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# Cadmium Resistance of *Staphylococcus aureus* Determined by Penicillinase Plasmids

Plazmidowa oporność *Staphylococcus aureus* na jony kadmu

Плазмидовая устойчивость *S. aureus* к ионам кадмия

It has been shown that penicillinase plasmids of *Staphylococcus aureus*, aside from penicillinase locus, can carry a number of genes conferring resistance to some inorganic ions (4, 5, 6). As was reported by Novick and Roth (4), and Tynecka and Żylińska (9), the highest resistance index — about 100, was obtained with cadmium salts. The biochemical basis of resistance to cadmium ions is unknown. Chopra (1) showed that there was a markedly decreased rate of the uptake of $Cd^{2+}$ ions by resistant *Staphylococcus aureus* when compared with its derivative that did not possess cad-r gene.

Very little is known about the effect of $Cd^{2+}$ ions on microorganisms. According to recent surveys (11), cadmium either activates or inhibits a number of enzyme systems *in vitro*. As was found by one of us (9). $Cd^{2+}$ inhibited oxygen uptake by *Staphylococcus aureus*, which may suggest that this inhibitor can obstruct —SH groups of some respiratory enzymes, since cysteine could protect cells against this effect. The respiration of growing cells of *Staphylococcus aureus* harbouring penicillinase plasmids was inhibited to a significantly lower extent than the respiration of plasmid negative derivative (9). The aim of the present paper was to study the effect of $Cd(NO_3)_2$ on oxidation of some substrates by plasmid harbouring *Staphylococcus aureus* strain and its plasmid negative variant.

## MATERIAL AND METHODS

Strains. *Staphylococcus aureus* 1014+, harbouring penicillinase plasmids and its plasmid negative variant 1014— were used in all experiments reported here.

Cultures. Both strains were maintained in a dry state and subcultured for each experiment. Nutrient broth and plain agar were used throughout experiments.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal

| Strain | Effect of $Cd(NO_3)_2$ | Concentration of $10^{-2}$ | $5\times10^{-3}$ | $2.5\times10^{-3}$ | $1.2\times10^{-3}$ | $6\times10^{-4}$ |
|---|---|---|---|---|---|---|
| 1014+ | bacterio-static | — | — | — | — | ng |
| | bactericidal | + | + | + | ++ | +++ |
| 1014— | bacterio-static | — | — | — | — | — |
| | bactericidal | — | — | — | — | — |

ng — normal growth in broth, as in the control tube, +++ — abundant growth on plates, ++ — weak growth, + — single colonies, — — no growth.

Stationary cells. Cells for manometric experiments were obtained as follows: plain agar in Roux bottles was inoculated with overnight broth culture of both strains and incubated for 21 hours at 37°. The cells were then washed in saline by centrifugation and then kept overnight in 0.1 M phosphate buffer ph 7.0 at 4°, to reduce endogenous respiration. To some cultures 1% glucose was added.

Exponential cells. Dry cells were incubated overnight in broth at 37°; then 100 ml of broth was inoculated with the overnight broth culture and then incubated for 5 hours at 37° on a shaker, until the optical density was equivalent to 1 mg/ml of dry cells. The resulting culture was cooled down and kept overnight

Tab. 2. The effect of $Cd(NO_3)_2$ on oxygen uptake by *Staphylococcus aureus* 1014+ in the presence of some substrates

| Substrates | $QO_2$ (µl $O_2$/hr/mg dry cells) Control | with $Cd(NO_3)_2$ in concentrations (M) $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
|---|---|---|---|---|
| Succinate | 26.62 | 10.98 (58.99%)* | 25.85 | 27.21 |
| Glucose | 62.63 | 33.47 (38.74%) | 60.83 | 62.08 |
| Malate | 29.10 | 2.39 (91.79%) | 30.01 | 29.58 |
| Lactate | 154.68 | 160.57 | 157.87 | 157.86 |
| Acetate | 9.32 | — (100%) | 9.71 | 9.89 |
| Pyruvate | 38.25 | 3.26 (91.48%) | 25.78 (32.60%) | 38.04 |
| Ribose | 20.26 | 11.79 (50.24%) | 19.90 | 20.14 |

