The distribution of homologues of the *Escherichia coli* KefC K⁺-efflux system in other bacterial species

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Using a variety of techniques the distribution of the glutathione-regulated KefC K⁺-transport system among bacterial species was investigated. The presence of similar systems in a number of Gram-negative bacteria was demonstrated. In contrast, the system appeared to be absent from most Gram-positive bacteria tested with the exception of *Staphylococcus aureus*. Using the cloned *Escherichia coli* *kefC* gene as a probe for Southern hybridization it was shown that only limited DNA sequence homology exists with other bacteria, even when closely related members of the enteric group were examined.

Introduction

K⁺ is accumulated to high concentrations in the bacterial cytoplasm and has been recognized to serve important roles in bacterial cells (Epstein, 1986). Although not proven for all bacteria, the role of K⁺ in turgor regulation is considered to be of central importance. In *Escherichia coli* control over K⁺ accumulation is the major mechanism of turgor regulation (Epstein, 1986) and the size of the cytoplasmic K⁺ pool is determined primarily by the osmotic pressure of the environment (Epstein & Schultz, 1965). Regulation of both influx and efflux processes for K⁺ has been demonstrated (Rhoads & Epstein, 1978; Meury *et al.*, 1985; Bakker *et al.*, 1987), which allows the cell to adapt to both low and high turgor. K⁺ uptake is accomplished by at least three systems, Kdp, Kup and Trk (Bossemeyer *et al.*, 1989*a*; Epstein, 1986). It has recently been suggested that the Trk system is the sum of two activities TrkG and TrkH (Dosch *et al.*, 1991). The Kup and Trk systems are constitutive while the Kdp system is induced by conditions that affect the ability of the cell to maintain turgor (Laimins *et al.*, 1981; Bossemeyer *et al.*, 1989*b*); the transport activity of each of the three systems is regulated by turgor pressure.

There are at least three K⁺-efflux systems in *E. coli* – KefB, KefC and a third system (now called KefA; W. Epstein, personal communication) that is evident in strains that lack KefB and KefC activity (Bakker *et al.*, 1987). The *kefB* and *kefC* (formerly *trkB* and *trkC*; Epstein & Kim, 1971; Booth *et al.*, 1985) loci encode two separate K⁺-efflux systems that are inhibited by glutathione and are activated by specific glutathione metabolites (Meury *et al.*, 1980; Booth *et al.*, 1985; Bakker *et al.*, 1987; Elmore *et al.*, 1990). The KefC system has been characterized in most detail at both the physiological and the molecular level (Elmore *et al.*, 1990; Munro *et al.*, 1991). The KefC system is activated by glutathione metabolites that possess hydrophobic additions to the sulphhydryl group of the cysteine of the peptide (Elmore *et al.*, 1990). Thus, *N*-ethylsuccinimido-*S*-glutathione (ESG), *N*-phenylsuccinimido-*S*-glutathione and 2,4-dinitrophenyl-*S*-glutathione activate the KefC system, but glutathione itself and *S*-carboxymethylglutathione do not produce significant activation and, indeed, reduce the activity of the KefC system (Elmore *et al.*, 1990; A. J. Lamb & I. R. Booth, unpublished data). The *kefC* gene from *E. coli* has recently been cloned and the DNA sequence determined (Munro *et al.*, 1991). The DNA sequence encodes a polypeptide of 620 amino acids, the sequence of which bears some similarity to bacterial enzymes that utilize glutathione (Munro *et al.*, 1991; K. T. Douglas, personal communication).

The present study was undertaken to determine the distribution of the KefC class of transport system among bacteria other than *E. coli*. We report that the transport

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Abbreviations: ESG, *N*-ethylsuccinimido-*S*-glutathione; GST, glutathione *S*-transferase; NEM, *N*-ethylmaleimide.

system appears to be widely distributed among Gram-negative bacterial species but is absent from all the Gram-positive bacteria tested with the exception of *Staphylococcus aureus*.

