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Short Communication

Energetics of Cd^{2+} efflux system in cadmium-resistant *Staphylococcus aureus* 17810R

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Energetics of ^{109}Cd efflux in resting cells of cadmium-resistant *Staphylococcus aureus* 17810R was assayed in 1 or 100 mM potassium/sodium phosphate buffer, pH 7 (PB). Experiments with the use of inhibitors and ionophores showed that Cd^{2+} extrusion in this organism required ATP and either a pH gradient (ΔpH) in 1 mM PB or membrane potential (Δψ) in 100 mM PB. The role of high phosphate ion concentration in Δψ-dependent Cd^{2+} efflux is discussed.

According to Tynecka *et al.* [1], plasmid-mediated cadmium resistance in *Staphylococcus aureus* is due to the presence of the *cadA*-coded Cd^{2+} efflux system. This system extrudes Cd^{2+} from the cytoplasm immediately after its entry *via* the Mn^{2+} porter down the membrane potential (Δψ), which prevents accumulation of Cd^{2+} and its toxic effects [1–3]. Nigericin, an ionophore which selectively collapses the pH gradient (ΔpH), caused enhancement of ^{109}Cd accumulation by growing cells of cadmium-resistant *S. aureus* 17810R which oxidized exogenous amino acids [1]. On the basis of this observation, Tynecka *et al.* [1] suggested that the Cd^{2+} efflux system operated as an electroneutral $Cd^{2+}/2H^+$ antiporter down the ΔpH generated by the respiratory chain.

Silver and coworkers [4, 5] suggested, on the basis of the predicted amino-acid sequence of CadA protein, that Cd^{2+} efflux system is the Cd^{2+}-ATPase, belonging to the P-type cation-translocating ATPases. Recently, Tsai & Linet [6] demonstrated, in membranes of *Bacillus subtilis* in which *cadA* determinant was subcloned and expressed, that the CadA protein formed a phosphorylated enzyme intermediate characteristic of the P-type ATPases. Tsai *et al.* [7], using everted membrane vesicles of the same organism, showed that Cd^{2+} efflux was energized solely by ATP. However, the experiments by these authors [7] with the use of inhibitors and ionophores suggested that Cd^{2+} extrusion could require the energy of ΔpH in addition to ATP and that Cd^{2+}-ATPase had unique properties.

In the present work we studied energization of Cd^{2+} efflux system in resting cells of cadmium-resistant *S. aureus* 17810R. In some experiments, a cadmium-sensitive derivative strain *S. aureus* 17810S which lacks the Cd^{2+} efflux system, was used as a control organism.

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[1]Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DCCD, dicyclohexylcarbodiimide; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; PB, potassium/sodium phosphate buffer, pH 7; $\Delta\mu_H^+$, electrochemical proton gradient; Δψ, membrane potential; ΔpH, pH gradient.

MATERIALS AND METHODS

Staphylococcus aureus 17810R, carrying a penicillinase plasmid pII17810 with the *cadA* gene, and its plasmidless, cadmium-sensitive variant strain 17810S were described previously [2]. Experiments were performed on resting, aerobically grown early-exponential phase cells of both strains, which were obtained according to [2, 8]. In most of the experiments, cells were suspended in 1 or 100 mM potassium/sodium phosphate buffer, pH 7 (further called PB), at a density of 0.2 mg dry wt. ml^{-1}. ^{109}Cd (carrier-free) uptake at its 10 μM concentration was assayed as described previously [2]. Steady-state ^{109}Cd efflux was studied according to [1]. Oxygen uptake was determined manometrically [1]. ATP content was measured by the luminescence method [9]. The experiments shown in each Figure were done in triplicates and representative data are presented.

RESULTS AND DISCUSSION

Resting cells of *S. aureus* suspended in buffers in the absence of exogenous energy donors, utilize endogenous energy reserves, such as the amino-acid pool or ATP for membrane energization [8, 10]. As shown in Fig. 1A, resting cells of cadmium-resistant *S. aureus* 17810R, similarly as growing cells of this organism [1, 2], incubated in 100 mM PB did not accumulate ^{109}Cd. This was due to the activity of the plasmid-coded Cd^{2+} efflux system [1]. Under similar conditions, the resting cells of cadmium-sensitive control strain 17810S accumulated ^{109}Cd *via* the high-affinity Mn^{2+} porter down the membrane potential ($\Delta\psi$), as indicated by inhibition of Cd^{2+} accumulation with Mn^{2+} or with valinomycin + K^+ (Fig. 1A).