* Percent inhibition of oxygen uptake.

concentration of $Cd(NO_3)_2$ for *Staphylococcus aureus* 1014+ and 1014—

| $Cd[No_3]_2$ [M] | | | | | | |
|---|---|---|---|---|---|---|
| $3\times10^{-4}$ | $1.5\times10^{-4}$ | $7.5\times10^{-5}$ | $3.8\times10^{-5}$ | $1.9\times10^{-5}$ | $9.5\times10^{-6}$ | $4.7\times10^{-6}$ |
| ng | ng | ng | ng | ng | ng | ng |
| +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| — | — | — | — | — | ng | ng |
| — | — | + | + | + | +++ | +++ |

at 4°. Next day the culture was diluted with the same volume of fresh broth and incubation continued until the optical density was again equivalent to 1 mg/ml of dry weight. Bacteria were then washed with saline by centrifugation.

Estimation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of $Cd(NO_3)_2$. Two rows of tubes with 2 ml of nutrient broth containing decreasing concentrations of cadmium nitrate were inoculated with 1 drop of overnight broth culture of both strains and then incubated at 37° for 24 hours. Next day one drop of culture was removed from each tube and plated out on agar plates to define the bactericidal effect of $Cd(NO_3)_2$.

Pyruvic acid estimation (2). 1 ml of supernatant from Warburg vessel was mixed with 4 ml of 25% trichloroacetic acid (TCA) to precipitate proteins. 1 ml of deproteinised supernatant was added to 1 ml of 2,4-dinitrophenylhydrazine (sa-

Tab. 3. The effect of $Cd(NO_3)_2$ on oxygen uptake by *Staphylococcus aureus* 1014— in the presence of some substrates

| Substrates | $QO_2$ (µl $O_2$/hr/mg dry cells) | | | |
|---|---|---|---|---|
| | Control | with Cd $(NO_3)_2$ in concentrations (M) | | |
| | | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| Succinate | 36.75 | 17.34 (52.82%) | 37.84 | 39.26 |
| Glucose | 72.00 | 41.37 (42.54%) | 70.02 | 70.50 |
| Malate | 23.09 | — (100%) | 18.87 (18.70%) | 25.38 |
| Lactate | 190.83 | 194.06 | 200.06 | 211.35 |
| Acetate | 16.00 | — (100%) | 13.73 (19.91%) | 17.03 |
| Pyruvate | 51.31 | — (100%) | 13.05 (74.62%) | 50.41 |
| Ribose | 25.47 | 10.97 (56.93%) | 24.56 | 23.38 |

* Percent inhibition of oxygen uptake.

Tab. 4. The effect of $Cd(NO_3)_2$ on glucose oxidation by *Staphylococcus aureus* strain 1014+ and 1014— grown on 1% glucose

| Strain | Number of experiments | $QO_2$ (µl $O_2$/hr/mg dry cells) | | | |
|---|---|---|---|---|---|
| | | Control | with $Cd(NO_3)_2$ in concentrations (M) | | |
| | | | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ |
| 1014— | 1 | 101.76 | 77.05 (24.28%)* | 102.35 | 99.78 |
| | 2 | 104.82 | 83.85 (20%) | 117.24 | 102.30 |
| | 3 | 94.04 | 73.48 (21.87%) | 109.28 | 99.47 |
| 1014+ | 1 | 89.25 | 70.90 (20.56%) | 88.53 | 83.62 |
| | 2 | 89.34 | 64.20 (28.14%) | 92.29 | 82.50 |

* Percent inhibition of oxygen uptake.

turated in 2 N HCl) and after 10 min vigorously shaken with 5 ml toluene for 3 min. Subsequently the bottom layer was removed and the upper layer was shaken with 6 ml of 10% sodium carbonate for 3 min. Then 5 ml of the carbonate extract was withdrawn and mixed with 5 ml of 1.5 N NaOH. After 5—10 min. the colour intensity was read colorimetrically (Spekol photocolorimeter, filter 530).

