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The Effect of the Elimination of Penicillinase Plasmids on the Sensitivity of *Staphylococcus aureus* to Some Penicillins and Cd^{2+} Ions

Wpływ eliminacji plazmidów penicylinazy na wrażliwość *Staphylococcus aureus* na niektóre penicyliny i jony kadmu

Влияние удаления плазмидов пенициллиназы на чувствительность *Staphylococcus aureus* к некоторым пенициллинам и ионам кадмия

Extrachromosomal genetic elements in *Staphylococcus aureus*, called penicillinase plasmids, carry determinants conferring resistance of the cell to penicillin and some heavy metal ions (3, 4, 5, 6, 7, 8, 9, 12). The mechanism of the plasmid born resistance to penicillin is based on the ability of the cell to produce penicillinase, an enzyme which destroys penicillin to an antibiotically inactive penicilloic acid (3, 4). The nature of the plasmid dependent resistance to inorganic ions it not fully understood (1, 2, 7, 12, 13). Elimination of plasmids from the cell results in a marked increase in its sensitivity to penicillin and the ions (3, 4, 6).

The aim of the present paper was to compare the sensitivity to some penicillins and Cd^{2+} ions between *Staphylococcus aureus* strains carrying penicillinase plasmids and their segregants which were cured of these genetic elements.

MATERIAL AND METHODS

Strains. 5 pairs of *S. aureus strains*, of which one was carrying penicillinase plasmid and the other was its plasmid-negative segregant, were obtained from Dr K. Dyke, Department of Biochemistry, University of Oxford, England. The strains were maintained in a dry state and subcultured on agar slants each month.

Cultures. Nutrient broth and nutrient agar were used throughout the experiments.

Estimation of M.I.C. (minimal inhibitory concentration) of penicillin G, ampicillin, carbenicillin, methicillin and $Cd(NO_3)_2$ for the strains studied. To series of tubes containing twofold dilutions of the antibiotics and cadmium nitrate in 2 ml broth, 0.02 ml of overnight broth cultures of the strains was added. The cultures were incubated for 24 hrs at 37°.

Manometric experiments. The effect of cadmium on respiration of *S. aureus* 17810+ and 17810− was studied in a Warburg respirometer (16). Exponential cells

were collected by centrifugation and resuspended in fresh broth to give approximately 1—1.2 mg/ml dry weight. The main Warburg vessel contained 2 ml of bacterial suspensions in broth, and into the sidearm bulb 0.5 ml of various concentrations of cadmium nitrate were added. 0.2 ml of 20% KOH was present in the central well. The suspensions were equillibrated for 10 min at 37°. Cadmium was added after 10 min. incubation and the readings were taken every 10 min. during 1 hr. Dry weight of cells was estimated by filtration of 5 ml of the suspensions through the previously dried and weighed milipore filters (0.6 μ), washed thoroughly with distilled water and then dried again to the constant weight.

Radioisotopic experiments. Uptake of $^{115m}Cd^{2+}$ by exponential cells of *S. aureus* 17810+ and 17810− in broth at 37°. Radioactive cadmium at concentrations: 10^{-3}M, 10^{-4}M, 10^{-5}M and 10^{-6}M was added to the prewarmed cultures and 5 ml removed after 0, 5, 10, 15, 20, 30, 40, 50 and 60 min, rapidly filtered through the milipore filters and then washed with 2 batches (5 ml each) of prewarmed broth. The radioactivity was measured by counting the dried filters with thin window G.M. counter. The uptake was expressed in moles of Cd^{2+} per mg of dry weight.

Chemicals. All chemicals were of analytical grade, obtained from POCH, Gliwice, Poland. Milipore filters were the product of Chempol, Praha, Chechoslovakia. $^{115m}CdCl_2$ (specific activity 50 μCi/μmol was obtained from the Institute of Nuclear Research, Świerk, Poland.

