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The Activity of the High-Affinity K^+ Uptake System Kdp Sensitizes Cells of *Escherichia coli* to Methylglyoxal

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Expression of the Kdp system sensitizes cells to methylglyoxal (MG) whether this electrophile is added externally or is synthesized endogenously. The basis of this enhanced sensitivity is the maintenance of a higher cytoplasmic pH (pHi) in cells expressing Kdp. In such cells, MG elicits rapid cytoplasmic acidification via KefB and KefC, but the steady-state pHi attained is still too high to confer protection. Lowering pHi further by incubation with acetate decreases the sensitivity of cells to MG.

Cytoplasmic homeostasis is essential for the growth and survival of bacteria. K^+ transport is believed to play an essential role in maintaining homeostasis by regulating cell turgor and the cytoplasmic pH (pHi). In order to regulate K^+ levels, *E. coli* cells possess multiple uptake (Trk, Kup, TrkF, Kch, and Kdp) (3, 6, 8, 10, 12, 23, 26) and efflux (KefA, KefB, KefC, and KefD) (4, 5) systems. The Trk transport system is the most complex, both at the genetic and physiological level (3, 8, 21, 26, 29), and in K^+-replete media, the Trk systems are thought to be responsible for the majority of K^+ uptake. Tight control over activity facilitates the role of the Trk system in turgor generation via control of the K^+ pool (22). In contrast to the other uptake systems, Kdp is a high-affinity (with a K_m in the micromolar range), inducible K^+ ATPase with a moderate activity (V_{max} = 100 to 150 μmol g^{-1} min^{-1}) (10, 18, 26, 28). Expression of the Kdp system occurs in media with low K^+ concentrations (0.2 mM or less), and it has been proposed that a reduction in cell turgor is responsible for inducing expression (25). The transport system is capable of sustaining very large K^+ gradients, greater than 10,000-fold, and its feedback control over activity is limited, unlike that of the Trk system.

Genetic analysis of the constitutive K^+ uptake and efflux systems was initiated with a mutant lacking the high-affinity Kdp system, since the activity of Kdp had previously been shown to mask K^+ efflux activity (11). Thus, a series of Kdp-deficient strains have been routinely used to study the activities and regulation of the K^+ efflux systems (4, 9, 13, 14, 24). Using these strains, we demonstrated that two of the K^+ efflux systems, KefB and KefC, are activated during the glutathione (GSH)-dependent detoxification of methylgloxal (MG), a toxic by-product of glycolysis (13–15). KefB and KefC are negatively regulated by GSH and are activated by GSH adducts (9, 14, 21). GSH-deficient strains have lost the ability to activate KefB and KefC (9, 14) and to carry out GSH-dependent MG detoxification and are greatly sensitized to MG (2). MG is thought to be synthesized in *Escherichia coli* and in other bacteria under conditions of phosphate limitation accompanied by sugar excess (7, 16). MG synthase converts dihydroxyacetone phosphate to MG with the release of P_i; the enzyme is inhibited by a high level of P_i and shows cooperativity with regard to dihydroxyacetone phosphate concentrations (17). Consequently, the enzyme is maximally active when glycolysis is proceeding under phosphate-limited conditions. Such conditions can be mimicked in *E. coli* by overriding catabolite repression with the addition of cyclic AMP (cAMP) during growth on poor carbon sources (1, 13).

We have previously demonstrated that the activation of the KefB and KefC K^+ channels during the detoxification of MG protects *E. coli* cells against MG-induced cell death (14). Activation of the KefB and KefC channels results in the rapid loss of K^+ and is partially balanced by the influx of H^+ ions, causing a fall in pHi (13). Mutants lacking KefB and KefC can be protected against MG by acidifying the cytoplasm with weak acids (13). Protection by KefB and KefC occurred only in media containing low levels of K^+ (0.2 mM K^+) and was reversed by 10 mM K^+ in the growth medium because of the elevation of pHi and reduction of the activity levels of the efflux systems (13, 14). Thus, KefB and KefC-mediated protection is observed most clearly in media with low concentrations of K^+, a condition that would be expected to elevate the level of expression of the Kdp system (18). However, in our previous analysis, the strains carried a Kdp deletion, and thus the aim of this study was to determine whether the activity of the high-affinity K^+ uptake system, Kdp, could influence protection of *E. coli* cells against MG.