In contrast to growing cells of strain 17810R [1], in resting cells of this organism nigericin did not cause enhancement of Cd^{2+} accumulation in 100 mM PB (Fig. 1A). This suggests that, in the absence of pH gradient (ΔpH) abolished by nigericin, $\Delta\psi$ generated by endogenous respiration (Fig. 1A, inset) could energize Cd^{2+} efflux.

A decrease of PB concentration from 100 to 1 mM caused some net ^{109}Cd accumulation by strain 17810R, which is insensitive to either Mn^{2+} or valinomycin + K^+ (Fig. 1B); this accumulation could occur *via* the low affinity Cd^{2+} uptake system without involvement of the Mn^{2+} porter [2]. DCCD, a known inhibitor of the ATP synthetase complex, stimulated Cd^{2+} accumulation *via* the Mn^{2+} porter (Fig. 1B). Under these conditions, the HQNO-sensitive respiratory $\Delta\psi$ energized Cd^{2+} entry *via* the Mn^{2+} porter (Fig. 1B), while ΔpH did not support Cd^{2+} efflux, since Cd^{2+} acted as an inhibitor of endogenous respiration (Fig. 1B, inset) and ΔpH formation. In the absence of DCCD, inhibition of respiration by Cd^{2+}, caused a reversal of the direction of the ATP synthetase

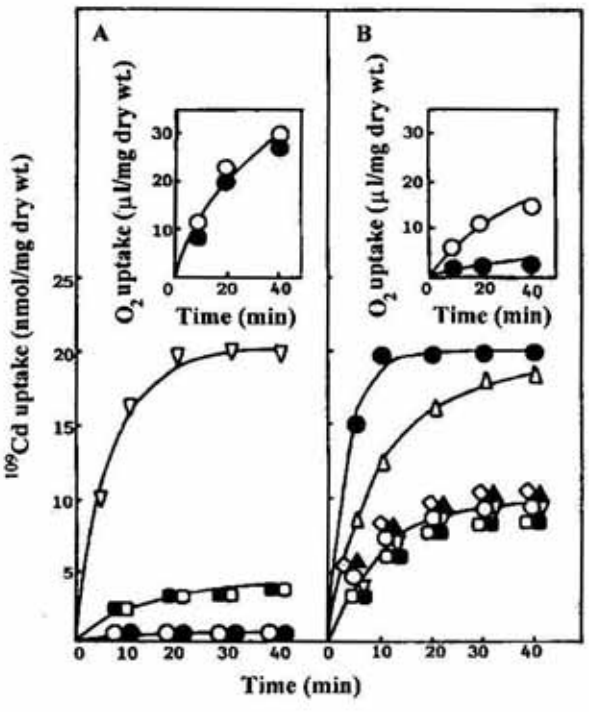

Fig. 1. ^{109}Cd uptake by resting cells of S. aureus 17810R and 17810S.

(A) Cells of both strains were suspended in 100 mM PB. Strain 17810R: ○, control cells; ●, cells pretreated with 0.5 μM nigericin. Strain 17810S: ▽, control cells; cells pretreated with: ❑, 1 μM valinomycin + 50 mM KCl; ■, 100 μM $MnCl_2$. (B) Cells of strain 17810R were suspended in 1 mM PB. ○, control cells; cells pretreated with: Δ, 100 μM DCCD; ●, 0.5 μM nigericin; ❑, 1 μM valinomycin + 100 mM KCl; ■, 100 μM $MnCl_2$; ◊, DCCD- or nigericin-pretreated cells + 1 μM valinomycin + 100 mM KCl; ▲, DCCD- or nigericin-pretreated cells + 100 μM $MnCl_2$; ▽, DCCD-pretreated cells + 100 μM HQNO. Cells were pretreated with inhibitors and ionophores for 10 min at 37°C before addition of ^{109}Cd. Insets: the effect of 10 μM Cd^{2+} on endogenous respiration of strain 17810R in 100 (A) or 1 mM PB (B). ○, control cells; ●, cells treated with $CdCl_2$.

activity into the hydrolysis [11, 12]. The $\Delta\psi$ generated *via* hydrolysis of endogenous ATP promoted Cd^{2+} influx *via* the Mn^{2+} porter, while ΔpH, not sensitive to Cd^{2+}, energized Cd^{2+} exit. Thus, by collapsing ΔpH, nigericin restored Cd^{2+} accumulation *via* the Mn^{2+} porter in strain 17810R (Fig. 1B). Our data are in agreement with those obtained by Tsai *et al.* [7] in everted membrane vesicles of *B. subtilis* containing the *cadA* determinant. These authors demonstrated that in the presence of ATP as a sole energy source, Cd^{2+} efflux was inhibited by either DCCD or nigericin [7].