RESULTS

As can be seen from table 1, strains endowed with penicillinase plasmids required a much higher concentration of penicillin G, ampicillin, and carbenicillin for inhibition of growth, while their plasmidless segregants were inhibited by much lower concentrations of the antibiotics. The RI (Resistance Index) values for penicillin G varied from one pair of

Table 1. Sensitivity of *Staphylococcus aureus* strains to penicillins

Strains	M.I.C. (Minimal inhibitory concentration) of penicillins μg/ml			
	Penicillin G	Ampicillin	Carbenicillin	Methicillin
17810 +	2	8	8	2
17810 −	0.007	0.015	0.06	0.5
	RI — 285×	RI — 533×	RI — 133×	RI — 4×
9033 +	64	256	16	0.5
9033 −	0.03	0.06	0.125	0.25
	RI — 2133×	RI — 4266×	RI — 128×	RI — 2×
9021 +	16	32	4	1
9021 −	0.07	0.06	0.06	1
	RI — 228×	RI — 533×	RI — 66×	RI — 0×
1051 +	128	512	32	2
1051 −	0.03	0.03	0.25	1
	RI — 4266×	RI — 17066×	RI — 128×	RI — 2×
1014 +	2048	512	64	2
1014 −	0.03	0.25	0.5	1
	RI — 68266×	RI — 2048×	RI — 128×	RI — 2×

the strains to another, the highest RI being 68.266× and the lowest — 228×. For ampicillin and carbenicillin the highest RI values were 17.066× and 128×, and the lowest 533× and 66× respectively. Almost no difference in sensitivity between plasmid carrying strains ant their sensitive segregants was observed with methicillin. Similar concentrations of this antibiotic inhibited growth both of resistant and sensitive organisms.

Table 2 presents the difference in sensitivity to Cd^{2+} ions between plasmid possessing strains and their sensitive variants. The highest RI values was 520× for strains 17810+ and 17810−, and the lowest was 31× for strains 9021+ and 9021−.

In order to see whether cadmium, as a heavy metal, will inhibit respiration of staphylococci, one pair of strains (with the highest RI for cadmium) was chosen for further studies. Fig 1 and 2 show the effect of cadmium ions on oxygen uptake by growing cells of both strains.

Table 2. Sensitivity of *Staphylococcus aureus* strains to $Cd(NO_3)_2$

Strains	Plasmid present	M.I.C. (minimal inhibitory concentration) of $Cd(NO_3)_2$ (M)	Resistance index (R. I.)
17810	+	1.25×10^{-3}	520×
	−	2.4×10^{-6}	
9033	+	1.25×10^{-3}	62×
	−	1.9×10^{-5}	
9021	+	7.5×10^{-5}	31×
	−	2.4×10^{-6}	
1051	+	1.25×10^{-3}	250×
	−	4.8×10^{-6}	
1014	+	6.2×10^{-4}	32×
	−	1.9×10^{-5}	

As can be seen, respiration of *S. aureus* 17810+ was only partially inhibited by 10^{-3}M of Cd^{2+}, while lower concentrations had no toxic effect on respiratory activity of this strain. In contrast to this, oxygen uptake by plasmid negative derivative was sensitive to all concentrations of cadmium used, except 10^{-7}M. Concentrations 10^{-3}M, 10^{-4}M and 10^{-5}M displayed a very similar degree of inhibition (about 80%) of sensitive organism, whereas at 10^{-6}M only partial inhibition of oxygen uptake was observed.

We were interested to know whether the insensitivity of plasmid carrying strain to cadmium, both in growth tests and in manometric experiments, was connected with the impermeability of the cell to the ions. To test this, we used radioactive $^{115m}Cd^{2+}$ and investigated the

