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THE DISTRIBUTION OF CADMIUM BETWEEN CELLULAR SUBFRACTIONS IN CADMIUM-SENSITIVE AND CADMIUM-RESISTANT STAPHYLOCOCCUS AUREUS

By

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KORKEALA, H.: *The distribution of cadmium between cellular subfractions in cadmium-sensitive and cadmium-resistant Staphylococcus aureus.* Acta vet. scand. 1979, *20*, 438—446. — The accumulation and distribution of cadmium (Cd) between cellular subfractions were studied in Cd-resistant Staphylococcus 3719+ and its plasmid-negative Cd-sensitive variant 3719—, grown in media containing 0.01, 0.1 or 50 mg of Cd/litre. In the media containing 0.01 and 0.1 mg of Cd/l the uptake of Cd by the Cd-resistant S. aureus was significantly less than that by Cd-sensitive S. aureus, both in toto and in terms of the cell wall fractions. Only variant 3719+ grew in the medium containing 50 mg of Cd/l. The finding of a lag phase in the growth of both variants is interpreted to suggest that the resistance is of inducible character, even in the sensitive S. aureus. The mechanism of resistance to Cd could consist of both barrier and clearance functions.

cadmium resistance; Staphylococcus aureus; plasmid; cellular subfractions; adaptation.

Novick & Roth (1968) have reported that certain strains of Staphylococcus aureus are resistant to inorganic ions, including cadmium (Cd). *Chopra* (1971) and *Tynecka et al.* (1975) have suggested that the resistance of S. aureus to Cd mediated by the penicillinase plasmid may be due to the permeability barrier, which retains the ion outside the cell. *Mitra et al.* (1975) and *Macara* (1978) have observed that Escherichia coli and Saccharomyces cerevisiae can accommodate to the presence of toxic but sublethal levels of Cd, and that this accommodation does not appear to result from a selection of mutant cells.

The aim of the present work was to make clearer the mechanism of this resistance, by studying the accumulation and distri-

bution of Cd between cellular subfractions, at an early stationary phase of growth in a Staphylococcus aureus strain which carries resistance to cadmium ions, and in its plasmid-negative Cd-sensitive variant, in media containing different Cd concentrations.

MATERIAL AND METHODS

The test organism

The microbial strain used in the study was Staphylococcus aureus strain 3719+ and its plasmid-negative variant 3719—. S. aureus 3719+ (phage type 52/80/81) is resistant to penicillin streptomycin and tetracycline; it carries a penicillin resistance plasmid and a resistance to Cd ions. Both variants were obtained from Dr. K. G. H. Dyke, the Department of Biochemistry, University of Oxford, England.

Chemicals and water

All chemicals were pro analysis grade. The NaCl and $Na_2HPO_4 \times 2H_2O$ were products of E. Merck, Darmstadt, German Federal Republic. $NaH_2PO_4 \times 2H_2O$ was obtained from BDH Chemicals, Poole, England and $CdCl_2 \times 2½ \times H_2O$ from J. T. Baker, Phillipsburg, N.J., USA. Lysozyme was obtained from the Sigma Chemical Co., St. Louis, Mo., USA.

The water used throughout the experiments was double-distilled and deionized. Before the experiments the Cd content of the water was determined by flameless atomic absorption spectrophotometry and found to be below the lowest limit of detection (0.2 ng).

Growth conditions

Cells from 20 h cultures of both variants of S. aureus were grown separately in a shaker at 35°C, in an autoclaved medium containing 10 g yeast extract (Difco Laboratories, Detroit, Mich., USA) and 1 g D-glucose (BDH Chemicals) per l of double distilled deionized water containing different amounts of Cd. Sterile $CdCl_2$ solutions were added to the medium after autoclaving. The final Cd concentrations added were 0.01, 0.1 and 50 mg of Cd/l medium. Growth was monitored with a Klett-Summerson photoelectric colorimeter (filter no. 42; Klett Manufacturing Co., New York, USA). At the early stationary phase

of growth cells were harvested by centrifugation, washed four times with an isotonic NaCl solution and lyophilized (Delta I, Martin Christ, Ostrode am Harz, German Federal Republic) according to the method of *Tornabene & Edwards* (1972).

Cellular subfractions

Portions of the lyophilized cellular preparations were first converted to protoplasts, as described by *Mitchell & Moyle* (1956) and *Brown* (1961). The cells were incubated for 1 h at 25°C in 0.05 M sodium phosphate buffer at pH 6.8, containing 1 M sodium chloride (P-S medium) and 50 μg lysozyme per ml. The suspension contained 3 mg of the lyophilized bacteria/ml. The protoplasts were harvested by centrifugation at 1200 × g for 5 min and washed twice with P-S medium. The combined supernatant solutions, which contained lysozyme and the products of its action on the cell wall, were designated "cell wall fraction". The sedimented protoplasts were broken by shaking with cold distilled water. The cytoplasmic fraction was then separated from the membrane fraction by centrifugation at 20000 × g for 15 min at 2°C, washed, and centrifuged again (*Brown, Tornabene & Edwards*). These washings were added to the cytoplasmic fraction. This procedure was carried out four times for each Cd concentration, except for S. aureus 3719— in the medium containing 50 mg of Cd/l, where the microbe failed to grow in 10 days.

