

Role of calcium in the efflux system of *Escherichia coli*

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ABSTRACT

Efflux of antibiotics by *Escherichia coli* AG100 is performed by a variety of efflux pumps, ensuring survival of the bacterium in widely diverse media. At pH 5, efflux is independent of metabolic energy during the period of time the assay is conducted; at pH 8 it is totally dependent upon metabolic energy. Because calcium ions (Ca^{2+}) are important for membrane transport channels and the activity of ATPases that provide energy functions, the role of Ca^{2+} in the extrusion of an efflux pump substrate under conditions that challenge the bacterium was investigated. Real-time accumulation and efflux of ethidium bromide (EtBr) by *E. coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ (gal-uvrB) supE44] was determined by a semi-automated fluorometric method in the presence and absence of Ca^{2+} and agents that are known to inhibit access of calcium to enzymes that provide energy. Chlorpromazine (CPZ), an inhibitor of calcium binding to proteins (calcium-dependent enzymes), and ethylene diamine tetra-acetic acid (EDTA), a chelator of Ca^{2+} , increased accumulation and efflux of EtBr at pH 8 but not at pH 5. Ca^{2+} reverses these effects when the assay is conducted at pH 8. In conclusion, the activity of the efflux pump system of *E. coli* is dependent upon metabolic energy at pH 8. Because at pH 8 hydrolysis of ATP is favoured and contributes protons for activation of the AcrAB–TolC efflux pump, CPZ is suspected of having its effects on accumulation/efflux of EtBr by indirectly affecting ATPase activity that is dependent upon Ca^{2+} .

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1. Introduction

The cell envelope of Gram-negative bacteria consists of an outer cell wall, a periplasmic space and the plasma membrane. Permeability of the cell envelope to antibiotics involves the interaction between antibiotics and components of the cell envelope. Antibiotics that are hydrophobic or contain hydrophobic components may penetrate the lipopolysaccharide (LPS) layer of the outer cell wall, which is a barrier to hydrophilic antibiotics [1,2]. Hydrophilic antibiotics gain access to targets beyond the outer cell wall via conduits that begin at the surface of the cell and end at the plasma membrane. These conduits, or porins, are selective diffusion channels that can be highly specialised for a given molecule, such as maltose, or allow diffusion of many unrelated compounds to reach the periplasmic space and even the cytoplasm [3]. Noxious substances that gain access to the periplasm or cytoplasm may be recognised and extruded to the outside of the cell by means of efflux pumps that are able to recognise a wide range of different molecular

structures [2]. The degree of permeability to a given noxious agent such as an antibiotic is therefore a balance between LPS, general porins and the activity of the main efflux pump of the organism [2,3].

The majority of Gram-negative clinical isolates that exhibit a multidrug-resistant phenotype owe their multidrug resistance to overexpressed efflux pumps [2,4]. In recent years, many compounds have been shown to be effective efflux pump inhibitors (EPIs); however, with the exception of some naturally occurring compounds [5,6], a derivative of an antibiotic [7], a designed EPI [8] and some compounds used for therapy of psychosis [8–11], most EPIs show serious cytotoxicity [2]. Because phenothiazines have been shown to express EPI activity against overexpressed efflux pumps of Gram-negative bacteria [8–13], they have been used in our investigations.

Phenothiazines have been shown to non-specifically inhibit enzymes that are involved in metabolism [14] as well as specifically by inhibiting binding of calcium to calcium-dependent enzymes [15]. The efflux pumps of Gram-negative bacteria, which are involved in the extrusion of a variety of non-related antibiotics, obtain their energy from the proton-motive force (PMF) [16]. The PMF is maintained by the metabolic activity of the bacterium and is the result of protons generated from the hydrolysis of ATP that are transported via channels to the surface of the bacterium,