Methods

Bacterial strains. *Escherichia coli* 3000, *Salmonella typhimurium* LT2, *Pseudomonas aeruginosa*, *Staphylococcus caseolyticus* NCIMB 11374, *Streptococcus faecalis* NCIMB 775, *Streptococcus lactis* NCIMB 6681, *Erwinia carotovora*, *Lactobacillus casei* ATCC 335, *Staphylococcus aureus*, *Bacillus cereus* and *Enterobacter aerogenes* were from the Departmental culture collection; *Azospirillum brasilense* NCIMB 11860 was obtained from the NCIMB, University of Aberdeen, UK. *Rhodobacter sphaeroides* NCIMB 8253 was obtained from Dr N. Hunter (University of Sheffield, UK).

Media for growth and transport assays. The minimal media K_x [where *x* indicates the K⁺ concentration (mM)] was used for the culture of cells and for transport experiments (Epstein & Kim, 1971). Complex medium LK (Rowland *et al.*, 1984) was used for cell growth for DNA manipulations.

Growth conditions. Cells were grown either in K₁₂₀ minimal medium (Epstein & Kim, 1971) at 37 °C containing glucose (0.2%) as sole carbon source or in LK medium (Rowland *et al.*, 1984). Some strains (*R. sphaeroides*, *Az. brasilense*, *Staph. caseolyticus*, *Strep. faecalis*, *Strep. lactis*, *Er. carotovora*, *L. casei*) were grown at 28 °C. *R. sphaeroides* was grown aerobically in the dark.

Measurement of K⁺ efflux. Overnight cultures of the appropriate strain were diluted into fresh growth medium and grown to an OD₆₅₀ of approximately 1.0. An aliquot of cells (25 ml) was then harvested by filtration (Millipore; 0.45 µm, 4.5 cm) and washed with 5 ml K₁₀ medium. The cells were then suspended by vortexing in a boiling-tube containing 25 ml K₀ medium and the cell suspension was divided equally between two thermally insulated glass vessels maintained at 37 °C. Zero time for experiments was taken from the time of suspension. Additions were made as required from 100-fold concentrated stock solutions. At intervals 1 ml samples were removed by Gilson pipette and transferred to Eppendorf tubes, and the cells collected by centrifugation at full speed for 30 s. The supernatant was then removed by aspiration, the pellet suspended in 1 ml of distilled water and K⁺ liberated from the cells by boiling for 5 min in a rack designed to keep the Eppendorf tubes closed. Cell debris was then removed by centrifugation and the potassium content of the supernatant measured by flame photometry (Corning 400 flame photometer).

Assay of glutathione. This was done as described previously (Elmore *et al.* 1990).

DNA manipulations. Chromosomal DNA was prepared by the method modified from Davis *et al.* (1980). Cells (400 ml) in early exponential phase (OD₆₅₀ 0.3) were harvested by centrifugation and suspended in 5 ml 25% (w/v) sucrose in 50 mM-Tris/HCl, pH 8, and 10 mg lysozyme was added. The cells were mixed and incubated at 30 °C for 10 min. [This procedure was modified for *Staph. aureus* by the addition of 1 mg lysostaphin (Sigma) in place of lysozyme and the incubation temperature was raised to 37 °C.] After a further 10 min incubation on ice 5 ml 0.2 M-Na₂EDTA, pH 8, was added and the tubes were shaken gently and placed on ice for 10 min. This incubation was followed by the addition of 1 ml SDS (10%, w/v) and the tubes were shaken until the cell suspension cleared. If the suspension remained viscous, 10 ml 50 mM-EDTA, pH 8.5, containing 0.2% (w/v) SDS was added and the suspension was heated to 65 °C for 10 min. Streptococcal

Fig. 1. Regions of the *kefC* gene used as probes in Southern hybridization. DNA probes were produced by restriction digestion of plasmids pkC8 and pkC6-16 (Munro *et al.*, 1991). The hatched area represents the limits of the *kefC* gene; X, *Xho*I site; S, *Sal*I site.

cells were difficult to lyse and cell disruption was found to be assisted by raising the temperature to 70–75 °C (Marmur, 1961). A 1/5th vol. of 5 M-potassium acetate (Maniatis *et al.* 1982) was added to the lysed cells and the solutions mixed by gentle inversion of the tubes. After incubating the mixture on ice for at least 30 min the protein precipitate was removed by centrifugation at 20000 *g* for 20 min at 4 °C. The supernatant was decanted into a fresh sterile tube and ethanol added, and precipitation of the DNA aided by incubation at –20 °C for 30 min. The DNA was spooled onto a fine glass-rod, dried and dissolved in 1–2 ml Tris/EDTA (TE) buffer, pH 7.5 (Maniatis *et al.*, 1982). RNA and residual protein were removed by treatment with RNAase A and proteinase K, respectively, followed by phenol extraction and ethanol precipitation as described by Maniatis *et al.* (1982); the purified DNA was dissolved in a small volume of TE buffer, pH 7.5.