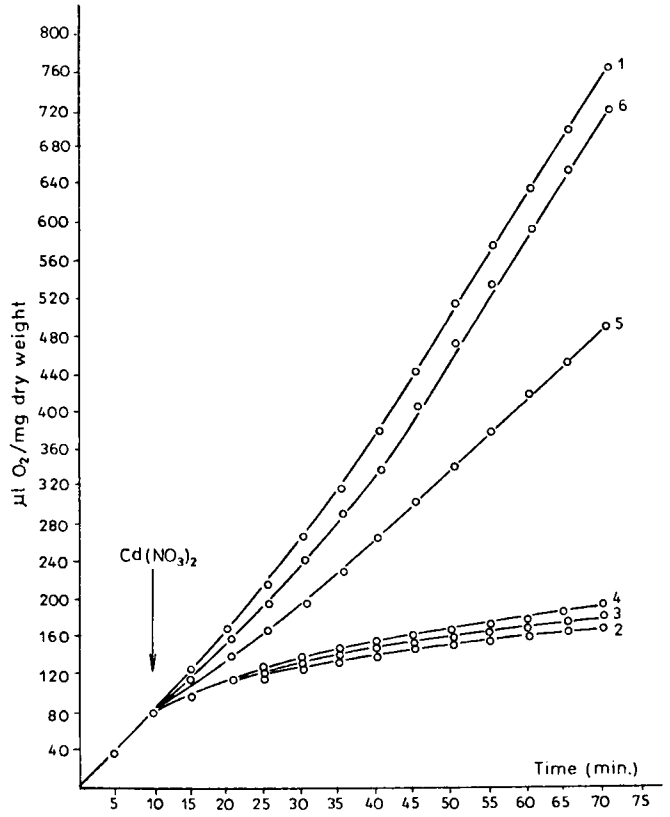

Fig. 1. The effect of $Cd(NO_3)_2$ on the respiration of *Staphylococcus aureus* strain 17810— in broth; 1 — control, 2 — 10^{-3}M of $Cd(NO_3)_2$, 3 — 10^{-4}M of Cd $(NO_3)_2$, 4 — 10^{-5}M of $Cd(NO_3)_2$, 5 — 10^{-6}M of $Cd(NO_3)_2$, 6 — 10^{-7}M of $Cd(NO_3)_2$

uptake of it by both strains. Fig. 3 presents the binding of $^{115m}Cd^{2+}$ by the strains studied. The plasmid harbouring *S. aureus* bound very little cadmium ions at concentrations 10^{-4}M—10^{-6}M, while at 10^{-3}M some marked uptake was observed. The plasmidless derivative took Cd^{2+} ions at all concentrations used, and this uptake proved to be concentration dependent.

DISCUSSION

As was mentioned earlier, the mechanism of plasmid born resistance of *S. aureus* to penicillin depends on the ability of the cell to destroy the antibiotic by penicillinase, before it can reach the sensitive sites (3, 4, 5, 6). According to resent knowledge, penicillin blocks an enzyme transpeptidase, located in the cytoplasmic membrane with the resultant inhibition of cross-linking of the cell wall peptidoglycan (11, 15, 18). The gene-

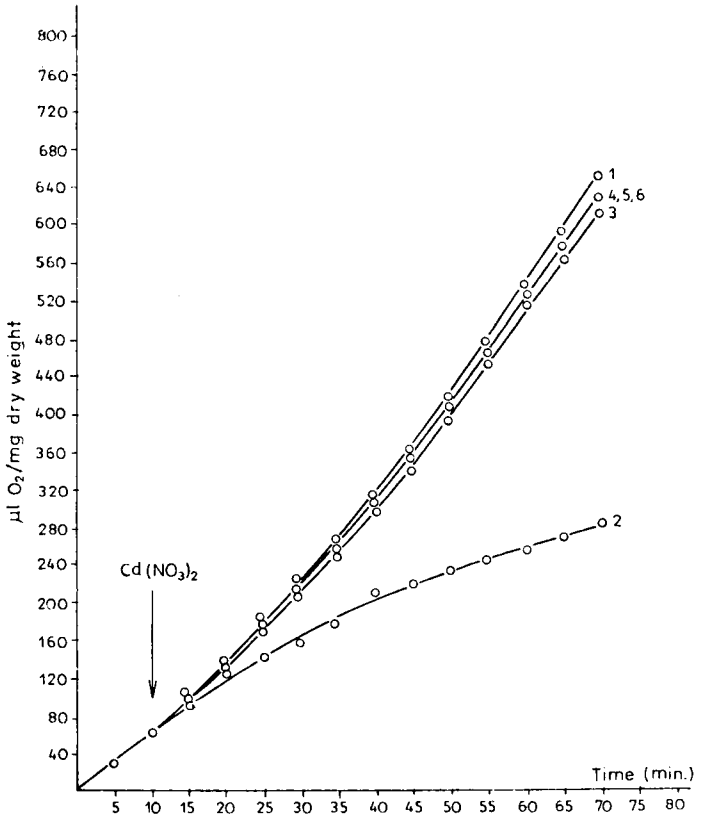

Fig. 2. The effect of $Cd(NO_3)_2$ on the respiration of *Staphylococcus aureus* strain 17810+ in broth; 1 — control, 2 — 10^{-3}M of $Cd(NO_3)_2$, 3 — 10^{-4}M of $Cd(NO_3)_2$, 4 — 10^{-5}M of $Cd/Cd(NO_3)_2$, 5 — 10^{-6}M of $Cd(NO_3)_2$, 6 — 10^{-7}M of $Cd(NO_3)_2$