Analysis method

The Cd content of the cellular subfractions was determined by flameless atomic absorption spectrophotometry. The apparatus used was the Perkin-Elmer 303 atomic absorption spectrophotometer (Norwalk, Conn., USA), equipped with a graphite furnace and graphite cell power supply HGA 72 (Überlingen, German Federal Republic).

RESULTS

The distribution of Cd between cellular subfractions of S. aureus 3719— and S. aureus 3719+, grown in media containing different amounts of Cd, is presented in Tables 1 and 2, respectively.

Table 1. Distribution of cadmium between cellular subfractions of plasmid-negative Cd-sensitive Staphylococcus aureus 3719 — at early stationary phase of growth. Values are means of four separate preparations from each culture.

	Cells of S. aureus 3719— grown in a medium containing 0.01 mg of added Cd per l		Cells of S. aureus 3719— grown in a medium containing 0.1 mg of added Cd per l	
	μg of Cd per 30 mg of lyophilized cells	percentage of total Cd	μg of Cd per 30 mg of lyophilized cells	percentage of total Cd
Cell wall fraction	1.005 ± 0.081 ($\bar{x} \pm s$)	77	2.355 ± 0.532 ($\bar{x} \pm s$)	85
Membrane fraction	0.280 ± 0.046 ($\bar{x} \pm s$)	21	0.305 ± 0.133 ($\bar{x} \pm s$)	11
Cytoplasmic fraction	0.023 ± 0.003 ($\bar{x} \pm s$)	2	0.105 ± 0.022 ($\bar{x} \pm s$)	4
Total	1.308 ± 0.093 ($\bar{x} \pm s$)		2.766 ± 0.605 ($\bar{x} \pm s$)	

The statistical significance of the results obtained on the total uptake of Cd and the distribution of Cd between cellular subfractions of S. aureus 3719— and 3719+, grown in media containing 0.01 or 0.1 mg of added Cd per l, is presented in Table 3.

When S. aureus 3719— was grown in the medium containing 0.1 mg of Cd/l, it exhibited a longer lag phase (> 3 h) than the same microbe grown in the medium containing 0.01 mg of Cd/l (Fig. 1). In the latter instance the lag phase was less than 1 h. Similarly S. aureus 3719+ exhibited a longer lag phase (> 15 h) in the medium containing 50 mg of Cd/l than the same microbe grown in the medium containing 0.01 or 0.1 mg of Cd/l. In both concentrations the lag phase was less than 1 h. S. aureus 3719— was unable to grow, within 10 days, in the medium containing 50 mg of Cd/l.

DISCUSSION

The results presented in Tables 1, 2 and 3 showed that the total uptake of Cd by plasmid-harbouring S. aureus 3719+ is significantly less than that of its plasmid-negative variant 3719—, when incubated in media containing 0.01 or 0.1 mg of Cd/l ($P < 0.001$ and $0.001 < P < 0.01$, respectively). This is in agreement with the theory of a permeability barrier by the resistant plasmid-containing cells, suggested by *Chopra* (1971) and *Tynecka et al.* (1975). The S. aureus 3719— and even the S. aureus 3719+, however, took up Cd moderately also at low Cd concentrations; the permeability barrier controlled by one or more plasmid genes is thus obviously, even at low concentrations of Cd, more of relative than of absolute character.

Table 2. Distribution of cadmium between cellular subfractions of Cd-resistant Staphylococcus aureus 3719 + carrying a penicillin resistant plasmid at early stationary phase of growth. Values are means of four separate preparations from each culture.

	Cells of S. aureus 3719 + grown in a medium containing 0.01 mg of added Cd per l		Cells of S. aureus 3719 + grown in a medium containing 0.1 mg of added Cd per l		Cells of S. aureus 3719 + grown in a medium containing 50 mg of added Cd per l	
	μg of Cd per 30 mg of lyophilized cells	percentage of total Cd	μg of Cd per 30 mg of lyophilized cells	percentage of total Cd	μg of Cd per 30 mg of lyophilized cells	percentage of total Cd
Cell wall fraction	0.500 ± 0.140 ($\bar{x} \pm s$)	70	0.984 ± 0.271 ($\bar{x} \pm s$)	74	19.977 ± 2.798 ($\bar{x} \pm s$)	88
Membrane fraction	0.197 ± 0.029 ($\bar{x} \pm s$)	28	0.336 ± 0.082 ($\bar{x} \pm s$)	25	2.494 ± 0.466 ($\bar{x} \pm s$)	11
Cytoplasmic fraction	0.016 ± 0.007 ($\bar{x} \pm s$)	2	0.012 ± 0.008 ($\bar{x} \pm s$)	1	0.290 ± 0.058 ($\bar{x} \pm s$)	1
Total	0.714 ± 0.129 ($\bar{x} \pm s$)		1.332 ± 0.340 ($\bar{x} \pm s$)		22.762 ± 3.210 ($\bar{x} \pm s$)	

