closed symbols) and without (open symbols) 0.05 mM IOA at time zero. (d) Cells of MJF274 ($KefB^+$ $KefC^+$) (●) and MJF276 ($KefB^-$ $KefC^-$) (○) were treated with 0.1 mM IOA at the time indicated by the arrow.

activation of the KefB system by IOA (4) prevented growth. To test this hypothesis, exponential-phase cells of MJF274 ($KefB^+$ $KefC^+$) were suspended in either $K_{0.2}$ or K_{10} and treated with 0.05 mM IOA (Fig. 3b). It has been shown previously that 10 mM potassium inhibits the activity of KefB (4). Raising the K^+ content from 0.2 to 10 mM restored growth to close to that of the control cells (Fig. 3b). Retention of the K^+ pool can also be effected by induction of the Kdp transport system, which has a high affinity for K^+ and is induced by growth in $K_{0.2}$ (6, 9). Exponential-phase cells of Frag1 (Kdp^+ $KefB^+$ $KefC^+$) and Frag5 (Kdp^- $KefB^+$ $KefC^+$) were grown in $K_{0.2}$ and treated with 0.05 mM IOA (Fig. 3c). Cells of Frag1 were slightly inhibited by the presence of 0.05 mM IOA but rapidly established a growth rate similar to that of the control cells. In contrast, Frag5 cells, which like MJF274 lack the Kdp system, were unable to grow throughout the experiment. In the absence of IOA, cells of Frag1 grew slightly faster than cells of Frag5, due to their higher potassium scavenging capacity. These data suggest that IOA inhibition of MJF274 and Frag5 cells, which possess KefB and KefC, is due to their inability to sustain a high K^+ pool. This was confirmed by direct measurement of the K^+ pool in MJF274 and MJF276 (Fig. 3d). In strain MJF276 there was a transient loss of K^+, possibly due to inhibition of the K^+ uptake system (data not shown), but in MJF274 the K^+ loss was more extensive and was sustained over the 150-min incubation period (Fig. 3d).

On exposure to MG or NEM, activation of KefB and KefC elicits rapid acidification of the cytoplasm, which results in enhanced survival of exposure to the electrophiles (5, 7). A similar lowering of pH_i was observed upon addition of CDNB and IOA to exponential-phase cells of MJF274 ($KefB^+$ $KefC^+$) (Fig. 4a and b, respectively). With both CDNB and IOA, the pH_i decreased rapidly (from 7.8 to 7.1 within 3 min after the addition of either CDNB or IOA). However, cells of MJF276 ($KefB^-$ $KefC^-$) also exhibited a lowering of pH_i in the presence of CDNB and IOA (Fig. 4c and d, respectively), but the rate of decline in pH_i was measurably lower. Cells of MJF276 required 7 min to achieve the same pH_i decline achieved after only 3 min in strain MJF274 (compare Fig. 4c and d with 4a and b). This difference in the rate of the pH_i drop between MJF274 and MJF276 was also observed with lower concentrations of IOA and CDNB, similar to those used for growth inhibition studies (data not shown).

The data presented demonstrate that the KefB and KefC systems are not essential for survival of *E. coli* cells during exposure to the electrophiles IOA and CDNB. This differs from our findings with two other electrophiles, NEM and MG, in which the KefB and KefC systems were seen to play a central role in protection. It was shown that protection, effected by activation of the KefB and KefC systems, was a consequence of the rapid decrease in the cytoplasmic pH in the presence of NEM and MG (5–7). Further, we have shown that for these electrophiles the rate at which pH_i declines is of equivalent importance for survival. Here we observed that activation of KefB and KefC by IOA and CDNB addition causes a rapid decrease in the cytoplasmic pH. However, neither of the two electrophiles tested here exhibited significant toxicity in the concentration range that activates KefB and