Analysis of growth and viability. Two isogenic *E. coli* strains were used for this study: Frag1 (*thi rha gal lacZ*) and Frag5 (Frag1, Δ(*kdpABC*)*5*). All cells were grown as stated below in K_x minimal medium (where *x* is the concentration of K^+ in millimolar units) (11) adjusted to pH 6.9 with concentrated HCl and supplemented with 0.2% glucose as the carbon source. In this study two media, $K_{0.2}$ and K_{120}, were utilized. The media were supplemented with thiamin (final concentration, 1 μg ml^{-1}). When experiments were conducted to stimulate MG production, cells were grown with 0.2% xylose as the sole carbon source and were supplemented with 2 mM cAMP during the exponential growth phase. Viability experiments, MG assays, experiments to measure K^+ efflux, pHi and K^+ uptake, and analysis of bound versus free K^+ were conducted exactly as described previously (9, 13, 14, 20, 28).

Cells for the pHi and K^+ efflux experiments were grown to late exponential phase (optical density at 650 nm = 0.8 to 1.0) in either $K_{0.2}$ or K_{120} medium (growth in K_{120} prevents Kdp expression [11]), filtered, and suspended to the same optical density at 650 nm in $K_{0.2}$ minimal buffer supplemented with 0.2% glucose. When the expression of Kdp was not desired, the protein synthesis inhibitor, chloramphenicol (25 μg ml^{-1}), was included in the suspension buffer. Kdp induction resulted in a

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TABLE 1. Effects of Kdp expression on intracellular K^+ pools and on pHi

Strain	Growth medium	Kdp expressed	Mean K^+ content ± SD of[a]: Initial pool	Mean K^+ content ± SD of[a]: Pool at 10 min[c]	Mean K^+ lost ± SD[a]	Mean pHi ± SD of[b]: Initial pHi	Mean pHi ± SD of[b]: Final pHi[c]	Mean drop in pHi ± SD
Frag1	$K_{0.2}$	Yes	617 ± 76	480 ± 17	137 ± 60	8.0 ± 0.1	7.6 ± 0.1	0.46 ± 0.1
Frag1	K_{120}[d]	No	550 ± 20	375 ± 35	175 ± 35	7.7 ± 0.1	7.2 ± 0.1	0.45 ± 0.1
Frag5	$K_{0.2}$	No	488 ± 41	321 ± 31	167 ± 21	7.75 ± 0.1	7.3 ± 0.1	0.46 ± 0.1

[a] K^+ contents are expressed in nanomoles per milligram of cell dry weight. Mean free K^+ (20) ± the standard deviation was approximately 53% ± 1% for Frag1 and 56% ± 4% for Frag5 grown in $K_{0.2}$ medium (n = 2), and these data are similar to those published previously (20). The numbers of experiments performed to determine K^+ contents were as follows: three for Frag1 cells in $K_{0.2}$ medium, two for Frag1 cells in K_{120} medium, and five for Frag5 cells in $K_{0.2}$ medium.

[b] The numbers of experiments performed to determine pHi values were as follows: four for Frag1 cells in $K_{0.2}$ medium, two for Frag1 cells in K_{120} medium, and five for Frag5 cells in $K_{0.2}$ medium.

[c] Measured 10 min after the addition of 3 mM MG.

[d] Cells grown in K_{120} medium were subsequently incubated in $K_{0.2}$ medium in the presence of 25 μg of chloramphenicol per ml. All incubations contained 0.2% (wt/vol) glucose.

twofold increase in the rate of K^+ uptake relative to that of Frag5 ($^-$) (75 and 33 nmol of K^+ min^{-1} mg^{-1} for Frag1 and Frag5, respectively). Cells induced for Kdp maintained a greater cytoplasmic K^+ pool than either K_{120}-grown Frag1 or $K_{0.2}$-grown Frag5 cells, and the potassium pools showed similar distributions of levels of free and fixed (20) K^+ (Table 1). Frag1 cells grown in K_{120} gave rates of K^+ transport identical to those observed for Frag5.