Table 3. Statistical significance of results obtained on total uptake of cadmium and distribution of Cd between cellular subfractions of Cd-resistant Staphylococcus aureus 3719+ and its plasmid-negative Cd-sensitive variant 3719— grown in media containing 0.01 or 0.1 mg of added Cd per l. The statistical evaluation was carried out by Student's t-test.

	Cells of S.aureus 3719— and 3719+ grown in a medium containing 0.01 mg of added Cd per litre, difference between the means	Cells of S.aureus 3719— and 3719+ grown in a medium containing 0.1 mg of added Cd per litre, difference between the means
Total uptake	$P < 0.001$	$0.001 < P < 0.01$
Cell wall fraction	$P < 0.001$	$0.001 < P < 0.01$
Membrane fraction	$0.01 < P < 0.05$	not significant
Cytoplasmic fraction	not significant	$P < 0.001$

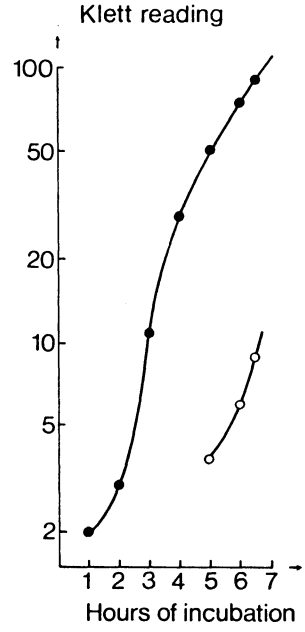

Figure 1. The beginning of the growth of the plasmid-negative cadmium-sensitive variant of Staphylococcus aureus 3719— incubated in the medium containing 0.01 and 0.1 mg of added Cd per l. Turbidities of the cultures were measured with the Klett-Summerson photoelectric colorimeter (filter no. 42). Each point represents means of four separate determinations. Cd-sensitive S. aureus incubated in the medium containing 0.01 mg of Cd/l is presented by closed circles and the same organism incubated in the medium containing 0.1 mg of Cd/l by open circles.

The results further showed that the Cd content of the cell wall fractions of S. aureus 3719— was significantly greater than the corresponding Cd content of S. aureus 3719+ when both were grown in media containing 0.01 or 0.1 mg of Cd/l ($P < 0.001$ and $0.001 < P < 0.01$, respectively). The Cd content of the membrane fraction or of the cytoplasmic fraction of S. aureus 3719— was not significantly greater than in the case of S. aureus 3719+ at either of these Cd concentrations. It thus seems that the plasmid-mediated Cd resistance is expressed in the structure and/or function of the cell wall of the Cd-resistant S. aureus. This resistance to Cd, the permeability barrier, could be due to proteins coded by one or more plasmid genes and ultimately located in outer parts of the cell wall. To what extent the results are influenced by the fact that the measurements were made in cells at the early stationary phase of growth, and not in the exponential phase, is not known. A background concentration of Cd in the cells of both variants could be due to the presence of dead cells, but the differences in Cd content of the cell fractions or the corresponding differences between the variants, which are the significant finding, would remain to be explained by characteristics of a resistance mechanism.

Chopra and *Tynecka et al.* studied the uptake of Cd during 60 min and found that there was an immediate and marked fall in the rate of uptake of Cd by resistant cells. According to these observations the resistance could be due more to constitutive synthesized than to inducible proteins, although the plasmid-coded inducible proteins could also have been immediately present in these experiments. Whether the discrepancies between the present results and those by *Chopra* and *Tynecka et al.*, who did not find any significant concentration of Cd in the resistant cells at the exponential phase of growth, could be due to methodological differences, remains to be clarified.