An increase of PB concentration from 1 to 100 mM at steady-state in the Cd^{2+}-preloaded, nigericin-pretreated cells of strain 17810R, caused a partial extrusion of Cd^{2+} (Fig. 2A). This extrusion was insensitive to CCCP (Fig. 2A) and probably represented an energy-independent loss of Cd^{2+} bound to the low affinity Cd^{2+} uptake system.

In 1 mM PB, Cd^{2+} inhibited endogenous respiration in strain 17810R, (Fig. 1B, inset) while nigericin deprived the cells of ATP (Fig. 2C, inset). Therefore L-lactate, the cadmium-insensitive respiratory substrate [13], was used as an exogenous energy donor. Addition of L-lactate to the Cd^{2+}-preloaded, nigericin-pretreated cells, restored respiration (Fig. 2B) and also caused some transient ATP resynthesis down $\Delta\psi$, sensitive to valinomycin + K^+ or to DCCD (Fig. 2C). However, L-lactate did not cause energy-dependent Cd^{2+} extrusion (Fig. 2A). This indicates that in the nigericin-pretreated cells the energy of $\Delta\psi$ generated by L-lactate oxidation could not energize Cd^{2+} efflux. Only simultaneous addition of L-lactate and 100 mM PB at steady-state resulted in energy-dependent Cd^{2+} extrusion, sensitive to valinomycin + K^+ or to CCCP (Fig. 2A). These data suggest an important role of high PB concentration in the presence of L-lactate in $\Delta\psi$ driven Cd^{2+} efflux in the nigericin-pretreated cells of strain 17810R.

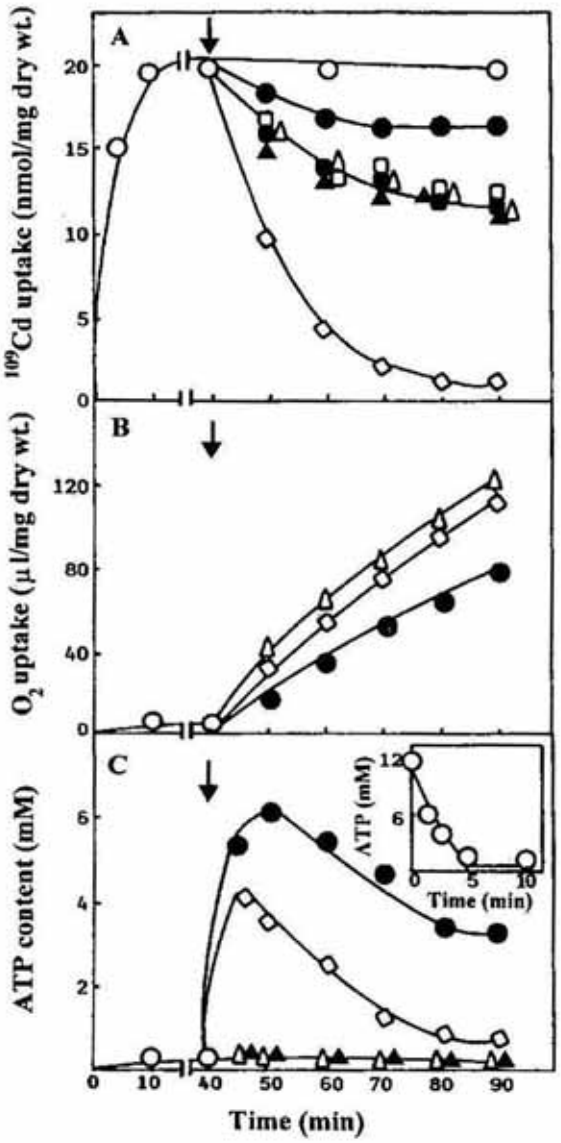

Fig. 2. (A) Steady-state ^{109}Cd efflux from the nigericin-pretreated, Cd^{2+}-preloaded cells of S. aureus 17810R.

O, Cells suspended in 1 mM PB and pretreated for 10 min at 37°C with 0.5 µM nigericin and for 40 min with 10 µM ^{109}Cd. At the time indicated by the arrow, cell suspensions were supplemented with: ●, 10 mM L-lactate; □, 100 mM PB; ■, 100 mM PB + 5 µM CCCP; ◊, 10 mM L-lactate + 100 mM PB; ▲, 10 mM L-lactate + 100 mM PB and 1 µM valinomycin + 50 mM KCl or 5 µM CCCP; Δ, 10 mM L-lactate + 100 mM PB and 100 µM DCCD.

