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Stress induced alterations in the outer membrane of *Escherichia coli K-12* strain

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Abstract

In the present study, gram-negative (*Escherichia coli K-12*) bacterial biomembrane involvement was studied in the presence of modulating factors such as EDTA, Mg^{+2} ions and EDTA and Mg^{+2} ions in combination. The release of proteins and their involvement during the transport of β-Lactams namely Ceftriaxone and Cefazolin were also studied. The broader applications of Ceftriaxone for pharmaceutical implications offer greater advantage as compared to pre-existing β-Lactams. Due to the availability of more signal molecules in the membranes there is enhanced toxicity at 5 mM EDTA concentration, and easy entrapment of antibiotics, thus enhanced sensitivity levels. A concentration of 15 mM Mg^{+2} ions was found to be toxic for *E.coli* whereas it exhibited luxuriant growth with decreasing Mg^{+2} ion concentration under antibiotic stress. On the contrary, when 5 mM EDTA is treated in combination with Mg^{+2}, it attributed reduced signals available on the membrane surface therefore, reduced drug sensitivity. To identify the involvement of specific proteins and to know the site of proteins released which are directly or indirectly involved in transport of antibiotics across the biological membrane, the protein release was monitored from intact cells, as well as, membrane vesicles derived from *E.coli* cells and studied upto a level of molecular weight determination and measured by using a high-pressure liquid chromatography (HPLC). The study confirms the induction of certain stress signal proteins from the outer membrane, thereby rendering the bacteria more susceptible to therapy.

Keywords:- *HPLC- High pressure liquid chromatography, CEF- Ceftriaxone Sodium, CEZ- Cefazolin Sodium, Outer Membrane Proteins- OMPs*

Introduction

Outer membrane protein A (OmpA) is located in the membrane of *Escherichia coli* and other gram-negative bacteria and plays a multifunctional role in bacterial physiology and pathogenesis.(Alfredo *et al.*, 2006). Also, many new outer membrane proteins have recently been identified by proteomics techniques in *Escherichia coli* (Marani *et al.*, 2006). In the outer membrane of gram-negative bacteria, the porins, are present in large amounts and form water-filled channels that permit the diffusion of small hydrophilic solutes across the membrane (Nikaido, 2003; Nikaido, 1994; Nikaido, 1985; Nikaido and Vaara, 1985) including bacterial nutrients and antimicrobials. The permeability of outer membranes of gram-negative bacteria to hydrophilic compounds is mostly due to presence of porin channels (Dela Vega and Delcour, 1995). Since β-Lactam antibiotics penetrate the outer membrane of gram-negative bacteria, resistance could also be caused by loss or deficiency of specific porins that reduce the outer membrane permeability to β-Lactam antibiotic. This might be an important factor in mediating β-Lactam resistance in multidrug *E.coli* (Nikaido, 2003). The Cephalosporins have been known to induce stress signal proteins, from the outer membrane, inhibiting cross-linking step of peptidoglycan biosynthesis in the cell wall of *E.coli* thereby rendering the bacteria more susceptible to therapy (Russel and Chopra, 1990). β-Lactams

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binding to these PBP's may be eliminated by the action of 3 enzymes, *viz.*, β-Lactamases, the acylases and the esterases. Bacteria may become more resistant to these β-Lactams by producing altered transpeptidases (PBP's) with greatly reduced affinity for the binding of these antibiotics (Nikaido *et al.*, 1990).

Materials and Method

Escherichia coli K-12, wild type strain was procured from IMTECH,Chandigarh. Cultures were grown on Nutrient Agar at 37 °C±1and stored at 4 °C. Ceftriaxone sodium (CEF) and Cefazolin Sodium (CEZ) was purchased from Aristo pharmaceutical lndia Ltd. (M.P.).

E.coli was grown for growth studies on Spencer and Guest (1973) media, pH 6.9. Incubation was carried out at 37°C±1 in a thermostatically controlled orbital shaker (Labline model No. 3521) under aerobic conditions with a platform rotation of 180 rev min^{-1}.

Growth was monitored under various conditions spectrophotometrically (Spectronics-20 Bausch and Lomb) at 550nm.