The activity of the Kdp system sensitizes cells to MG. Cells of Frag1 (Kdp^+) were more sensitive to 0.7 mM MG than Frag5 cells prepared in the same manner (Fig. 1a). Cells surviving a 200-min incubation with MG do so by virtue of detoxification of the electrophile rather than by selection of a resistant subpopulation, since addition of further MG leads to killing with kinetics similar to those occurring over the first 50 min (14). Growth of Frag1 in K_{120} medium, to suppress Kdp expression, enhanced the survival of this strain in the presence of MG to levels similar to those observed with Frag5 (Fig. 1b). These data clearly demonstrated that Frag1 cells, induced for the Kdp system, had an increased level of sensitivity to MG when compared with that of Frag5 cells.

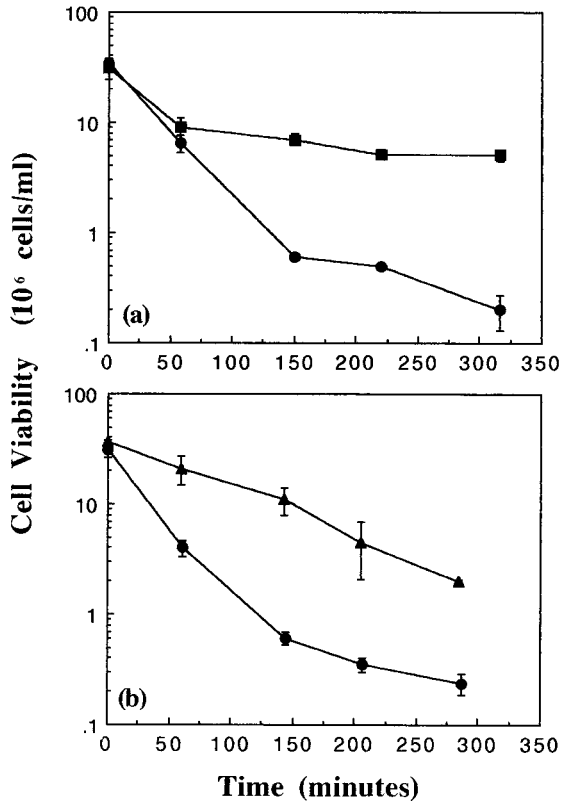

FIG. 1. The activity of the Kdp system greatly sensitizes cells to MG poisoning. Exponential-phase cells were prepared, and cell viabilities were determined exactly as described previously (13, 14). Time zero represents the time of dilution and of the addition of 0.7 mM MG. MG was added from a 0.65 M aqueous solution. (a) Cells of Frag1 (Kdp^+) (●) and Frag5 (Kdp^-) (■) grown in $K_{0.2}$ medium. (b) Cells of Frag1 grown in $K_{0.2}$ (●) and K_{120} (▲) media.

E. coli cells can be stimulated to synthesize MG by growth on rare carbon sources in the presence of cAMP (1). Under such conditions, the activities of the KefB and KefC systems provide transient protection against the toxicity of MG (14). We determined that Kdp expression reduced survival during endogenous production of MG. Frag1 and Frag5 cells were grown to exponential phase on xylose (0.2%) as the sole carbon source and diluted into fresh growth medium containing 2 mM cAMP and xylose (1, 14). Cell growth slowed in the presence of cAMP, and this correlated with a rise in the concentration of external MG (Fig. 2). Once the external-MG concentration exceeded 0.3 mM, there was no further growth, and as the concentration reached 0.7 to 0.8 mM, the viabilities of the cultures declined. The threshold concentration for the decline in cell viability was lower for Kdp^+ (Frag1) cells than for Kdp^- (Frag5) cells, indicating again that Kdp activity sensitized cells to MG. It was notable that the rise in MG concentration continued unabated after substantial cell death had occurred, indicating that nonviable cells were still metabolically active.