Manometric experiments (10). To the main Warburg vessel the following were added: 1 ml of cell suspension (4—5 mg of dry cells), 1 ml of 0.1 M phosphate buffer pH 7.0, 0.5 ml of substrates (glucose — 0.2 M; pyruvate — 0.3 M; lactate — 1.0 M; and the other substrates — 0.1 M). To the sidearm bulbs 0.5 ml of $Cd(NO_3)_2$ in concentrations ranging from $10^{-4}$ M to $10^{-6}$ M was added. The central well contained 0.2 ml of 20% KOH. After 20 min. incubation, cadmium nitrate was transferred from the sidearm bulbs to the main vessels and incubation continued for 60 min. Readings were taken every 10 min. To determine the quotiens $QO_2$, suitable portions of the suspensions were dried (10 hours, 110°) and weighed.

## RESULTS

Table 1 shows MIC and MBC of cadmium nitrate for growth of both strains. A hundredfold difference in sensitivity to cadmium ions between strains — 1014+ and 1014— could be observed. As can be seen from table 2, oxidation of all substrates in phosphate buffer by washed cells of *S. aureus* 1014+ was inhibited by $10^{-4}$ M of $Cd^{2+}$ to a various degree. Oxygen uptake in the presence of lactate was not inhibited at all, while oxidation of glucose, succinate and ribose showed only partial inhibition — about 50%. Pyruvate, malate and acetate oxidation was nearly completely inhibited. Concentrations $10^{-5}$ M and $10^{-6}$ M did not show any significant inhibition on the oxidation of either substrate, except pyruva-

te. It is interesting to note that the degree of inhibition of oxygen consumption in the presence of all substrates by cadmium sensitive strain 1014— was nearly identical with that for strain 1014+ (Table 3). When cells were allowed to respire in the growing medium, a hundredfold difference in sensitivity to $Cd^{2+}$ ions between both strains was observed, which is illustrated in Figs. 1 and 2.

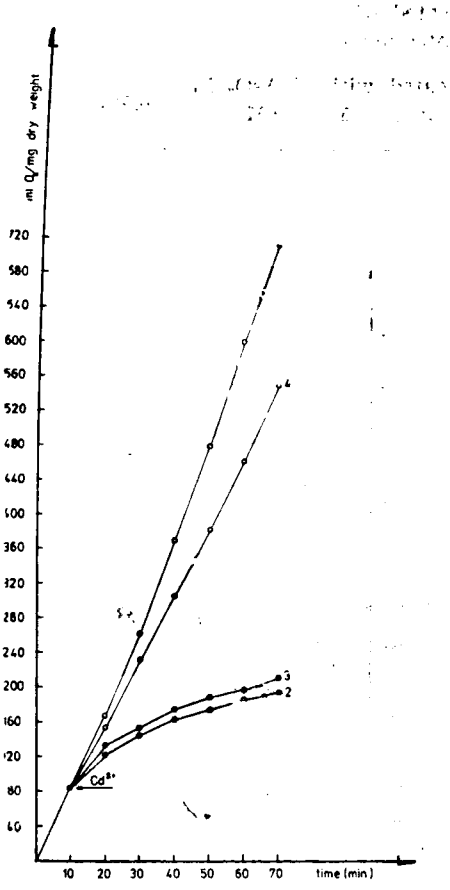


Fig. 1. The effect of $Cd^{2+}$ on respiration of *Staphylococcus aureus* 1014— under growing conditions; 1 — control, 2 — $10^{-4}$ M of $Cd(NO_3)_2$, 3 — $10^{-5}$ M of $(Cd(NO_3)_2$, 4 — $10^{-6}$ M of $(Cd(NO_3)_2$

Table 4 shows the effect of $Cd^{2+}$ on oxidation of glucose by strains grown on agar containing 1% glucose. It can be seen that the percent of inhibition of glucose oxidation by $10^{-4}$ M of cadmium nitrate dropped from about 50% (cells grown without glucose, table 2) to 20%. Table 5 shows to accumulation of pyruvate when lactate was used as a substrate.