Additional DNA from *R. sphaeroides* and *Anacystis nidulans* was supplied by N. Hunter (University of Sheffield, UK) and E. Padan (University of Jerusalem, Israel), respectively.

DNA fragments for use as probes were derived from plasmids pkC8 and pkC6-16 (Munro *et al.*, 1991) (Fig. 1). The fragments were labelled with [α -³²P]dCTP [specific activity 3000 Ci mol^{–1} (111 TBq mol^{–1})] using the random oligonucleotide primer method (Feinberg & Vogelstein, 1983). The labelled DNA was purified by column chromatography (Stratagene Push column) to give probes with specific activities of 10⁸–10⁹ c.p.m. (µg DNA)^{–1}.

Restriction enzyme digestion, gel electrophoresis and Southern hybridization were performed according to Maniatis *et al.* (1982). DNA was transferred to GeneScreen Plus nylon membranes (Dupont). The blotted and hybridized membranes were given two final washes in 0.2 × SSC, 0.1% SDS at 50 °C. Washed blots were exposed to Fuji NIF RX X-ray film at –70 °C for 48 h prior to development.

Results

Activation of K⁺ efflux by *N*-ethylmaleimide

The activation of the KefC system can be achieved by the reaction of *N*-ethylmaleimide (NEM) with cellular glutathione to form the activator *N*-ethylsuccinimido-S-glutathione (ESG) (Elmore *et al.*, 1990). We therefore sought to elicit K⁺ efflux from a range of bacteria by the addition of NEM (0.5 mM final concentration) to cells washed and suspended in K⁺-free medium (Fig. 2*a, b*). A typical efflux curve is shown for *P. aeruginosa* (Fig. 2*a*)

Fig. 2. NEM-elicited K⁺ efflux from bacteria. (a) K⁺ efflux from *P. aeruginosa* was elicited by addition of 0.5 mM-NEM 3 min after transfer of the cells to K₀ medium (zero time). The initial K⁺ content of the cells was 350 ng-ion (OD₆₅₀ unit)⁻¹. □, No NEM; ■, 0.5 mM-NEM. (b) NEM-elicited K⁺ efflux was measured in experiments similar to that shown in (a) and the K⁺ lost by the cells 2 min (solid bars) and 18 min (stippled bars) after addition of 0.5 mM-NEM is shown. The K⁺ content of the cells in each case was approximately 350 ng-ion (OD₆₅₀ unit)⁻¹.

and the extent of K⁺ efflux 2 min and 18 min after NEM addition is shown for the other species investigated (Fig. 2b). In control incubations, to which NEM was not added, K⁺ efflux over the 18 min incubation was less than 4%. Rapid K⁺ efflux was observed in *S. typhimurium*, *Ent. aerogenes*, *Er. carotovora* and *P. aeruginosa*, and the rate of K⁺ efflux was similar to that observed in *E. coli*. However, the K⁺ efflux systems of *R. sphaeroides* and *Az. brasilense* were much less active than for the above group of micro-organisms. NEM did not elicit K⁺ efflux from the Gram-positive organisms studied with the exception of *Staph. aureus*, which exhibited an activity that was similar to that observed in *R. sphaeroides* and *Az. brasilense*. The related species *Staph. caseolyticus* did not show the efflux of K⁺ exhibited by *Staph. aureus* (Fig. 2b).