The activity of the Kdp system results in a more alkaline intracellular pH but does not suppress K^+ efflux via KefB and KefC. We recently demonstrated that the pHi is a critical determinant of MG sensitivity (13) and that a reduction in the pHi from 7.7 ± 0.1 to 7.4 ± 0.1 was sufficient to protect *E. coli* cells against MG poisoning (13). We sought to determine whether the increased sensitivity to MG of cells expressing the Kdp system was caused by elevated steady-state pHi prior to exposure to MG or by reduced acidification of the cytoplasm during detoxification. Exponential-phase cells of Frag1 (Kdp^+) and Frag5 (Kdp^-) were prepared and assayed in $K_{0.2}$ medium in the presence and absence of 3 mM MG for KefB and KefC activities and for changes in pHi (Table 1). MG-induced activation of KefB and KefC leads to moderately rapid K^+ efflux via KefB and KefC (14). Kdp activity has been suggested to be sufficiently active to suppress K^+ efflux via these systems (11). However, in Frag1 cells induced for Kdp, levels of MG-activated KefB and KefC activity were only slightly reduced and

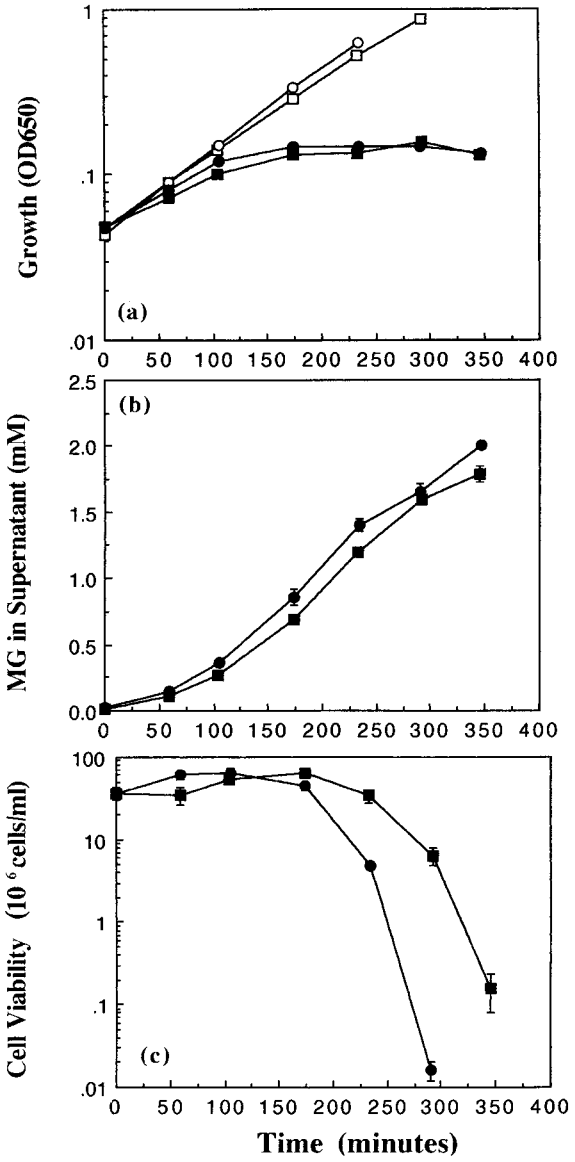

FIG. 2. Survival during endogenous synthesis of MG. Cells were grown overnight in $K_{0.2}$ medium with xylose as the sole carbon source. After outgrowth into mid-exponential phase, the cultures were diluted into prewarmed fresh-growth $K_{0.2}$ medium containing xylose (0.2% [wt/vol]) as the carbon source and 2 mM cAMP. Growth and viability were monitored as described previously. (a) Cell growth of strains Frag5 (■ and □) and Frag1 (● and ○). Open symbols indicate the control, and closed symbols indicate cAMP was added. OD650, optical density at 650 nm. (b) MG production. MG in the supernatant was assayed as described previously (13). Symbols are as described for panel a; (c) Cell viability in the presence of cAMP. Symbols are as described for panel a.