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distributed on its surface and bound to reactive groups of the outer cell wall (LPS and $-\text{CO}_2^-$) [17–19]. The difference in proton concentration between the surface of the cell and the medial side of the plasma membrane establishes a pH gradient, resulting in an electrochemical potential that drives protons from the surface to the periplasm of the cell [17–19]. The protons that are mobilised via channels to the periplasm are used by efflux pump(s) of the bacterium for extrusion of antibiotics prior to reaching their intended targets. Although it was initially proposed that these protons activate the efflux pump, it was demonstrated that the protons create a localised pH that is acidic enough to promote the dissociation of the substrate from the transporter component of the pump [19–22]. Binding of protons on the surface of the bacterium results in a pH that is 2 to 3 units lower than that of the bulk medium [19]. Consequently, when the pH of the medium is near or slightly above 7, protons do not readily dissociate into the bulk medium and therefore the PMF is maintained [19,20]. However, if the bacterium is challenged with a noxious substance, more protons are needed in the periplasm and, because the concentration of protons at the surface of the cell must be maintained if the PMF is to be maintained, additional protons must be made available by metabolic activity for subsequent transport to the cell surface. Therefore, when the pH of the medium is near neutral and the cell is exposed to a noxious agent for a prolonged period of time, any interference with the process of metabolic energy would be expected to decrease the effectiveness of the efflux pump [16]. It is under these conditions that phenothiazine is expected to indirectly express its effects on the activity of the efflux pump and hence render the bacterium increasingly susceptible to the antibiotic to which it was initially resistant as a consequence of an overexpressed efflux pump. Because the targets of phenothiazines are Ca^{2+} -dependent, we have studied the effect of phenothiazines on the accumulation and efflux of ethidium bromide (EtBr) as well as the role of calcium on modulation of that effect.

2. Materials and methods

2.1. Materials

Mueller–Hinton and trypticase soy in powder form for the preparation of broth and agar were purchased from Oxoid Ltd. (Basingstoke, UK). Phosphate-buffered saline (PBS), glucose, EtBr, chlorpromazine (CPZ), CaCl_2 and ethylene diamine tetra-acetic acid (EDTA) were purchased from Sigma-Aldrich Quimica S.A. (Madrid, Spain).

2.2. Bacteria

Wild-type *Escherichia coli* K-12 AG100 strain [argE3 thi-1 rpsL xyl mtl Δ (gal-uvrB) supE44] [22] was kindly provided by Hiroshi Nikaido (Department of Molecular and Cell Biology and Chemistry, University of California, Berkeley, CA).

2.3. Methods

Detection of efflux pump activity in *E. coli* AG100 was conducted by a semi-automated fluorometric method as described previously [16,23]. Briefly, this method follows the real-time accumulation of EtBr inside the cell with the aid of a Rotor-GeneTM 3000 thermocycler (Corbett Research, Sydney, Australia) programmed for 30–40 cycles of 1 min each (ca. 30–40 min) at a constant temperature of 37 °C. Bacteria were grown in Mueller–Hinton broth until they reached an optical density at 600 nm (OD_{600}) of 0.6. Cells were centrifuged, washed twice with PBS (pH 8) and the OD_{600} was adjusted to 0.6 with PBS (pH 8). Then, aliquots of 0.045 mL were transferred to microtubes of 0.2 mL volume.

2.3.1. Accumulation assay

Immediately, aliquots of 0.045 mL of saline (pH 8) containing EtBr and glucose to yield final concentrations of 1 mg/L and 0.4%, respectively, with and without varying concentrations of CPZ with and without varying concentrations of Ca^{2+} , and varying concentrations of EDTA with and without varying concentrations of Ca^{2+} , were added to the 0.2 mL tubes containing 0.045 mL of cells in PBS (pH 8). The instrument was started and accumulation of EtBr (amount of relative fluorescence emitted) was followed for 25 min. It is important to note that although accumulation of EtBr and the effects of CPZ, EDTA, etc. could be increased to very high levels, it is critical that accumulation does not reach levels that result in the binding of EtBr to DNA and hence it would no longer be subject to efflux [11].