Involvement of glutathione in NEM-activated K⁺ efflux

SH reagents, such as NEM, could cause K⁺ efflux either by their action on protein thiols or by their forming an activator of the KefC system. We have previously shown that the involvement of glutathione in the NEM-activation of K⁺ efflux can be demonstrated by converting the glutathione pool to S-carboxymethyl-

glutathione by treatment with iodoacetate. S-Carboxymethylglutathione is not a strong activator of the *E. coli* KefC system and subsequent incubation with NEM does not elicit K⁺ efflux because there is no free glutathione to react with NEM and form ESG (Elmore *et al.*, 1990). Re-incubation of iodoacetate-treated *E. coli* cells with glutathione leads to the re-establishment of the cytoplasmic glutathione pool and restoration of sensitivity of K⁺ efflux to NEM (Elmore *et al.*, 1990). We therefore sought to apply this protocol to the above group of organisms.

Iodoacetate treatment prevented the NEM-activation of K⁺ efflux in each of the organisms that showed KefC-type activity. Incubation of cells with iodoacetate (1 mM final concentration) for 10 min prevented significant K⁺ efflux upon addition of NEM. Incubation of iodoacetate-treated cells of *Er. carotovora* and *Staph. aureus* with 5 mM-glutathione for 20 min in the presence of chloramphenicol to inhibit protein synthesis restored K⁺ efflux upon addition of NEM (Fig. 3a, b). The rate and extent of K⁺ loss was similar to that observed in cell suspensions that had not been treated with iodoacetate (Fig. 2b). Similarly, in the other Gram-negative bacteria tested (*R. sphaeroides*, *Az. brasilense*, *P. aeruginosa*, *S. typhimurium* and *Ent. aerogenes*), iodoacetate inhibited

Fig. 3. Restoration of K^+ efflux in iodoacetate-treated cells by glutathione. Cells of *Staph. aureus* (a) and *Er. carotovora* (b) were treated with iodoacetate (1 mM final concentration) and NEM (0.5 mM final concentration) added at 3 min (○). Alternatively, cells were treated as above with iodoacetate for 10 min and then washed and incubated for 20 min with 5 mM-glutathione. The cells were then harvested by filtration and resuspended in K_0 medium. After incubation for 3 min 0.5 mM-NEM was added (■). The K^+ content of the cells at the time of NEM addition was approximately 350 ng-ion (OD_{650} unit) $^{-1}$ in control cells (□) and was 15% and 30% lower in iodoacetate-treated cells of *Staph. aureus* and *Er. carotovora*, respectively.

NEM-elicited K^+ efflux by 73–84%. Thus the behaviour of the cells is consistent with the presence of a transport system analogous to KefC in these organisms.

The NEM-activation of K^+ efflux in *E. coli* can be reversed, in the absence of protein synthesis, by treatment of the cells with a reducing agent such as dithiothreitol (DTT) (Meury *et al.*, 1980; Bakker & Mangerich, 1982). This reversal arises from the breakdown of ESG to release glutathione (Elmore *et al.*, 1990) and from the release of glutathione from cellular dithiols (A. J. Lamb & I. R. Booth, unpublished data). We have found that a similar reversal of the effects of iodoacetate can be effected by incubation with DTT. Thus iodoacetate-treated cells of *Er. carotovora* and *Staph. aureus* were incubated with 10 mM-DTT for 10 min in the presence of chloramphenicol to inhibit new protein synthesis; the cells were then filtered and suspended in fresh incubation medium. Addition of NEM (0.5 mM) provoked immediate loss of K^+ similar to that shown in

Table 1. Formation of *S*-carboxymethylglutathione and *N*-ethylsuccinimido-*S*-glutathione in bacteria

The formation of *N*-ethylsuccinimido-*S*-glutathione and *S*-carboxymethylglutathione was measured as described previously (Elmore *et al.*, 1990); cells were labelled with *N*-ethyl[2,3- ^{14}C]-maleimide or iodo[2- ^{14}C]acetate and extracted with trichloroacetic acid. The acid-soluble pool was fractionated by thin-layer chromatography and the radioactive spots identified by autoradiography. Radioactive areas were removed from the plates and counted by liquid scintillation counting. All values are triplicate determinations and each experiment was repeated twice except those marked with an asterisk (*) which represent single determinations. ND, Not determined.