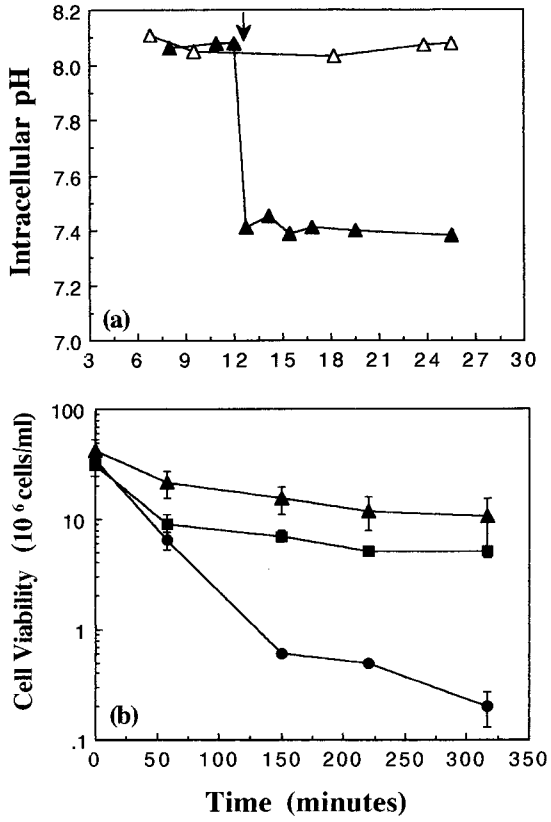

FIG. 3. A more alkaline intracellular pH is responsible for the increased sensitivity to MG. (a) Exponential-phase cells of Frag1 (Kdp^+) were prepared in $K_{0.2}$ medium, and the pHi values were measured exactly as described previously (13). Time zero represents the time at which the radioactivity was added. MG (3 mM) was added from a 0.65 M aqueous stock solution along with 25 mM sodium acetate, added from a 2 M stock, to the cell suspension (filled symbols) at the time indicated by the arrow. No additions were made to the control suspension (open symbols). (b) Exponential-phase cells were prepared in $K_{0.2}$ medium, and the viabilities were measured exactly as described in the text. Time zero represents the time of dilution and of the addition of 0.7 mM MG from a 0.65 M aqueous stock solution. Symbols: ●, Frag1 (Kdp^+); ▲, Frag1 treated with 25 mM sodium acetate at time zero; ■, Frag5 (Kdp^-).

the total change in the pools was similar to that of Frag5 cells (Kdp^-) (Table 1) or to Frag1 cells grown in K_{120} to suppress Kdp expression (Table 1). Thus, Kdp expression only slightly masked MG-induced K^+ loss via KefB and KefC (Table 1).

The steady-state pHi maintained in Frag1, cultured in $K_{0.2}$ medium, was poised at a more alkaline value when compared with that of Frag5. The values observed were pHi 8.0 ± 0.1 for Frag1 and pHi 7.7 ± 0.1 for Frag5 (Table 1) (13). Consistent with the above K^+ efflux data, MG elicited a fall in the pHi of 0.4 ± 0.1 units, irrespective of the strains used or the growth conditions (Table 1). Consequently, the pHi attained after MG addition were 7.6 ± 0.1 and 7.3 ± 0.1 for Frag1 and Frag5, respectively. Preventing the expression of the Kdp system in cells of Frag1 by growth on K_{120} medium yielded an intracellular pH profile for Frag1 that resembled that of Frag5 (in the absence of MG, at steady state, the pHi values were 7.75 ± 0.1 and 7.7 ± 0.1 for Frag5 and Frag1, respectively [Table 1]). Thus, the higher value of intracellular pH correlates with the expression of the Kdp system in Frag1 and with higher sensitivity to MG.