tic determinants which confer resistance upon staphylococci to heavy metal ions — mercury, cadmium, arsenic, lead and zinc — are also located on penicillinase plasmids (7, 8, 9). The mechanism of resistance of staphylococci to these metals is not clear yet. Some evidence suggests that resistance of bacteria to Hg^{2+} might be based upon the ability of the cell to reduce mercuric chloride to metallic mercury which evaporates from the cell (10). As far as resistance to other heavy metal ions is concerned, it has been suggested that resistance of staphylococci to cadmium might be connected with the inability of this metal to penetrate the cell (1, 2, 14).

The present results demonstrate the effect of plasmid elimination from staphylococcal cell on its sensitivity to penicillin and cadmium. Plasmidless segregants were far more sensitive to these toxic agents than their plasmid carrying parent organisms. The RI values were very high

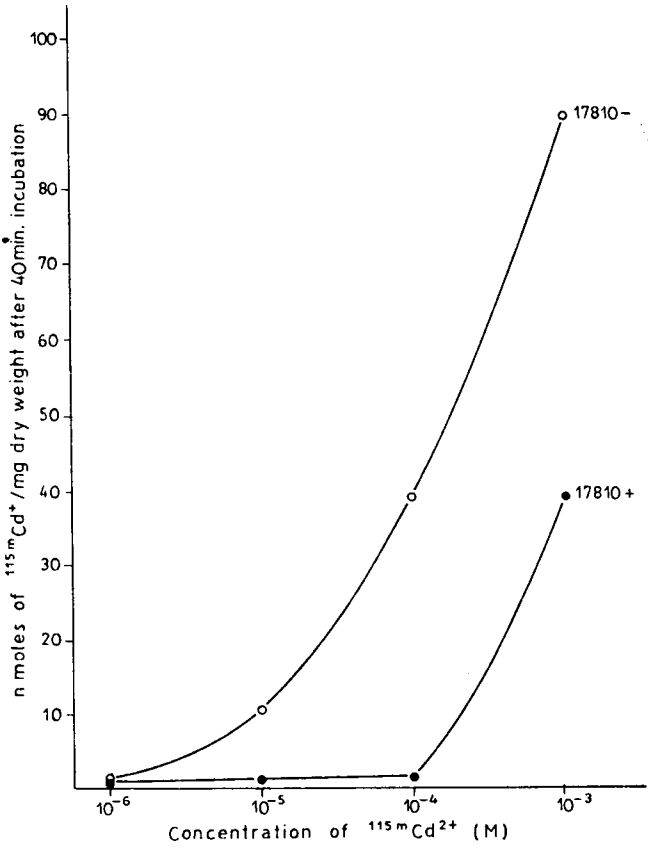

Fig. 3. Concentration—dependent binding of $^{115m}Cd^{2+}$ to *Staphylococcus aureus* 17810+ and 17810−

for penicillin G and ampicillin and lower for carbenicillin and cadmium salt. No significant difference in sensitivity to methicillin between parent organisms and their segregants was noted. This observation confirms the insensitivity of methicillin to staphylococcal penicillinase. Further studies were conducted on one pair of the strains which showed the highest RI for cadmium salt. Radioisotopic and manometric experiments have shown that binding of Cd^{2+} ions resulted in inhibition of respiration of both strains, but the plasmid born difference in sensitivity of respiration between these strains was closely dependent upon the concentration of the ions used. Cadmium sensitive variant took up $^{115m}Cd^{2+}$ ions at all concentrations used and this always resulted in a high inhibition of its respiration. Even at concentration 10^{-6}M when very small number of Cd ions were bound by this strain, partial inhibition of oxygen uptake was

observed. In contrast to this, the resistant parent organism was able to take up $^{115m}Cd^{2+}$ ions only when the external concentration was 10^{-3}M and this uptake also resulted in inhibition of respiration, like in the sensitive variant. At lower concentration, cadmium was not taken up by this organism and no inhibition of its respiration took place. It may be thus suggested that plasmid genes provide the resistant cell with a protective barrier against Cd^{2+} ions, but only up to the concentration 10^{-3}M. The chemical nature of this barrier is still unknown.