When Cd concentrations in the growth media were increased, the total uptake of Cd by both variants of S. aureus increased also. Correspondingly the Cd concentrations of all cellular subfractions increased except those of the cytoplasmic fractions of S. aureus 3719+ incubated in media containing 0.01 and 0.1 mg of Cd/l. At these medium concentrations the barrier seems to inhibit the increase in the influx of Cd into the cytoplasm of the cell. When the Cd concentrations in the growth media increased, the observed increase in the Cd content was always greatest, in both absolute and relative terms, in the cell wall fractions. In

the medium containing 50 mg of Cd/l, where S. aureus 3719— was unable to grow, the total uptake of Cd and the Cd content of the cell wall of S. aureus 3719+ were relatively high. In addition, the cell wall fraction contained the highest share of the total Cd in the present experiments. Although at lower Cd concentrations the total uptake of Cd and the Cd content of the cell wall fraction were smaller in the case of the Cd-resistant S. aureus than in that of the Cd-sensitive variant, this does not exclude the possibility that the cell wall of a resistant S. aureus, under extreme Cd conditions, could immobilize and tolerate relatively higher concentrations of Cd than the Cd-sensitive variant, without fatal disturbance in its metabolic functions necessary for growth and survival. The other possibility is that the Cd concentration of the cell wall of the sensitive variant could reach Cd levels, at the maximum external Cd concentrations tolerable to it, as high as those reached by the cell wall of the resistant S. aureus at its respective maximum external Cd concentration.

Lag phases were observed when S. aureus 3719— was incubated in the medium containing 0.1 mg of Cd/l (Fig. 1) and S. aureus 3719+ in the medium containing 50 mg of Cd/l. Both variants can thus evidently accommodate to the presence of toxic but not immediately lethal levels of Cd. The phenomenon of adaptation suggests, as perhaps the most likely explanation, that a higher level of resistance is built up through the inducible synthesis of one or more proteins specific for the purpose. The presence of a lag period would further agree with a theory of a primary, merely bacteriostatic effect by Cd, and a subsequent clearance of Cd from the bacterial body inside the cell wall to sub-bacteriostatic levels, possibly through the action of the freshly synthesized proteins just hypothesized. These proteins could also participate in the construction of the barrier in the cell wall. It is also at least thinkable that the adaptation could be due to inducible proteins somehow "inactivating" Cd within the cell. The studies of *Mitra et al.* (1975) on Escherichia coli showed that the share of the Cd in the cell wall was less, and the share of the Cd in the membranes greater, in terms of percentage of total Cd, in unaccommodated than in accommodated cells. *Mitra et al.* also found that the highest Cd content in the cell wall was recorded at the late lag phase. These data would also fit the hypothesis presented above, that the inducible proteins synthesized during the later phase of the adaptation period transport Cd out of the bacterial body.

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REFERENCES

Brown, J. W.: Composition of fractions prepared from Sarcina lutea protoplasts. Biochim. biophys. Acta 1961, *52*, 368—374.

Chopra, I.: Decreased uptake of cadmium by a resistant strain of Staphylococcus aureus. J. gen. Microbiol. 1971, *63*, 265—267.

Macara, I. G.: Accommodation of yeast to toxic levels of cadmium ions. J. gen. Microbiol. 1978, *104*, 321—324.

Mitchell, P. & J. Moyle: Liberation and osmotic properties of the protoplasts of Micrococcus lysodeikticus and Sarcina lutea. J. gen. Microbiol. 1956, *15*, 512—520.

Mitra, R. S., R. H. Gray, B. Chin & I. A. Bernstein: Molecular mechanisms of accommodation in Escherichia coli to toxic levels of Cd^{2+}. J. Bact. 1975, *121*, 1180—1188.

Novick, R. P. & C. Roth: Plasmid-linked resistance to inorganic salts in Staphylococcus aureus. J. Bact. 1968, *95*, 1335—1342.

Tornabene, T. G. & H. W. Edwards: Microbial uptake of lead. Science 1972, *176*, 1334—1335.

Tynecka, Z., J. Zajac & Z. Goś: Plasmid dependent impermeability barrier to cadmium ions in Staphylococcus aureus. Acta. microbiol. polon., Ser. A 1975, *7*, 11—20.

SAMMANFATTNING

Distributionen av kadmium mellan bakteriecellens subfraktioner i kadmiumkänslig och kadmiumresistent Staphylococcus aureus.

Ackumulationen och distributionen av kadmium (Cd) mellan bakteriecellens subfraktioner undersöktes på en Cd-resistent Staphylococcus aureus 3719+ och dess plasmid-negativa, Cd-känsliga variant 3719— i medier som innehöll 0,01, 0,1 eller 50 mg Cd/liter. I medier som innehöll 0,01 eller 0,1 mg Cd/l var både det totala upptaget av Cd och ackumulationen av Cd i cellväggen signifikant större hos den Cd-känsliga än hos den Cd-resistenta varianten. Endast den Cd-resistenta varianten växte i mediet, som innehöll 50 mg Cd/l. Förekomst av lagfas under växten vid bägge varianterna ansågs som ett bevis för den inducerbara karaktären av resistensen även på den Cd-känsliga S. aureus. De undersökta bakteriernas resistens mot Cd ansågs kunna bestå av både barriär- och eliminationsfunktioner.

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