We have previously shown that mutants lacking KefB and KefC can be protected against MG by lowering the pHi with weak acids (13). To be certain that the observed differences in viabilities between cells of Frag1 (Kdp^+) and Frag5 (Kdp^-) were due to the differences in the absolute values of pHi obtained after MG addition, we investigated the effect of further reducing pHi by weak acid addition. The pHi of cells of Frag1 was reduced to 7.4 ± 0.1 by the addition of 25 mM sodium acetate (Fig. 3a). Exponential-phase cells of Frag1 (prepared in $K_{0.2}$ medium) were treated with and without 25 mM sodium acetate and then exposed to 0.7 mM MG, and the viabilities were determined (Fig. 3b). These results clearly showed that reducing the pHi of cells of Frag1 with 25 mM

acetate protected cells against MG poisoning at a level similar to that of Frag5 cells (Fig. 3). These data provide strong evidence that the differences in the sensitivities of cells of Frag1 and Frag5 to MG were due to the higher pHi values sustained by cells expressing Kdp activity.

These data demonstrate that the activity of the high-affinity K^+ uptake system, Kdp, sensitizes cells of *E. coli* to MG because of the higher steady-state value of pHi in cells expressing this transport system. K^+ efflux and the change in pHi during KefB and KefC activation were similar in Frag5 and Frag1. This was contrary to our expectation, since the high scavenging capacity of Kdp could have suppressed the K^+ efflux and confer sensitivity to MG by this mechanism. Indeed, some suppression of K^+ efflux was observed (Table 1), but this was insufficient to prevent the change in pHi (Table 1). High-level survival after exposure to MG was correlated with the fall in pHi to below 7.5. Thus, cells of Frag1, induced for Kdp, exhibited pHi 7.6 $\pm$ 0.1 and rapidly lost viability in the presence of MG. In contrast, Frag1 cultured in K_{120} medium, to suppress Kdp expression, and Frag5 cells grown in $K_{0.2}$ medium exhibited tolerance of MG and their pHi values in the presence of MG were no greater than 7.4 $\pm$ 0.1. Finally, Kdp-induced Frag1 cells that had been treated with 25 mM sodium acetate to lower the pHi to below 7.5 were resistant to MG. Therefore, the absolute value of pHi attained upon activation of KefB and KefC, during the GSH-dependent detoxification of MG, is clearly an important determinant of protection. The findings of these data support our earlier findings (13) and highlight once again that relatively minor differences in pHi values have profound effects on cell survival upon exposure to MG.

Relatively little is known about the precise environmental conditions that provoke MG production (7). Most studies with *E. coli* have either utilized specific mutants with metabolic blocks (16) or strains affected in catabolite repression (1). Overcoming catabolite repression by supplying cAMP causes MG production in *E. coli* cells (1). Transient exposure to high concentrations of sugar after periods of starvation, which can lead to release from catabolite repression (20), may elicit MG production. In *Prevotella ruminicola,* it has been demonstrated that phosphate starvation, in the presence of high glucose concentrations, causes MG excretion that is accompanied by K^+ efflux and cell death (27). It seems probable that this is an example of the imbalance in glycolysis caused by starvation for phosphate, and the K^+ efflux observed is consistent with the operation of efflux systems equivalent to those of KefB and KefC. Sustained production of MG overwhelms the protective effect of the efflux systems in *E. coli* (14), and this may be the equivalent of the observations of *Prevotella* spp. (27). Thus, the efflux systems must be seen as a survival mechanism that operates during the transient production of MG rather than during its sustained production.

In conclusion, we have shown that the activity of the Kdp system counters the protective roles of the KefB and KefC systems by setting the pHi to a more alkaline value. The Kdp system plays an important role in K^+ uptake, particularly in cells growing in media with low K^+ concentrations (10, 18, 25, 26). We have shown that there can be growth conditions that impose K^+ limitation and stimulate MG production (Fig. 2). Clearly, if K^+ limitation itself predisposes cells to produce MG, there would be the apparent paradox that the possession of the transport system required to counteract K^+ deficiency also sensitizes cells to MG. However, it is known that there is a complex relationship between K^+ accumulation and phosphate transport, wherein inhibition of the former leads to limitations on the latter (30). Thus, while a low level of K^+ might lead to phosphate limitation, a condition that could stimulate MG production, the expression of Kdp would counter K^+ limitation and also establish favorable conditions for phosphate uptake, an explanation that resolves the apparent paradox.

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