Table 5. Accumulation of pyruvate by strain 1014— during of lactate in the presence of $Cd(NO_3)_2$ and $NaAsO_2$

| $E_{530}$ | Cells grown without glucose | $E_{530}$ | Cells grown on 1% glucose |
|---|---|---|---|
| 0.375 | Control cells | 0.385 | Control cells |
| 0.590 | Cells treated with $Cd(NO_3)_2$ in concentration $10^{-4}$ M | 0.540 | Cells treated with $Cd(NO_3)_2$ in concentration $10^{-4}$ M |
| 0.580 | Cells treated with $NaAsO_2$ in concentration $5\times10^{-3}$ M | 0.510 | Cells treated with $NaAsO_2$ in concentration $5\times10^{-3}$ M |

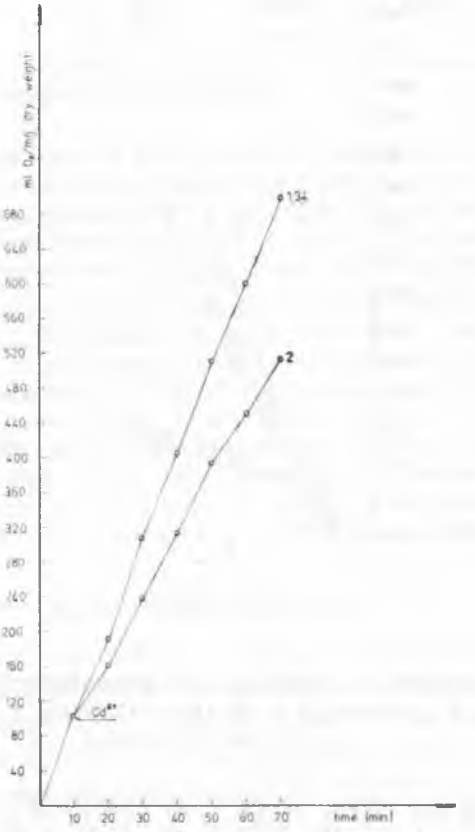


Fig. 2. The effect of $Cd^{2+}$ on respiration of *Staphylococcus aureus* 1014+ under growing conditions; 1 — control, 2 — $10^{-4}$ M of $Cd(NO_3)_2$, 3 — $10^{-5}$ M of $(Cd(NO_3)_2$, 4 — $10^{-6}$ M of $(Cd(NO_3)_2$

One can notice that $Cd^{2+}$ in concentration $10^{-4}$ M causes the same degree of accumulation of pyruvate as did $As^{3+}$ ions. No difference in accumulation of pyruvate could be seen between cells grown on glucose, and in the absence of this sugar.

## DISCUSSION

Observations of Chopra (1) suggest that the uptake of 115 m $CdCl_2$ in concentration $10^{-4}$ M by cadmium sensitive *Staphylococcus aureus* was about 15 times that found with the plasmid harbouring organism. As was shown in the present paper, the same concentration of $Cd^{2+}$ was bactericidal for strain 1014—, while cadmium resistant organism — 1014+ grew normally under those conditions. The same rate of difference in sensitivity between both strains to $Cd^{2+}$ was seen when organisms respired in the growing medium. When washed cells of both strains were allowed to oxidize some of the carbohydrate substrates in the buffer in the presence of the same concentration of cadmium nitrate, no significant difference could be seen in sensitivity to the inhibitor between both strains.