For transport and protein release studies, membrane vesicles were prepared by Koning and Kaback (1973) with some modifications. Membrane protein analysis was carried out by Warburg and Christian (1941) method. The qualitative study of protein release was done by HPLC (Waters, USA) at 4,000 psi pressure, at absorbance of 280 nm. The solid phase was Protein-PAK column (Waters,USA).

Results and Discussion

A highly significant and negative correlation between concentration of CEF and CEZ with percent survival was found to be r = -0.646 (pd" 0.02) and r = -0.757 (pd" 0.02) respectively. *E.coli K-12* wild type strain was found to be ten times sensitive to CEF (LD_{50} of 0.005 ppm), than with CEZ (LD_{50} of 0.05 ppm) (Fig. 1). This high sensitivity of *E.coli* to CEF as compared to CEZ was attributed to the high Na^+ ion concentration outside *E.coli* (Na^+ ion

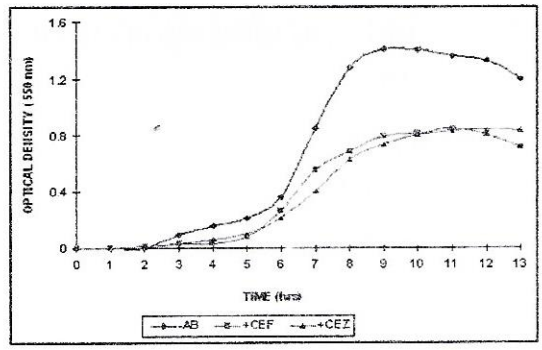

Fig. 1: Effect of LD_{50} concentrations of CEF and CEZ on the growth of *E.coli*

content of CEF-Na^+ ion, 83 mg/gm) cell wall as compared to its interior, leading to Na^+-H^+ ion antiporter activity and thus dissipation of pH gradient, ultimately interrupting the pmf and increase in the CEF uptake through its porins. Loss of porin-mediated resistance mechanism against cephalosporin has been observed among the multidrug resistant *E.coli* (Ananthan and Subha, 2005). In relation to sensitivity of *E.coli* cells to β-Lactams in the presence of varying H^+ ion concentrations, the protein thus, obtained in suspension medium shows variations with respect to time of incubation. The protein content present in this suspension medium (Tris-HCl Buffer), in the presence of β-Lactams as well as the release of proteins under antibiotic stress shows variations with respect to time of treatment (Fig. 2a, 2b).

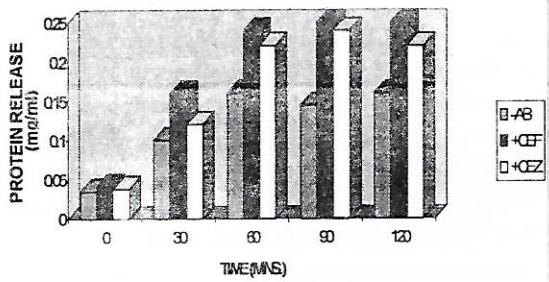

Fig. 2a: Protein release from intact cells of *E.coli* at varying time intervals

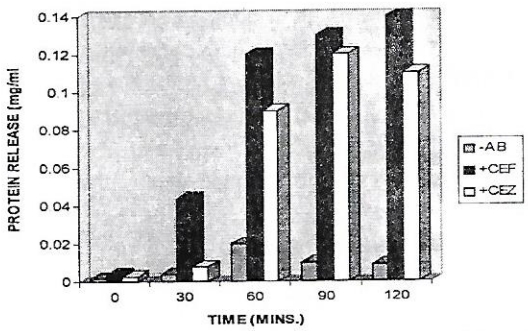

Fig. 2b: Protein release from membrane vesicles of *E.coli* at varying time intervals

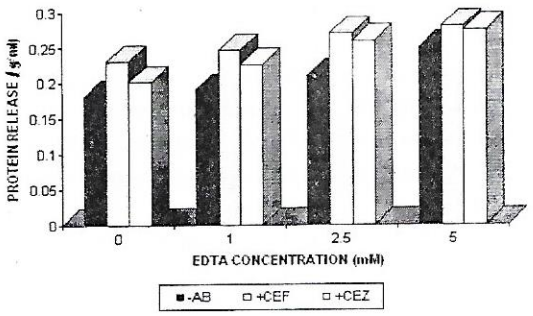

Fig. 3a: Protein release from intact cells of *E.coli* in presence of varying conc. of EDTA