The fact that very low concentration of Cd^{2+}, comparable to those required for growth inhibition, caused inhibition of respiration of the sensitive cell, may indicate that the primary site of Cd^{2+} action could be respiratory enzymes (cysteinyl and histidyl side chains) located on or in the cytoplasmic membrane (17). The observed by us ability of the sensitive variant to bind the increasing number of Cd^{2+} ions in response to the increasing external concentration, indicate that Cd^{2+}, apart from blocking the respiratory enzymes, can bind to a variety of chemical groups of cell components and cause additional disturbance to its metabolism (17).

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STRESZCZENIE

Badano wpływ eliminacji plazmidów penicylinazy ze szczepów *Staphylococcus aureus* na ich wrażliwość na niektóre penicyliny i jony kadmu. Wykazano, że bezplazmidowe warianty wymagały do zahamowania wzrostu znacznie mniejszych stężeń penicyliny G, ampicyliny, karbenicyliny i azotanu kadmu aniżeli ich plazmidowe szczepy macierzyste. Podobnie oddychanie jednego ze szczepów bezplazmidowych było hamowane przez znacznie niższe stężenia jonów Cd^{2+} niż oddychanie wyjściowego szczepu opornego. Badania izotopowe wykazały, że zahamowanie oddychania gronkowców było rezultatem wiązania się tego metalu z komórkami, lecz determinowana przez plazmidy penicylinazy różnica we wrażliwości na kadm pomiędzy szczepami zależna była od stężenia tego metalu w środowisku. Bezplazmidowy wariant wiązał $^{115m}Cd^{2+}$ we wszystkich zastosowanych stężeniach (od 10^{-3}M do 10^{-6}M), co zawsze prowadziło do zahamowania jego oddychania. Natomiast szczep plazmidowy wiązał te jony tylko wtedy, gdy stężenie w środowisku wynosiło 10^{-3}M. Kadm w niższych stężeniach nie wiązał się z komórkami szczepu opornego i jego oddychanie również nie było hamowane w tych warunkach. Należy więc wnosić, że geny plazmidu obdarzają komórkę jakąś barierą chroniącą ją przed wniknięciem jonów kadmu, ale że bariera ta działa tylko w zakresie stężeń niższych od 10^{-3}M.

РЕЗЮМЕ

Исследовалось влияние удаления плазмидов пенициллиназы из штаммов *Staphylococcus aureus* на их чувствительность к некоторым пенициллинам и ионам кадмия. Установлено, что бесплазмидовые варианты требовали от заторможения роста значительно меньших концентраций пенициллина, ампицеллина, карбенициллина и нитрата кадмия, чем их выходные штаммы. Также и дыхание одного из бесплазмидовых штаммов тормозилось значительно меньшими концентрациями ионов Cd^{2+}, чем дыхание выходного антибиотико-устойчивого штамма.

При помощи изотоповых исследований установлено, что заторможение дыхания *Staphylococcus* было результатом связывания этого металла с клетками, а разница устойчивости к кадмию между штаммами, обусловленная плазмидами пенициллиназы, зависела от концентрации этого металла в среде. Бесплазмидовый вариант связывал 115 м Cd^{2+} во всех примененных концентрациях (от 10^{-3} М до 10^{-6} М), что всегда приводило к заторможению его дыхания. Зато плазмидный штамм связывал эти ионы только тогда, когда концентрация в среде равнялась 10^{-3} М. Кадмий меньших концентраций с клетками антибиотико-устойчивого штамма не связывался и его дыхание в этих условиях также заторможено не было. Следует предположить, что гены плазмида наделяют клетку каким-то барьером, охраняющим ее от проникания ионов кадмия, но этот барьер действует только в области концентраций, меньших 10^{-3} М.