One can assume that under growing conditions plasmid harbouring strain may produce and excrete some protective substance of protein nature into the medium, which could bind $Cd^{2+}$ ions rendering this organism more resistant than the plasmid negative variant. Another possibility might be that cadmium resistant strain may contain such protective substance bound either to the cell wall or cell membrane, which may render these structures impermeable to $Cd^{2+}$ ions. According to the uptake experiments of Chopra (1), those bound $Cd^{2+}$ ions could be washed out from the resistant strain. This author (1) suggested recently (personal communication) that the plasmid born resistance to $Cd^{2+}$ is most probably connected with the impermeability at the level of the cell membrane, but the precise mechanism is still obscure. Experiments along this line are in progress in our laboratory.

As the target of $Cd^{2+}$ in *Staphylococcus aureus* cell is concerned, it has been shown (9) that the inhibition of growth of staphylococci by cadmium could be reversed by cysteine. This may suggest that this inhibitor can act on respiratory enzymes by combining with their —SH groups. From the data presented in this paper it is evident that cadmium may inhibit some enzymes catalyzing oxidation of pyruvate, acetate and some intermediates of the tricarboxylic acid cycle (TCA), since with pyruvate, acetate and malate, inhibition was complete. Oxidation of glucose and ribose was inhibited only in about 50%, while oxygen uptake in the presence of lactate was not inhibited at all. Higher contents of pyruvate

during lactate oxidation in the presence of cadmium nitrate, as compared with the control cells, may indicate that this inhibitor can cause pyruvate accumulation, similarly to $As^{3+}$. The 50% inhibition of glucose and ribose oxidation may thus reflect inhibition of oxidation of those sugars in TCA cycle via pyruvate, which is a common pathway in staphylococci under aerobic conditions (7).

When cells were grown on 1% glucose and then allowed to oxidize glucose in Warburg apparatus, cadmium ions in concentration $10^{-4}$ M caused only 20% inhibition. As was pointed out by Strasters and Winkler (7), and by Tynecka and Rusin (8), growth of staphylococci on glucose decreased their ability to oxidase pyruvate, acetate, ribose and TCA cycle intermediates. The observed by us lower rate of cadmium inhibition of glucose oxidation by glucose grown staphylococci may reflect the diminished rate of glucose oxidation via TCA cycle and hence the diminished rate of cadmium inhibition of oxygen uptake is understandable. It has been shown (7, 8) that glucose grown staphylococci respired mainly glycolytically and conversion of pyruvate to lactate is possible under such conditions. As the oxidation of lactate is not inhibited by cadmium ions at all, it is interesting whether the observed by us 20% inhibition of glucose oxidation in cells which respired glycolytically means partial inhibition of glycolytic route, or that even under these conditions cells can oxidize some pyruvate via TCA cycle.

Further investigation on cadmium inhibition of respiratory enzymes in a cell free system is necessary, which may throw some light on the precise mode of action of $Cd^{2+}$ ions on respiration of staphylococci.

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OBJAŚNIENIA RYCIN I TABLIC

Ryc. 1. Wpływ $Cd(NO_3)_2$ na pobór tlenu przez *Staphylococcus aureus* 1014— w warunkach wzrostowych

Ryc. 2. Wpływ $Cd(NO_3)_2$ na pobór tlenu przez *Staphylococcus aureus* 1014+ w warunkach wzrostowych

Tab. 1. Najmniejsza dawka hamująca (MIC) oraz najmniejsza dawka bakteriobójcza (MBC) $Cd(NO_3)_2$ dla szczepu 1014+ i 1014—.

Tab. 2. Utlenianie niektórych substratów przez *Staphylococcus aureus* 1014+ w obecności różnych stężeń $Cd(NO_3)_2$.

Tab. 3. Utlenianie niektórych substratów przez *Staphylococcus aureus* 1014— w obecności różnych stężeń $Cd(NO_3)_2$.