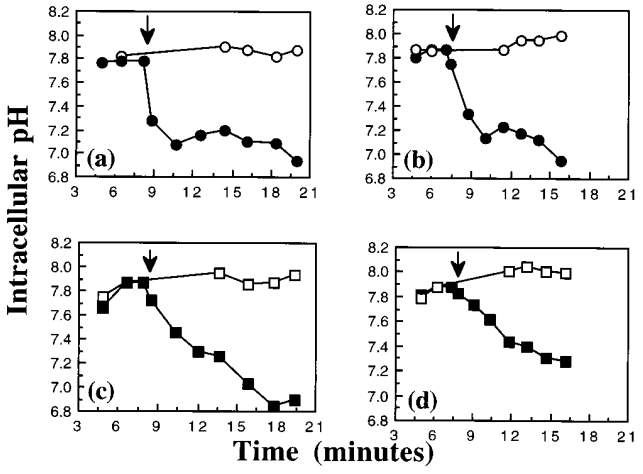

FIG. 4. Acidification of the cytoplasm by the addition of CDNB and IOA. Exponential-phase cells were prepared, and pH_i experiments were conducted exactly as described in the text. Cells of MJF274 ($KefB^+$ $KefC^+$) (● and ○) and MJF276 ($KefB^-$ $KefC^-$) (■ and □) were suspended in $K_{0.2}$, and the pH_i was measured in the presence (closed symbols) or absence (open symbols) of either 0.6 mM CDNB (a and c) or 1 mM IOA (b and d). Additions were made at the time indicated by the arrow. Time zero represents the time of addition of [^{14}C]benzoic acid.

KefC. The fluoro analog of CDNB is a potent mutagen (10), and it is likely that the substitution of chlorine for fluorine is sufficient to lower the toxicity of this compound. The data presented here are consistent with adaptation to the presence of CDNB, and possibly to its metabolism, since cells recovered their normal growth rate after a short period of growth inhibition that was extended by increasing the concentration of the electrophile. Similarly, IOA, though reactive, is less toxic than its fluoro and chloro analogs. CDNB is a known substrate for glutathione *S*-transferases (11), and the activation of KefB and KefC is consistent with the formation of a glutathione adduct (2). The fate of the adduct is not known, although others have suggested that some adducts are exported from the cell and degraded by γ-glutamyl transpeptidase (12) in the periplasm, leading to the accumulation of the cysteine adduct in the medium. The yellow-brown color formed in the medium of *E. coli* cells incubated with CDNB may indicate a similar fate for the 2,4-dinitrobenzyl-*S*-glutathione formed from reaction with glutathione.

IOA, though reactive, is less toxic than its fluoro and chloro analogs. It was notable that at each IOA concentration tested rapid exponential growth of a $KefB^-$ $KefC^-$ strain, MJF276, was established at a submaximal level soon after the addition of the compound. This growth pattern is not consistent with the detoxification of the electrophile, since recovery is extremely rapid and the subsequent growth rate is always at a lower value, suggesting sustained inhibition. These data suggest that the glutathione adduct, carboxymethyl-*S*-glutathione, may be a poor substrate for the detoxification systems of the cell.

At high concentrations, IOA and CDNB do reduce viability, but we have shown that there is a KefB-KefC-independent mechanism by which acidification can be achieved, and this may override the need for the K^+ efflux systems at these high concentrations of electrophile. Thus the data do not preclude a role for changes in pH_i in the survival of *E. coli* cells during exposure to IOA and CDNB, but they suggest that KefB and KefC are not essential components of the survival mechanism in this instance. We have previously suggested that KefB and KefC might be valuable targets for antibacterial therapy. The data presented here suggest that not all activators are severely toxic and that this might be utilized to inhibit the growth of bacteria without human tissue damage. However, the physiological K^+ concentrations may be too high for such a strategy to be effective, since we have shown here that as little as 10 mM K^+ overcame the inhibition caused by IOA. The studies with MG and NEM suggest that effective antibacterial compounds would need to be sufficiently toxic to require the activation of KefB and KefC to ensure cell survival.

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