2.3.2. Efflux assay 1

Fluorescence of samples containing 0.045 mL of cells in PBS (pH 8), no glucose and EtBr to yield a final concentration of 1.0 mg/L was followed for 40 min at 37 °C, the instrument was paused and 0.045 mL of PBS (pH 8) containing glucose to yield 0.4% with and without varying concentrations of CPZ alone and in combination with varying concentrations of Ca^{2+} , and with varying concentrations of EDTA alone and in combination with varying concentrations of Ca^{2+} were added. The instrument was restarted and fluorescence was followed for an additional 10 min. To demonstrate the role of glucose in the efflux assay, a set of controls that lacked glucose and any other agent were added to the accumulation phase of the assay and, after 40 min, the instrument was paused and 10 μL of PBS (pH 8) containing glucose to yield 0.4% final concentrations was added. Fluorescence was followed for 10 min.

2.3.3. Efflux assay 2

EtBr accumulation by 0.090 mL of cells in PBS (pH 8) with EtBr and glucose to yield final concentrations of 1.0 mg/L and 0.4%, respectively, with and without concentrations of 20 mg/L CPZ and 5 mM EDTA was followed at 37 °C over 10 min using a Rotor-GeneTM 3000 thermocycler. The instrument was paused and aliquots of 0.010 mL of PBS (pH 8) containing 0.4% glucose with and without 5 mM Ca^{2+} were added. The instrument was restarted and fluorescence was followed for 20 min. It is important to note that for the accumulation period in the presence of CPZ, a low concentration of CPZ (20 mg/L) was used in order to ensure that accumulated EtBr was subject to efflux, i.e. not bound to DNA [11].

The experiments described above were conducted as sets. All experiments were conducted at least three times. Data presented in the figures of the text represent typical data obtained from any one set of experiments.

3. Results

Phenothiazines such as CPZ and thioridazine (TZ) inhibit the binding of Ca^{2+} to enzymes involved in furnishing energy from the hydrolysis of ATP [24]. Ca^{2+} plays a crucial role in the biochemical pathways of the cell and is of extreme importance for cell signalling, membrane transport channels and also for the activity of some types of ATPases [25,26]. Because the phenothiazines CPZ and TZ have major effects on the accumulation and efflux of EtBr at pH 7 [23], and these effects are modified by metabolic energy, the role of Ca^{2+} in the modulation of EtBr accumulation and efflux was evaluated. Because CPZ had a greater effect on accumulation of EtBr by *E. coli* AG100 strain than other phenothiazines such as TZ (data not shown), CPZ was used in this study of the role of Ca^{2+} on the efflux of EtBr by this strain. Moreover, because EDTA is a chelating agent and has the ability to 'sequester' metal ions such as Ca^{2+} and Fe^{3+} , this agent was also used to study the role of Ca^{2+} .