Tab. 4. Wpływ $Cd(NO_3)_2$ na utlenianie glukozy przez *Staphylococcus aureus* 1014+ i 1014— namnażane na podłożu z 1% glukozy.

Tab. 5. Akumulacja pirogronianu przez *Staphylococcus aureus* 1014— podczas utleniania mleczanu w obecności $Cd(NO_3)_2$ i $NaAsO_2$.

## STRESZCZENIE

Niektóre szczepy *Staphylococcus aureus* posiadają pozachromosomowe elementy genetyczne będące nośnikami genów oporności na penicylinę oraz jony metali. Najwyższy indeks oporności uzyskuje się w przypadku soli kadmu. Mechanizm tej oporności nie jest jeszcze wyjaśniony.

Założeniem pracy było wykazanie różnic we wrażliwości na azotan kadmu pomiędzy szczepem *S. aureus* posiadającym plazmidy penicylinazy oraz jego wariantem pozbawionym tych plazmidów. Badania prowadzono nad wpływem $Cd(NO_3)_2$ na wzrost i oddychanie w aparacie Warburga.

Wykazano duże różnice we wrażliwości badanych szczepów na kadm w warunkach wzrostowych; indeks oporności wynosił około 100. Takie same różnice we wrażliwości pomiędzy szczepami obserwowano w doświadczeniach manometrycznych, gdy komórki oddychały w podłożu wzrostowym, natomiast przemyte komórki obydwu szczepów zawieszone w buforze fosforanowym były niemal identycznie wrażliwe na stosowany inhibitor. Sugeruje to, że podczas wzrostu w komórkach szczepu posiadającego plazmidy może odbywać się na matrycy genów oporności synteza substancji warunkujących oporność szczepu na sole kadmu.

Mechanizm działania soli kadmu na mikroorganizmy jest stosunkowo mało poznany. W niniejszej pracy wykazano, że jony kadmu działają hamująco na utlenianie pirogronianu, octanu, rybozy oraz niektórych metabolitów pośrednich cyklu Krebsa. Należy przypuszczać, że jony kadmu inaktywują niektóre enzymy oddechowe przez blokowanie ich grup —SH, ponieważ cysteina znosi to hamujące działanie.

## РЕЗЮМЕ

У некоторых штаммов *Staphylococcus aureus* выступают позахромосомальные генетические элементы, являющиеся носителями генов сопротивления к пенициллину и ионам металлов. Самый большой индекс устойчивости можно получить употребляя соли кадмия. Механизм этой устойчивости еще не выяснен.

Целью настоящей работы было выявление разницы в чувствительности к $Cd(NO_3)_2$ между *S. aureus*, имеющими плазмиды и его вариантом, который этими плазмидами не обладал.

Исследования над влиянием $Cd(NO_3)_2$ на рост и дыхание проводились в аппарате Варбурга. Обнаружены большие разницы в чувствительности исследованных штаммов к кадмию в ростовых условиях. Индекс устойчивости равнялся приблизительно 100. Такие же разницы в чувствительности между штаммами мы наблюдали в манометрических опытах, когда клетки находились в ростовой среде. В то же время промытые клетки обоих штаммов, суспендированные в фосфорном буфере, были чувствительны к применяемому ингибитору почти одинаково. Возможно, что во время роста в клетках штамма, содержащего плазмиды, на матрице генов сопротивляемости происходит синтез веществ, обусловливающих сопротивление штамма к солям кадмия.

Механизм действия солей кадмия на микроорганизмы пока еще мало известен.

В настоящей статье говорится о тормозящем действии кадмия на окисление прувота, ацетата, рибозы и некоторых метаболлитов ЦТК. Следует предположить, что ионы кадмия ингибируют некоторые дыхательные ферменты, блокируя группы SH этих ферментов, т. к. цистеин уничтожает это тормозящее влияние.