Bacterial adaptation to various environmental stresses has been extensively investigated (Yohannes *et al.*, 2004; Yohannes *et al.*, 2005). Chelation property of EDTA, thus, facilitates antibiotic transport, thereby resulting in antibiotic toxicity (Yamada *et al.*, 1978). It has also been demonstrated that the effect of Mg^{+2} ions at high concentrations include reduction in the growth rate, extension of lag phase and perturbation in morphology and physiology (Hughes and Poole, 1989). The cell extract of *E.coli* exhibited a significant degradation of EDTA only in the presence of Fe^{+3} ion. There have been some reports regarding the interaction of non-toxic metals with toxic metals or metal chelators diminishing or enhancing the toxicity of the latter by the former. The ion antagonism has been studied but not sufficiently. Leive (1968) found that the concentration of Na^{+} ion and K^{+} ion do not affect EDTA action but the divalent cation like Mg^{+2} ion prevent its action. Attempts to revive EDTA-treated cells with cation such as Mg^{+2} ion have met with slight success.

It was observed that with varying concentrations of EDTA (1.00, 2.50 and 5.00 mM), antibiotic-treated cells were inhibited more than normal cells. The protein release in CEF-treated cells along with 1.00, 2.50 and 5.00 mM EDTA concentrations was 0.245, 0.270 and 0.280 µg/ml; whereas with CEZ-treated cells was observed as 0.225, 0.260 and 0.275 µg/ml respectively, after 60 min of incubation, as illustrated in Fig. 3a.

These observations were found consistent with the existing evidence (Matzushita *et al.*,1978). The enhanced protein release with higher concentration of EDTA explained the pronounced activity of antibiotics against the EDTA treated cells at higher concentration. The effect of varying concentrations of EDTA on the membrane vesicles of *E.coli* is illustrated in Fig. 3b.

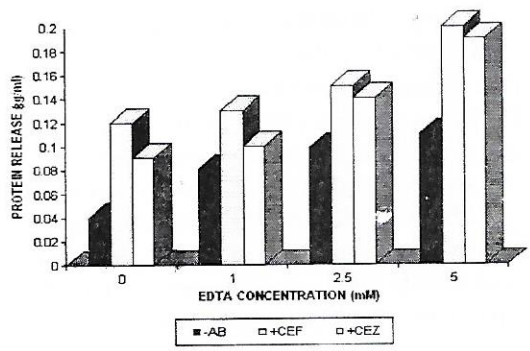

Fig. 3b: Protein release from membrane vesicles of *E.coli* in the presence of varying conc. of EDTA

In the presence of 1.00 mM EDTA, the release of protein was quite less *i.e.*, 0.13 and 0.10 µg/ml with CEF and CEZ, respectively in comparison to 2.50 mM and 5.00 mM EDTA (in combination with antibiotics) releasing 0.15 and 0.20 µg/ml in the presence of CEF; whereas, 0.14 and 0.19 µg/ml in the presence of CEZ respectively, in the medium after 60 mins of incubation. In contrast to that, the

inhibition of 5.00 mM EDTA was more pronounced than 1.00 mM EDTA in membrane vesicles of *E.coli*. The less quantity of protein released with 1.00 mM EDTA supports the earlier observations that in growing cultures of *E.coli*, 5.00 mM EDTA along with cephalosporins showed maximum inhibition of growth.

In case of intact cells, the amount of protein released in the presence of 5.00 mM EDTA was four-fold greater than the protein from membrane vesicles derived from gram-negative bacteria. It is very obvious that outer membrane comprises of low protein content in contrast to intact cells which has all the cellular proteins in combination with structural component, as well as, functional components such as enzymes. But with intact cells, as well as, membrane vesicles the speciation of proteins differs with respect to their molecular size as apparent from the Fig. 6 (a), (b). As far as molecular size is concerned, EDTA with cephalosporin resulted in release of similar group of proteins which had molecular size more than 67.00 kD. In case of EDTA also, peak C at 5 min was very prominent and differs in concentration reflecting high peak in case of EDTA and CEF, in contrast to EDTA and CEZ; after 60 min of incubation. In all the cases, peak height of the proteins were greater in the presence of CEF and CEZ. It is evident from growth, as well as, enzyme studies, that gram-negative cells were more sensitive to CEF treatment in contrast to CEZ as a counterpart. EDTA although potentiated the cephalosporin toxicity by many folds in case of CEF than CEZ. It is clear from the figure that the protein leaked out at 10 min run was at much higher conc. in case of CEF than CEZ. The protein release was found to be directly dependent upon the increasing concentration of EDTA from the cell. The amount of cellular protein, thus calculated by carrying a difference between protein release from intact cells and membrane vesicles shows a constant trend with a similar concentration at 1.00, 2.50 and 5.00 mM concentration, in case of both the cephalosporins. However, the difference in toxicity can be then considered as involvement of OM proteins exclusively in conferring sensitivity towards antibiotics.