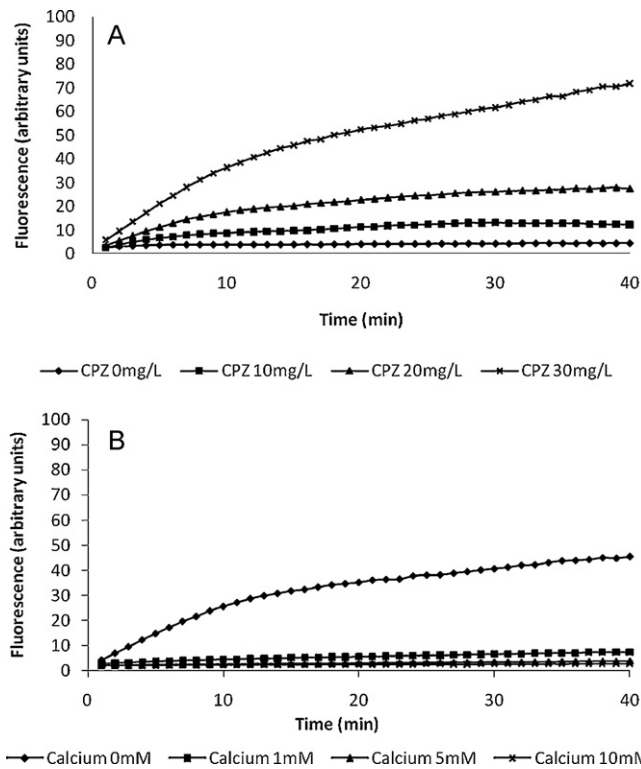


Fig. 1. (A) Effect of chlorpromazine (CPZ) on accumulation of ethidium bromide (EtBr) and (B) modulation of that effect by calcium ions (Ca^{2+}). (A) EtBr accumulation by *Escherichia coli* AG100 strain with EtBr at 1 mg/L, glucose at 0.4% and CPZ to yield final concentrations of 0.0, 10, 20 and 30 mg/L. (B) EtBr accumulation by *E. coli* AG100 strain with EtBr at 1 mg/L, glucose at 0.4%, CPZ at 20 mg/L and calcium chloride to yield final concentrations of 0.0, 1, 5 and 10 mM.

As shown in Fig. 1A, increasing concentrations of CPZ promote comparable increases in the amount of relative fluorescence, which is associated with increased accumulation of EtBr by *E. coli* AG100 at pH 8, as demonstrated previously at pH 7 [23] and considered to result from inhibition of the efflux pump of the organism [23]. Since CPZ is known to inhibit access to Ca^{2+} of Ca^{2+} -dependent enzymes [24], the potential modulating role of Ca^{2+} on the activity of CPZ on the efflux pump system was investigated. As seen in Fig. 1B, the activity of CPZ on the accumulation of EtBr can be completely abolished by addition of 5 mM Ca^{2+} to the assay. Ca^{2+} plays a role in the mechanism by which the efflux pump system functions; as seen in Fig. 2A, addition of 5.0 mM and 10 mM EDTA to the assay promotes accumulation of EtBr, which can be reversed by the simultaneous presence of Ca^{2+} as seen in Fig. 2B(i) and (ii). That indeed it is the efflux pump that is affected by the presence of CPZ, as seen in Fig. 3, the accumulation of EtBr that takes place in glucose-free PBS and subsequent efflux of EtBr can be modulated by the addition of glucose, which affords complete efflux for the duration of the assay; by addition of glucose and CPZ at two concentrations that do not affect replication of the organism (data not shown), each one of which inhibits efflux of EtBr in a concentration-dependent manner; and by addition of glucose and Ca^{2+} to the CPZ-containing PBS tubes resulting in the abolishment of the effect of CPZ alone on the efflux of EtBr.

Although the above results prove that Ca^{2+} plays an important role in the efflux machinery of *E. coli* reference strain AG100, the question of whether the initial effects of CPZ on the efflux pump system of *E. coli* AG100 are reversible required study. As shown by Fig. 4A, prior exposure of the reference strain to CPZ in PBS (pH 8) containing glucose and EtBr results in marked accumulation of EtBr. Addition of Ca^{2+} reduces the accumulation effects of CPZ, but not