Organism	<i>S</i> -Carboxymethylglutathione [nmol (mg cells) $^{-1}$]	<i>N</i> -Ethylsuccinimido- <i>S</i> -glutathione [nmol (mg cells) $^{-1}$]
<i>Escherichia coli</i>	12.9 ± 0.1	4.8 ± 0.5
<i>Salmonella typhimurium</i>	9.7 ± 0.3	8.1 ± 0.6
<i>Enterobacter aerogenes</i>	7.4 ± 0.8	10 ± 0.6
<i>Erwinia carotovora</i>	8.8 ± 0.1	11 ± 0.7
<i>Pseudomonas aeruginosa</i>	3.7 ± 0.4	6.9 ± 0.6
<i>Rhodobacter sphaeroides</i>	21 ± 0.2	6.7 ± 0.4
<i>Azospirillum brasilense</i>	6.8 ± 0.6	3.1 ± 0.1
<i>Staphylococcus aureus</i>	3.4 ± 0.1	3.1*
<i>Bacillus cereus</i>	7.3 ± 0.2	ND
<i>Streptococcus faecalis</i>	1.3 ± 0.3	5.0*
<i>Streptococcus lactis</i>	0.3 ± 0.1	3.9 ± 0.6
<i>Lactobacillus casei</i>	1.8 ± 0.1	ND

Fig. 3 (data not shown). Similarly, in *P. aeruginosa* the prevention of NEM-activated K^+ efflux by iodoacetate could be overcome by treatment of the cells with DTT (data not shown).

Thus by these criteria the NEM-elicited K^+ efflux in these bacteria occurs via a KefC-type of transport system.

Generation of glutathione adducts

The activation of a KefC homologue requires the formation of the glutathione metabolites. The presence of glutathione in each of the species was investigated by assaying for the formation of *S*-carboxymethylglutathione when cells were incubated with iodoacetate. Metabolites were extracted from cells incubated with iodo[2- ^{14}C]acetate [1 mM final concentration; 54 μ Ci μ mol $^{-1}$ (2 MBq μ mol $^{-1}$) specific activity] with trichloroacetic acid and subjected to chromatography on PEI-cellulose plates (Elmore *et al.*, 1990). In each case a single major metabolite which had the same R_F value as *S*-carboxymethylglutathione (Elmore *et al.*, 1990) was detected by autoradiography and the amount of the metabolite was determined (Table 1). All of the Gram-negative species tested showed the formation of

S-carboxymethylglutathione, but the levels of the metabolite were very low in the Gram-positive species with the exception of *Staph. aureus* and *B. cereus* (Table 1). Similarly, when cells were incubated with *N*-ethyl-[2,3-¹⁴C]maleimide all the organisms able to form S-carboxymethylglutathione with iodoacetate formed ESG (Table 1). Thus all species that showed NEM-elicited K⁺ efflux possessed significant glutathione pools. There was no correlation between the rate of K⁺ efflux and the size of the glutathione pool (compare Table 1 and Fig. 2). However, the two streptococci and *B. cereus* possess glutathione but did not show NEM-elicited efflux, and thus must lack a KefC-type transport system.

Distribution of genes homologous to *kefC*

The presence of genes homologous to the *kefC* gene was investigated using Southern hybridization. Three probes were used to detect sequences homologous to *kefC* (Fig. 1). Probe A is a full length probe (approximately 2.1 kb) that encodes all the information needed for KefC activity. The 5'-end of the probe lies just upstream of the *kefC* gene sequence and includes approximately 200 bp of the downstream region (this extra sequence is non-coding DNA; Smith & Calvo, 1980). Probe B extends from the *Xho*I site 400 bp 5' to the start of the *kefC* gene and terminates at a *Sal*I site within the *kefC* gene and is thus lacking the coding region for the last 121 amino acids and the downstream region of probe A. Probe C carries the 0.5 kb *Sal*I–*Sal*I region encoding the last 121 amino acids of the KefC protein and the downstream non-coding region (Munro *et al.*, 1991). When DNA digested with *Pst*I was probed with probe A strong hybridization was observed for *E. coli*, *S. typhimurium* and *Ent. aerogenes*; the hybridization to *P. aeruginosa* and *Er. carotovora* DNA was very weak (Fig. 4). The single band observed with *E. coli* DNA was 7 kb as predicted from the restriction map of this region (Smith & Calvo, 1980). This suggests that the homology to the *kefB* gene is too weak to be detected by Southern hybridization. With the *S. typhimurium* DNA two bands of approximately 1.4 kb and 2.8 kb were observed in the *Pst*I-digested DNA. To determine whether this represented two homologous sequences or a *Pst*I site internal to the *kefC* gene, DNA digested with *Eco*RI was investigated. A single band of approximately 8 kb was observed in *Eco*RI digests of *S. typhimurium* DNA indicating that the two bands observed in the *Pst*I digest arose from digestion at an internal *Pst*I site. Two bands were also observed in the *Pst*I digest of *Ent. aerogenes* (approximately 1 kb and 2 kb; Fig. 4). However, the origin of these two bands was not investigated further. No hybridization was observed to DNA from Gram-

positive bacteria or from the other Gram-negative species.