In *E.coli*, when intact cells were treated with varying concentrations of Mg^{+2} ions, it was observed that the extent of protein release was quite higher as the concentration of Mg^{+2} ions was increased . The protein release from the cell wall of *E.coli* when treated with 1.00 mM, 5.00 mM, 10.00 mM and 15.00 mM Mg^{+2} ion was observed as 0.160, 0.175, 0.225 and 0.247 µg/ml; in the absence of antibiotics after 60 min of incubation. Thus, increasing concentration of Mg^{+2} ions had released more proteins. The inhibitory effects induced by high Mg^{+2} ion concentration which release more protein were explained in terms of stress-induced injury of OM. On the other hand in the presence of antibiotics, 15.00 mM Mg^{+2} ions drastically inhibits the transport of antibiotics, therefore resulting in fewer protein release. The protein release at 1.00, 5.00, 10.00 and 15.00 mM Mg^{+2} ions in the presence of CEF was found to be 0.229, 0.225, 0.215 and 0.190 µg/ml; whereas, with CEZ was observed as 0.202, 0.206, 0.20 and 0.185 µg/ml respectively after 60 min of incubation (Fig. 4a). However, the protein release from the membrane vesicles (OM) of *E.coli* was observed as 0.042, 0.05, 0.072 and 0.097 µg/ml when the cells were suspended in the presence of 1,5,10 and 15.00 mM Mg^{+2} ion concentration respectively.

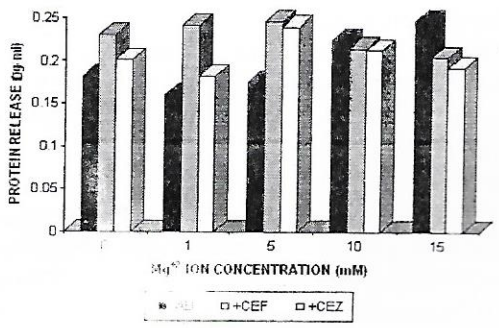

Fig. 4a: Protein release from intact cells of *E.coli* with and without antibiotics in presence of varying concentrations of Mg^{+2} ions

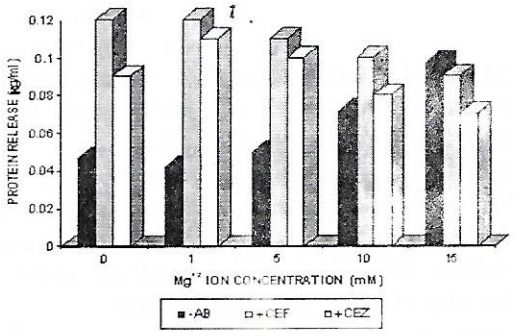

Fig. 4b: Protein release from membrane vesicles of *E.coli* in presence of varying conc. of Mg^{+2}

after incubation period of 60 mins (Fig. 4b). The variation in protein release at varying concentrations of Mg^{+2} ions could be attributed to the differential behaviour of Mg^{+2} ions at different concentrations i.e., at lower concentration Mg^{+2} ions acted as a potentiator and a stabilizing agent of the membrane while at higher concentrations they are inhibitory. With β-Lactams, the contrast behaviour of Mg^{+2} ions has been observed in relation to protein release.

At MIC_{50} conc. of CEF and CEZ, the protein released in the medium when the cells were suspended with 1.00, 5.00, 10.00 and 15.00 mM Mg^{+2} ion concentrations was observed as 0.12, 0.11, 0.10 and 0.09 µg/ml with CEF, whereas with CEZ, the protein release was 0.11, 0.10, 0.08 and 0.07 µg/ml, when the aliquots were drawn after 60 min of incubation. This might be due to the fact that the antibiotic accessibility to the inside of the cell is reduced at higher concentration of Mg^{+2} ions which releases fewer proteins at high concentrations (15.00 mM Mg^{+2} ions).