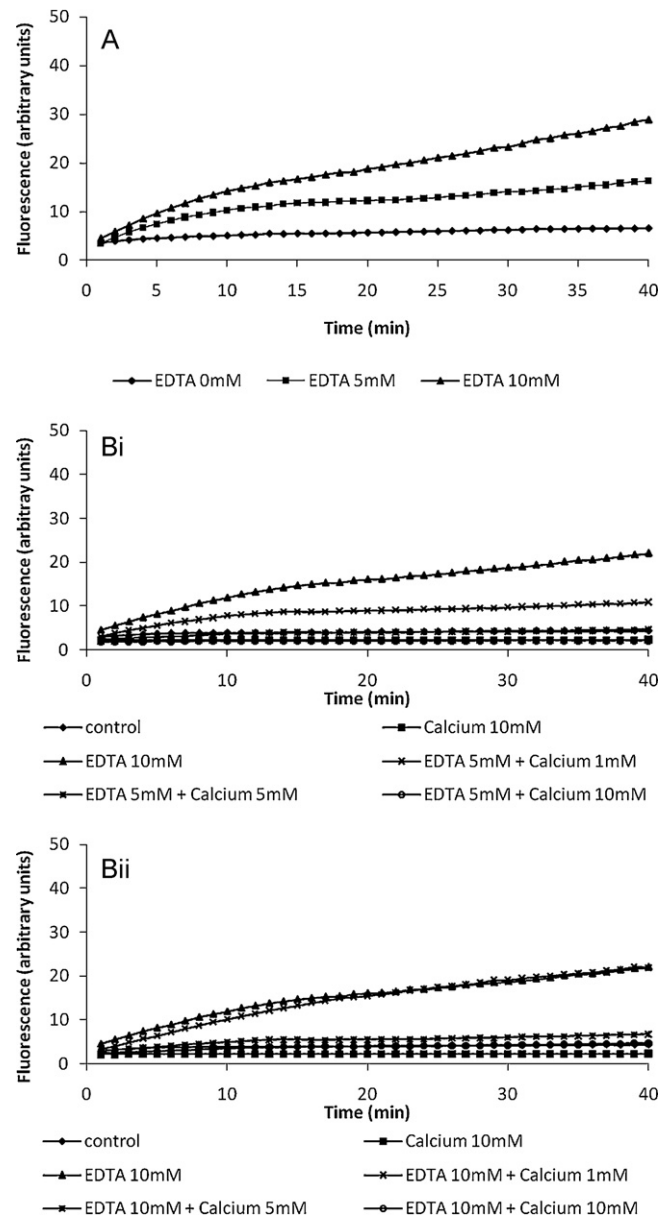


Fig. 2. (A) Effect of ethylene diamine tetra-acetic acid (EDTA) on the accumulation of ethidium bromide (EtBr) and (B) modulation of that effect by calcium ions (Ca^{2+}). (A) EtBr accumulation by *Escherichia coli* AG100 strain with EtBr at 1 mg/L, glucose at 0.4% and EDTA to yield final concentrations of 0.0, 5 and 10 mM. (B, i) Ca^{2+} modulation of the effects of 5 mM EDTA: EtBr accumulation by *E. coli* AG100 strain with EtBr at 1 mg/L, glucose at 0.4%, EDTA at 0.0, 5 and 10 mM and calcium chloride to yield final concentrations of 0.0, 1, 5 and 10 mM. (B, ii) Ca^{2+} modulation of the effects of 10 mM of EDTA: EtBr accumulation by *E. coli* AG100 strain with EtBr at 1 mg/L, glucose at 0.4%, EDTA at 0.0 mM and 10 mM and calcium chloride to yield final concentrations of 0.0, 1, 5 and 10 mM.

when Ca^{2+} and EDTA are added. These results suggest that addition of Ca^{2+} restores the efflux capacity of the cell that had been reduced by the presence of CPZ. When CPZ and EDTA are both present in the accumulation period (Fig. 4B), the accumulation is significant. Addition of Ca^{2+} at a concentration that would reverse the effects of CPZ alone is not sufficient to reverse the effect produced by the presence of CPZ and EDTA. Addition of glucose does not obviate the CPZ effect on efflux. The initial effects of CPZ and EDTA, namely the level of EtBr accumulated by the cell, can be maintained as long as CPZ and EDTA remain without the addition of Ca^{2+} . When CPZ and Ca^{2+} were present in the accumulation period, addition of EDTA promotes accumulation as observed in Fig. 4C.

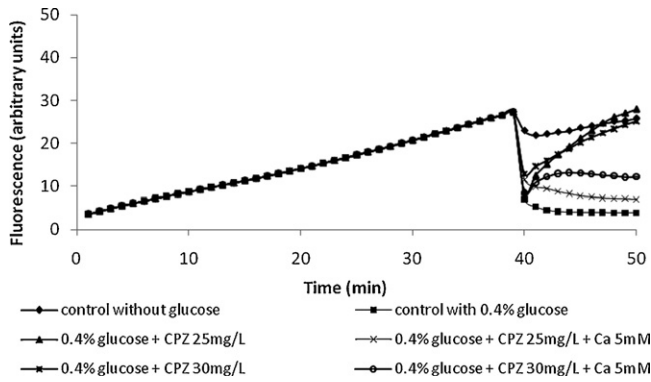


Fig. 3. Effects of chlorpromazine (CPZ) with and without Ca²⁺ on efflux of ethidium bromide (EtBr) by *Escherichia coli* AG100 strain with EtBr at 1 mg/L. Addition of CPZ at 25 mg/L and 30 mg/L, with and without 5 mM calcium chloride.