(B) L-Lactate oxidation by the nigericin-pretreated, Cd^{2+}-preloaded cells of S. aureus 17810R.

O, Endogenous respiration in cells suspended in 1 mM PB and pretreated with nigericin and Cd^{2+}. At the time indicated by the arrow, cell suspensions were supplemented with: ●, 10 mM L-lactate; ◊, 10 mM L-lactate + 100 mM PB; Δ, 10 mM L-lactate + 100 mM PB and 100 µM DCCD. Oxygen consumption by the cells was measured manometrically.

(C) ATP resynthesis in the nigericin-pretreated, Cd^{2+}-preloaded cells of S. aureus 17810R.

O, Endogenous ATP level in cells suspended in 1 mM PB and pretreated with nigericin and Cd^{2+}. At the time indicated by the arrow, cell suspensions were supplemented with: ●, 10 mM L-lactate; ◊, 10 mM L-lactate + 100 mM PB; ▲, 10 mM L-lactate + 1 µM valinomycin + 50 mM KCl ± 100 mM PB; Δ, 10 mM L-lactate + 100 µM DCCD ± 100 mM PB. The cellular ATP content was determined by the luminescence method. Inset: the effect of 0.5 µM nigericin on the endogenous ATP content in cells of *S. aureus* 17810R suspended in 1 mM PB (O).

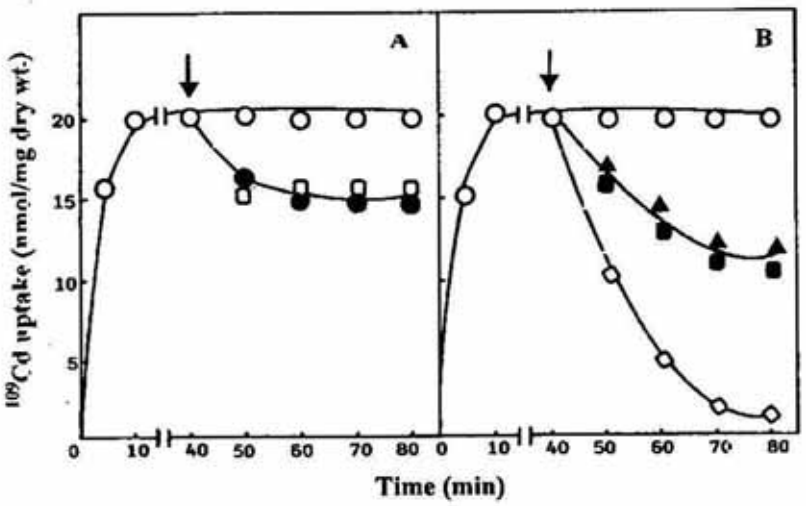

Fig. 3. Steady-state ^{109}Cd efflux from the nigericin-pretreated, Cd^{2+}-preloaded cells of S. aureus 17810R in various media.
○, Cells suspended in 1 mM PB and pretreated for 10 min at 37°C with 0.5 μM nigericin and for 40 min with 10 μM ^{109}Cd. (A) Cell suspensions were supplemented with: ●, 100 mM KCl, 100 mM NaCl, 100 mM Tris/HCl buffer, pH 7, 100 mM Mops/NaOH buffer, pH 7 + 10 mM L-lactate; or with ❐, the same media + 10 mM L-lactate + 5 μM CCCP. (B) Cell suspensions were supplemented with: ■, 100 mM triethanolamine-phosphate buffer, pH 7; ◊, 100 mM triethanolamine-phosphate buffer, pH 7 + 10 mM L-lactate; ▲, 100 mM triethanolamine-phosphate buffer, pH 7 + 10 mM L-lactate and 1 μM valinomycin + 50 mM KCl. The efflux was initiated at the time indicated by the arrow by addition of L-lactate.

Figure 3A shows that neither K^+ nor Na^+, present in PB, played any role in the L-lactate-induced Cd^{2+} efflux from the nigericin-pretreated cells of strain 17810R. Similarly, other 100 mM buffers, pH 7, Tris/HCl or Mops/NaOH, did not cause energy-dependent Cd^{2+} extrusion in the presence of L-lactate (Fig. 3A). The above data point to an important role of phosphate ions in Cd^{2+} efflux. This was confirmed by the energy-dependent Cd^{2+} extrusion caused by 100 mM triethanolamine-phosphate buffer supplemented with L-lactate (Fig. 3B).