The effect of β-Lactams on the protein release of *E.coli* in the presence of EDTA as well as, Mg^{+2} ions have been studied. The extent of protein release was less when intact cells were suspended in 15 mM Mg^{+2} (in combination with 5 mM EDTA) than in 1.00 mM (in combination with 5.00 mM EDTA), after 60 min of incubation. The protein released in the medium when cells were suspended in 1.00, 5.00,10.00 and 15.00 mM Mg^{+2} ion concentration (each in combination with 5.00 mM EDTA) was observed as 0.250,0.242, 0.234 and 0.224 µg/ml respectively, in the presence of CEF, whereas protein release was 0.234, 0.241, 0.240 and 0.238 µg/ml respectively, in the presence of CEZ; after 60 min of incubation (Fig. 5a).

This must be attributed again to the differential behaviour of different concentrations of Mg^{+2} ions in relation to release of proteins. The enhanced protein release by antibiotics and EDTA had been limited by high concentrations of Mg^{+2} ions *i.e.*, 15.00 mM to a great extent. The protein release was observed as 0.19, 0.18, 0.154 and 0.13 µg/ml in the presence of CEF; and in the case of CEZ, the protein

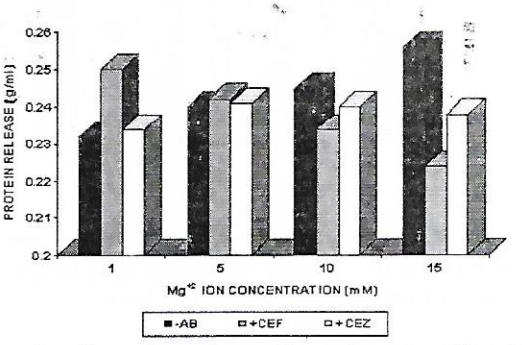

Fig. 5a: Protein release from intact cells of *E.coli* in the presence of varying conc. of Mg^{+2} each followed by the addition of 5mM EDTA

release from the membrane vesicles of *E.coli* was observed as 0.18, 0.17, 0.13 and 0.10 µg/ml, when the cells were suspended in 1.00 mM, 5.00 mM, 10.00 mM and 15.00 mM Mg^{+2} ion concentration (each supplemented with 5 mM EDTA) respectively, after 60 min of incubation (Fig. 5b). The variation in protein release might be attributed to the fact that the inhibitory effect of antibiotics was potentiated with the help of EDTA, which has resulted in more protein release, however, Mg^{+2} ions would have retained the stability of OM thus protecting the synergistic behaviour of EDTA in combination with β-Lactam. Contrary to that Mg^{+2} ions at higher conc. when supplemented would have saturated the

molecules of EDTA present into the medium thereby, protecting possible chelation of OM ions by EDTA; thus, maintaining integrity of cell wall.

As far as, speciation of protein leakage is concerned magnesium in combination with EDTA and cephalosporins resulted in release of possibly similar group of proteins that were greater than 67 kD which appeared after 11 min of run. In the absence of EDTA, Mg^{+2} ion causes effectively release of protein A and B in case of CEF; whereas with CEZ peak B was omitted.(Fig 6c). A remarkable phenomenon observed with CEF when treated with

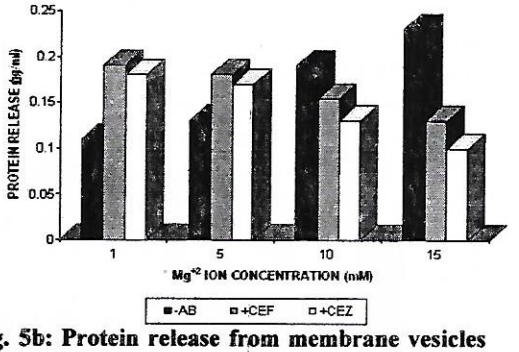

Fig. 5b: Protein release from membrane vesicles of *E.coli* in the presence of varying conc. of Mg^{+2} each in combination with 5 mM EDTA