4. Discussion

Calcium is important for signalling [25] and for activating genetic systems [26] as well as for a wide variety of metabolic and energy-deriving pathways within the cell. Central to these pathways are ATPases that hydrolyse ATP and furnish protons for the activation of ABC-type transporters.

CPZ is noted for its inhibitory effects on the binding of Ca²⁺ to enzymes that are involved in the provision of energy (ATP) [14]. The results of this study demonstrate that CPZ, a phenothiazine noted

for its inhibitory effects on the activity of efflux pump systems [13], also promotes the accumulation of a bacterial efflux pump substrate (EtBr) that results from the inhibition of efflux. Because the inhibitory effects of CPZ on efflux of EtBr can be obviated by the addition of Ca²⁺, the role of calcium in efflux by a bacterium is demonstrated for the first time. The role of Ca²⁺ on efflux is further demonstrated by the addition of EDTA, a chelator of divalent cations; the inhibition of efflux by EDTA, as is the case for CPZ, is also obviated by the addition of Ca²⁺. Because the effects of CPZ on accumulation can be obviated by the addition of Ca²⁺, the inhibitory effects of this phenothiazine on the efflux pump system of the bacterium are fully reversible.

The effects of a phenothiazine and, for that matter, by agents that others have claimed to be inhibitors of efflux by bacteria, are not evident at pH 5 regardless of whether metabolic energy is present [16]. It has therefore been postulated that for the duration of the assay period, conditions that favour maintenance of the PMF are sufficient to maintain efflux [16]. Unlike the situation at pH 5, when the assay is conducted at pH 8 maintenance of the PMF requires metabolic energy [16]. This requirement reflects conditions at the surface of the cell where protons in the form of hydronium ions resulting from metabolic activity are stored, and that due to the dissociation of these ions into the bulk medium at pH 8 must be replenished via metabolic activity. Hence, the dependency on glucose for efflux at pH 8 is evident, whereas at pH 5 for the full duration of the assay glucose is not required for maintenance of efflux.

The assay system employed for the assessment of efflux does not contain Ca²⁺. Because medium that is devoid of Ca²⁺ as well as

