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A Simple Method for the Determination of Bacterial Resistance to Metals

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Bacterial resistance to heavy metals in the environment can result in bioaccumulation, biotransformation, changes in ecological diversity and co-selection of resistance factors for antibiotics (STERRIT & LESTER 1980). The use of microorganisms for the removal of heavy metals from industrial effluents and as indicator organisms in bioassays have also been investigated recently (REMACLE & HOUBA personal communication; ANDERSON & ABDELGHANI 1980). Fundamental to all such studies is the determination of the susceptibility of different bacterial species to a wide range of heavy metals.

Many of the techniques used previously are time consuming (e.g. agar dilution (OLSON & THORNTON 1981) and continuous flow cultures (MAYFIELD et al. 1980)) or require specialised instrumentation (e.g. radioactive marker techniques (TAN 1980)). As a result research has often been limited to selected metal compounds and bacterial species. In order to estimate bacterial resistance patterns to a large number of metals, a simple, rapid but reliable method is required.

The toxicities of five elements to bacteria were determined by SMITH et al. (1982) who modified the antibiotic susceptibility test of BAUER et al. (1965). The present study describes some further modifications to this method, including the standardization of experimental parameters and the quantification of the metal concentrations across the inhibition zone.

MATERIALS AND METHODS

Oxoid CM3 (nutrient agar) was reconstituted and heated to dissolve prior to bottling in 100 ml volumes which were autoclaved at 121°C for 15 min. Oxoid CM115 (MacConkey agar) was reconstituted and treated as for the nutrient agar. Tryptone (Oxoid L42) was prepared as a 1% solution in 0.5% sodium chloride and distributed in 5 ml volumes in capped tubes and autoclaved at 121°C for 15 min.

Whatman AA discs (diameter 13 mm) were sterilized in a hot air oven at 160°C for 1 h in glass petri dishes. Stock solutions (1000 µg/ml) of copper and cadmium were prepared in distilled water using analar grade metal chlorides. A stock culture of E. coli (NCTC 10148) was grown in MacConkey agar for 18 h to determine purity. A few representative colonies were subcultured into

tryptone water and incubated at 37°C for 18 h. The standard inoculum was prepared by transferring 0.1 ml of this subculture to a fresh 5 ml volume of tryptone water immediately prior to the test. Eppendorf pipette tips and 10 ml glass pipettes were wrapped in aluminium foil and autoclaved at 121°C for 15 min. Pre-sterilized plastic petri dishes (85 mm diameter) were used.

In order to prepare metal diffusion gradients, nutrient agar was melted and cooled to 50°C and 10 ml volumes were transferred aseptically to each petri dish. Metal-impregnated discs were prepared in duplicate by dropping 0.1 ml of the stock metal solution onto the disg (100 µg metal per disc). The discs were placed on the agar surface and the plates incubated at 37° C for 18 h.

After incubation, twenty strips of agar (15mm x 2 mm) were removed sequentially from the edge of the disc to the edge of the plate. Each strip was weighed, digested with 2 ml concentrated nitric acid in a glass vial and evaporated to dryness. A 2 ml aliquot of 10% v/v nitric acid was added to dissolve the sample residue. The metal concentrations in these solutions were determined by atomic absorption spectrometry. The results were calculated as µg/g metal in agar and the diffusion gradient plotted in terms of metal concentration and distance from the edge of the disc.

Agar diffusion was used to test for bacterial sensitivity to metals. The nutrient agar was melted and cooled to 50°C and 5 ml standard inoculum per 100 ml agar was added. Even distribution of the inoculum was achieved by gentle rotation and 10 ml volumes added to each petri dish. Metal-impregnated discs, prepared in duplicate as for the diffusion gradients, were placed on the agar surface within 30 min of the preparation of the seeded agar plates and the plates incubated at 37°C for 18 h.

After incubation the zones of inhibition of bacterial growth as visible to the naked eye, were measured with a micrometer and recorded in mm zone size. The zones were measured from the outer edge of the disc to the inner edge of the normal growth zone and included areas of diffuse growth when these were present. Agar strips were then removed from the plate and metal concentrations determined as described previously.

RESULTS AND DISCUSSION

The results of these experiments are illustrated in Figures 1 and 2. It is apparent that both cadmium and copper diffuse from the disc into the agar medium and that metal concentrations in the agar decrease exponentially with increasing distance from the disc (Figs. 1A and 2A). Diffusion with depth was also investigated by placing a metal-impregnated disc on 20 mm deep agar and cutting serial sections beneath the disc. The decrease in metal concentrations with increasing distance from the disc at the surface was the same as for the horizontal diffusion gradient.