EDTA and Mg^{+2} ion the potentiality of third generation cephalosporin can be possibly related by the appearance of peak G after 20 min of run resembling exactly with cytochrome c with a molecular weight of 12.5 kD (Fig. 6d). The appearance of cytchrome "c" peak into the medium under the stress of CEF might have resulted in making gram-negative cells sensitive towards CEF by reducing cellular energy level required to carry out possible cellular metabolism. Omp's located in the outer membrane of *E.coli* plays a multifunctional role in bacterial physiology and pathogenesis (Alfredo *et al.*, 2006). Recently it has been identified by proteomics techniques that there are many new *E.coli* outer membrane proteins, out of eight predicted outer membrane proteins, the outer membrane localization for five- YftM, YaiO, YfaZ, CsgF, and YliI- are confirmed and also the sixth one- YfaL- may be an outer membrane autotransporter. Since β-Lactam antibiotics penetrate the outer membrane, resistance could also be caused by loss or deficiency of specific porins that reduce the outer membrane permeability to β-Lactam antibiotic. Also, the cause for antibacterial drug resistance has been known to be the active efflux.The study of protein release from intact cells, as well as, membrane vesicles of *E.coli* shows the direct involvement of outer membrane components in developing sensitivity towards β-Lactams. The observations, thus, confirms the greater effectiveness of CEF as compared to CEZ towards *E.coli*.

The extent of EDTA- induced outer membrane losses from cells of wild- type *Escherichia coli* K-12 were concentration dependent. An additional Mg^{+2} ions immediately following the EDTA treatment decreased the release of outer membrane proteins and reduced the leakage of periplasmic proteins, suggesting that the temporary increase in outer membrane "permeability" caused by EDTA treatment was rapidly reversed by the redistribution of outer membrane components, a process which is favored by a low Mg^{+2} ion concentration. The envelope alterations caused by EDTA reveals that the biochemical, as well as, cellular disturbances were found to be more easily related to the toxic action of β-Lactams, CEF and CEZ. The susceptibility of bacterial culture for specific β-Lactams can be assessed by gradual exploration of membrane/cellular proteins responsible for making possible transport of antibiotics, across the cellular membrane against concentration gradient.

The study thus reveals that some of the membrane proteins as, well as some of the intracellular proteins get affected by the β-Lactam transport, revealing the mediation of direct antibiotic-protein interaction. The study confirms the orientation of the outer membrane and the energy status of the cell, determining the therapeutic value of cephalosporins.

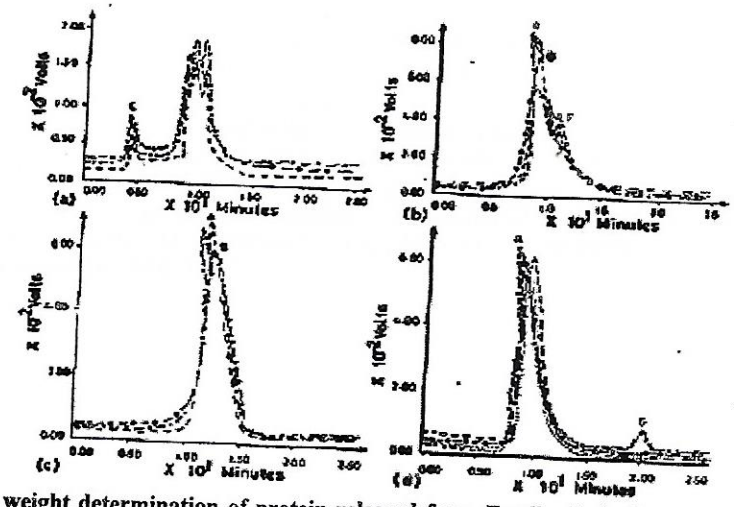

Fig. 6: Molecular weight determination of protein released from *E.coli* cells in the presence of antibiotic stress as monitored by HPLC, in the presence of 5mM EDTA, 5mM Mg^{+2} ions and 5 mM EDTA in combination with 5 mM Mg^{+2} ions, separately after 60 mins of incubation. (a) Membrane vesicles- EDTA (5 mM) (b) Intact cells – EDTA (5 mM) (c) Intact cells – Mg^{+2} ions (5 mM) (d) Intact cells - EDTA (5 mM) + Mg^{+2} ions (5mM). Here —— indicates –AB; -x-x indicates +CEF; -o-o- indicates + CEZ; ..... indicates control conditions

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