When the cells of strain 17810R, devoid of endogenous energy reserves by starvation and not pretreated with nigericin, were energized by L-lactate in 1 mM PB, they accumulated Cd^{2+} only *via* the Mn^{2+} porter (Fig. 4A) at the same rate as the control strain 17810S (Fig. 4B). This indicates that in 1 mM PB neither $\Delta\psi$ nor ΔpH generated by L-lactate oxidation could energize Cd^{2+} efflux in strain 17810R, although CCCP-sensitive ATP synthesis was driven by the total electrochemical proton gradient — $\Delta\mu_{H^+}$ (Fig. 4A, inset). Only in 100 mM PB, the $\Delta\psi$ generated by L-lactate oxidation, but not ΔpH, energized Cd^{2+} efflux. This was evidenced by the lack of enhancement of ^{109}Cd accumulation with nigericin (Fig. 4A). Our data indicate that, the ΔpH generated by L-lactate oxidation can not energize Cd^{2+} extrusion, while $\Delta\psi$ can do it but only in combination with 100 mM PB.

The question arises as to what is the role of high phosphate ion concentration in the $\Delta\psi$-dependent Cd^{2+} efflux in strain 17810R? It should be stressed, that according to our polarographic studies, 100 mM PB complexed Cd^{2+} only in about 15% (not shown). We suggest that phosphate ions may provide extra protons during formation of cadmium phosphate in the cytoplasm. These protons pumped out *via* the

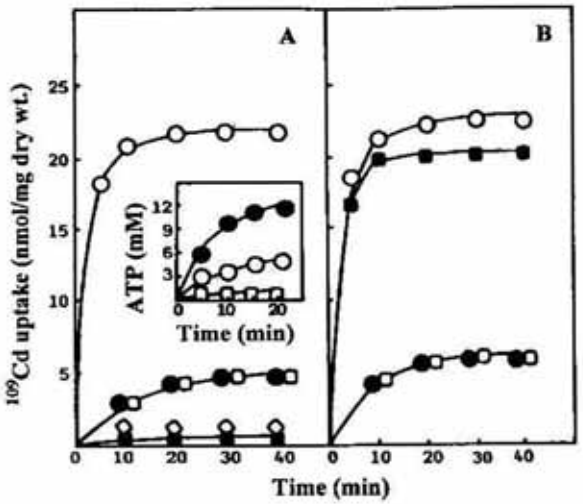

Fig. 4. ^{109}Cd uptake by starved cells of S. aureus 17810R (A) and 17810S (B) oxidizing L-lactate.
Cells of both strains were deprived of endogenous energy reserves in 100 mM PB, containing 5 mM $MgCl_2$ at 37°C for 3 h on a shaker. Starved cells were suspended in 1 or 100 mM PB and preincubated with 10 mM L-lactate for 20 min at 37°C. In 1 mM PB: ○, control cells; cells pretreated with: ●, 1 μM valinomycin + 100 mM KCl; ❑, 100 μM $MnCl_2$. In 100 mM PB: ■, control cells; ◊, cells pretreated with 0.5 μM nigericin. Inset: ATP synthesis in starved cells of *S. aureus* 17810R oxidizing L-lactate. ATP content was estimated in starved cells suspended in 1 (○) or 100 mM PB (●) after addition of L-lactate. ❑, Cells pretreated with 5 μM CCCP in 1 or 100 mM PB before addition of the substrate.

respiratory chain may return through the Cd^{2+} efflux system down the $\Delta\psi$ generated by L-lactate oxidation. Our suggestion is in agreement with a model of Nicholls & Akerman [14] explaining the role of "phosphate" protons in Ca^{2+} efflux in mammalian mitochondria.

As shown in Fig. 2A, DCCD, which stopped ATP resynthesis (Fig. 2C) but not L-lactate oxidation (Fig. 2B), inhibited the energy-dependent Cd^{2+} efflux from the nigericin-pretreated cells of strain 17810R oxidizing L-lactate in 100 mM PB. This suggests that, besides $\Delta\psi$ and phosphate ions, ATP was also needed for Cd^{2+} efflux in strain 17810R. The ATP requirement for Cd^{2+} extrusion is in agreement with data by other authors [4–7].

To summarize, the activity of the Cd^{2+} efflux system in *S. aureus* strain 17810R depends not only on ATP required for phosphorylation of CadA protein but also on either $\Delta\psi$ or ΔpH needed for proton circulation. Our suggestion is in accord with the data by Serrano [15] that some cation translocating P-type ATPases require for their activity both ATP and the proton current.

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