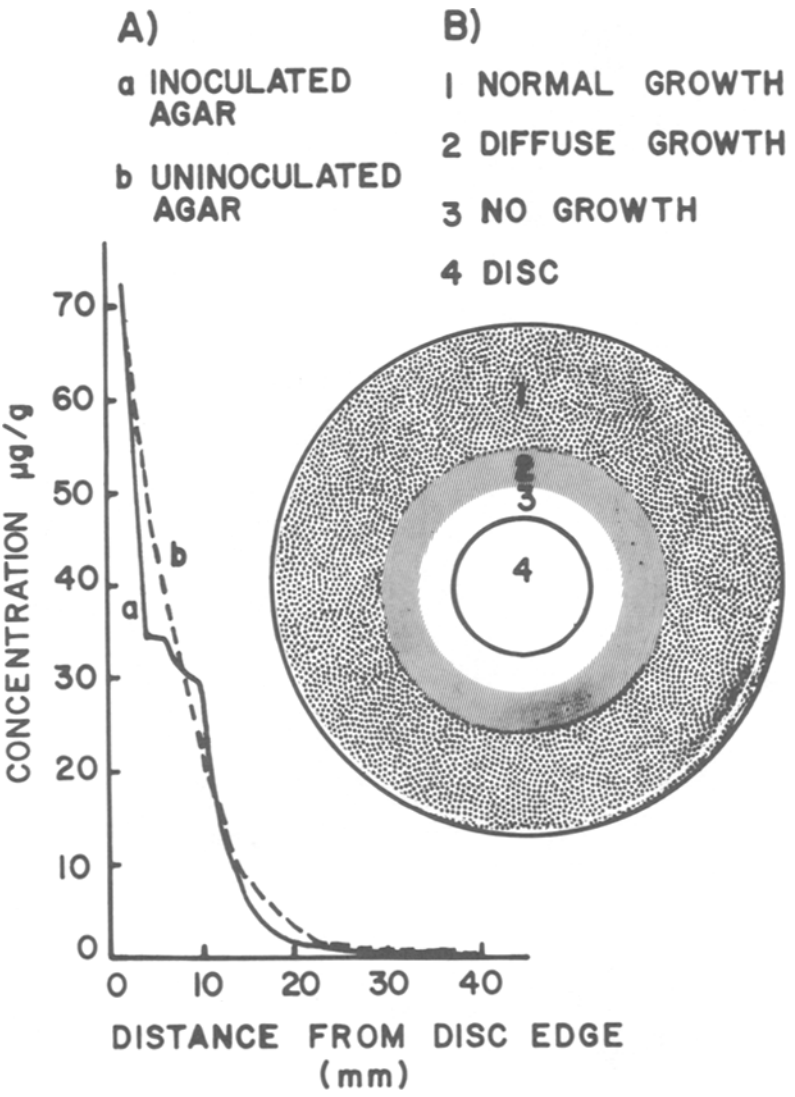

Fig. 1. Cadmium. A) Diffusion gradients
B) Bacterial zones.