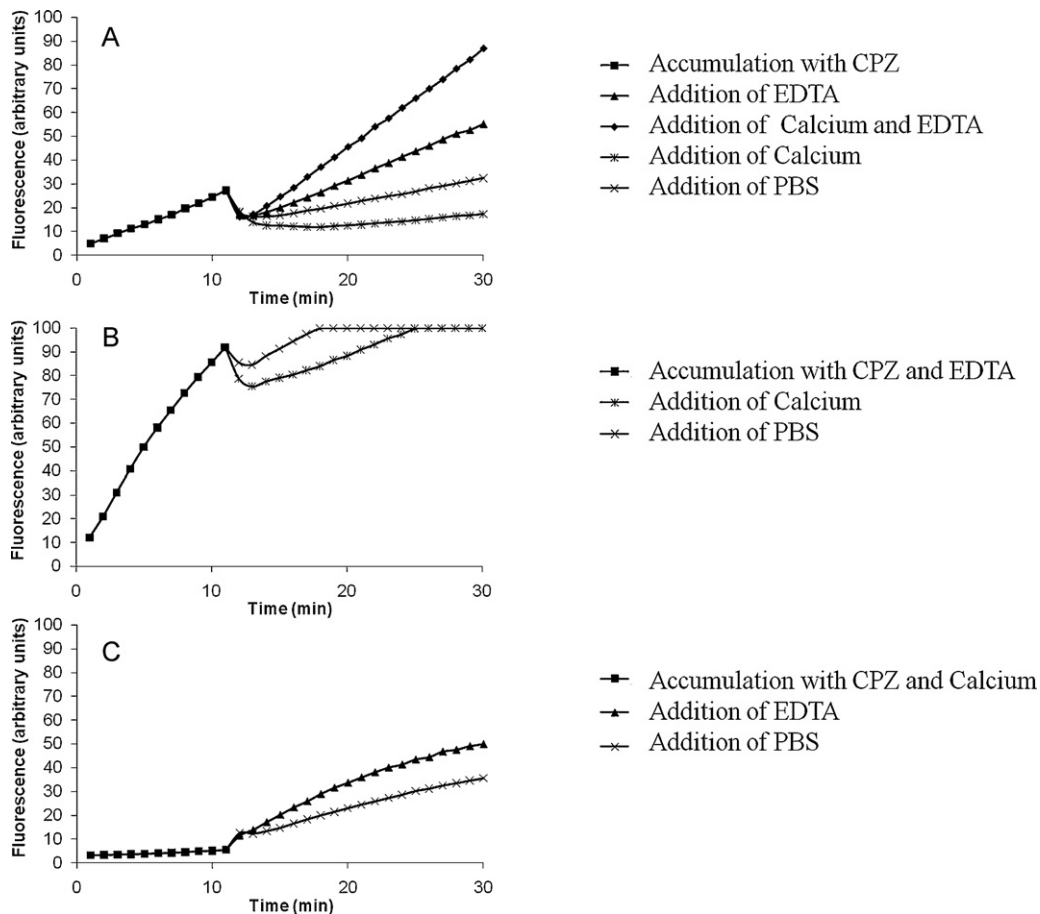


Fig. 4. Effect of addition of ethylene diamine tetra-acetic acid (EDTA), calcium, and EDTA with calcium on efflux of ethidium bromide (EtBr) that had accumulated due to CPZ alone and to CPZ and EDTA. Note: accumulation phase (first 10 min) conducted as described in Section 2.3.3 and the agents present in the accumulation phase identified by the legends of each figure. The instrument was paused, the agents were added as identified in the legends of the figures, and the instrument restarted. Efflux of EtBr was followed for 20 min.

other divalent and trivalent ions does not support bacterial growth (replication), we assume that Ca^{2+} that is subject to recycling for binding to calcium-dependent enzyme functions is inhibited by CPZ. Because at pH 8 CPZ has a strong inhibitory activity on the efflux system of the bacterium, as shown by the current study, we further assume that this activity is the result of inhibition of enzymes involved in the furnishing of energy, a process that is dependent upon Ca^{2+} . If this assumption is correct, CPZ cannot be considered to be an inhibitor of an efflux pump system. It should be noted that metabolic energy does not reverse the inhibitory effects of CPZ on the efflux pump system of the bacterium.

The activity of efflux is now considered to be dependent upon metabolic energy [16]. However, it is doubtful that the generation of protons from metabolic energy energises an efflux pump. The dissociation of a substrate is pH dependent [21] and at pH near neutral dissociation of the substrate is very very slow, and hence the activity of the efflux pump is also very very slow. If the efflux pump is to function at near neutral pH, the internal part of the trimeric transporter of an RND efflux pump such as AcrAB–TolC of *E. coli* must bind the substrate at neutral pH, where the association constant is high, and must then release it to the TolC channel. To do this, the internal pH of the tripartite transporter must be rapidly lowered. It is therefore postulated that immediately after the recognition and binding of a substrate (EtBr), the internal pH of the transporter is decreased by passage of the hydronium ion through the cavity containing the substrate [20,27].

This promotes release of the substrate, which is then carried by the flow of the hydronium ion from the transporter to TolC and then to the outside of the cell. These are then distributed on the surface of the cell and subject to re-use for proton force-dependent transport activities as suggested by others [17–19,28]. It remains for studies currently on-going to determine whether the above sequence of events actually take place in the efflux machinery of a bacterium responding to the presence of an obnoxious agent in the environment.

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Competing interests: None declared.

Ethical approval: Not required.

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