Both *S. typhimurium* and *Ent. aerogenes* exhibited strong homology to *E. coli kefC* using probes A and B which cover the 5'-end of the gene, but only *S. typhimurium* exhibited strong hybridization to probe C (data not shown). This suggests that the 3'-end of the gene has diverged significantly more than the 5'-end.

These data suggest that although the KefC activity is conserved among unrelated Gram-negative bacteria there is little conservation of the DNA sequences. Further, even within the enteric group, the DNA sequence appears to have diverged significantly. No DNA sequence homology was detected by hybridization with any of the Gram-positive species even though *Staph. aureus* displayed KefC-type activity.

Discussion

The data presented confirm the presence of a KefC-class of transport system in a range of bacteria and thus suggests that the regulation of K⁺ efflux by glutathione is a widespread phenomenon. There are two other reports of organisms that possess this system; *Vibrio alginolyticus* (Nakamura *et al.*, 1986) and *Anacystis nidulans* (E. Padan, personal communication). DNA from *V. alginolyticus* does not hybridize to the *E. coli kefC* gene (T. Nakamura, personal communication). The system appears to be widespread among Gram-negative bacteria, but is absent from Gram-positive bacteria, with the exception of *Staph. aureus*. The system is also absent from *Saccharomyces cerevisiae*, although this organism does have significant pools of glutathione (unpublished data).

The lack of activity in most of the Gram-positive bacteria tested could have been explained by the previously noted failure of these organisms to synthesize significant quantities of glutathione (Fahey *et al.*, 1978). This objection was overcome in our work by growing the cells in complex media that contained glutathione. Thus many of the organisms accumulated glutathione from their surroundings and consequently the absence of KefC activity represents the lack of the relevant transport components.

The formation of ESG and S-carboxymethylglutathione are likely to be spontaneous events arising from the nucleophilic attack on NEM and iodoacetate, respectively, by glutathione. Other activators of the KefC system can only be synthesized through the intervention of the glutathione S-transferase (GST), for example 2,4-dinitrophenyl-S-glutathione formed from chlorodinitrobenzene (Elmore *et al.*, 1990). While it is known that GSTs are widespread in Gram-negative bacteria

Fig. 4. Detection of genes homologous to *E. coli* *kefC*. Southern hybridization was carried out as described in Methods. Approximately 3–5 µg DNA was loaded onto each track of the gel.

there does not appear to have been a systematic evaluation of their presence in Gram-positive bacteria (Piccolomini *et al.*, 1989). The role of GST in Gram-negative bacteria is not completely resolved since there are no mutants lacking this enzyme at the present time. However, mutants lacking glutathione, an essential co-substrate for GST, grow normally except when challenged with reactive chemical species such as NEM and iodoacetate (Apontowiel & Berends, 1975). Further, bacterial glutathione and GST activity have been implicated in reducing the toxicity of both 3-fluoro- and 3-chloro-2,4-dinitrobenzene (Summer & Goggelmann,

1980). This implies that while the GST function is not essential for growth and survival of bacterial cells it is essential for detoxification of reactive chemical species.

The absence of an obvious phenotype, other than loss of NEM-elicited K⁺ efflux, in *kefB* and *kefC* mutants of *E. coli* (Booth *et al.*, 1985) raised the possibility that these glutathione-regulated transport components have no significant role in cell physiology. The presence of this system in such a wide group of bacteria, illustrated by this study, suggests that the system has a function and that an understanding of the role of the system may come from a wider investigation of the metabolism of

glutathione and the physiology of mutants under conditions of stress associated with chemical challenge. Such studies are now under way in our laboratory.

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