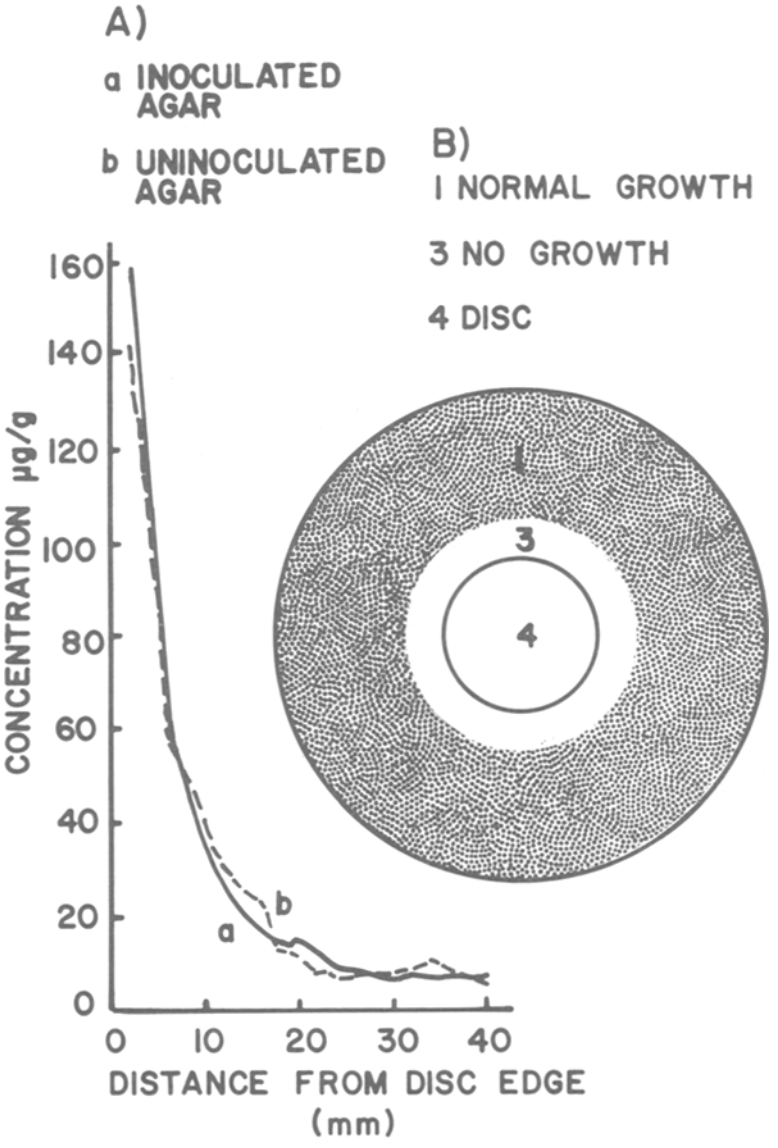

Fig. 2 Copper. A) Diffusion gradients
B) Bacterial zones.

These diffusion gradients are reproducible under the standardized conditions but are element specific; copper chloride diffuses with greater ease than cadmium chloride, as indicated by the metal concentration present in the agar at any given distance from the disc.

In the presence of bacteria, the gradient of the slope is altered due to interactions between the metal and the microorganism and irregularities in the curve profile are apparent. This is particularly so for cadmium (Fig. 1B) where, in the region of the diffuse zone, the decrease in cadmium concentration with distance from the disc is temporarily interrupted and in this region there appears to be retention of the cadmium by the bacteria. Bacterial accumulation of cadmium has been described by BABICH & STOTZKY (1978) and is also discussed by GADD & GRIFFITHS (1978). In the normal growth area of the plate the two curves are almost identical and it is probable that little cadmium accumulation is taking place.

The concentration of the metal at the edge of the zone is read off the diffusion gradient graph to give the minimum inhibitory concentration (MIC) for that metal compound and microorganizm. Results for cadmium and copper are listed in Table 1.

TABLE 1. Zone sizes and MIC values for copper and cadmium

Metal	Zone size (mm)	MIC (µg/g)
Cu	2.6	148
	2.8	144
Cd	9.4	29.5
	10.0	29.5

The zones of inhibition of bacterial growth are well defined for both elements. Two distinct zones are evident for cadmium, one of total inhibition (no growth) and one of partial inhibition (diffuse growth) extending beyond the total inhibition zone (Fig. 1B). The MIC of cadmium was determined at the outer edge of the zone of diffuse growth (29.5 µg/g).

The principal aim of this study was to develop a simple, rapid method for the determination of bacterial resistance to a wide range of metals. The method described is a modification of the antibiotic susceptibility test used by SMITH et al. (1982).

Preliminary studies showed that several factors influenced the reproducibility of zone sizes. In an international interlaboratory study on single disc diffusion for antibiotic testing (ERICSSON & SHERRIS 1971) it was concluded that non standardization of the inoculum concentration was the biggest single factor in the variability of the results. Tests in this laboratory, using metals instead of antibiotics, have shown that the standard

inoculum detailed here yields reproducible results for Gram-negative bacteria but that further modification may be required for Gram-positive strains.

Metal concentrations in individual discs varied by up to 200% when a known volume of standard metal solution is added to several discs together. The addition of the metal solution to each disc using an Eppendorf pipette immediately prior to placing the disc on the seeded agar surface ensures that accurate metal concentrations are achieved for each test.

The nutrient agar media was selected for this method because it yielded clear, well-defined zones and allowed the metals to diffuse evenly, as shown by the diffusion gradients (Figs. 1A and 2A). The volume of agar used (and hence depth) was a critical factor in achieving reproducible results, since zone sizes decreased proportionately with increased agar volumes

Another factor which influenced zone size was the time lapse between seeding the agar with the inoculum and placing the discs upon the plate. One possibility arising out of this factor is that the effects of metals on different phases of bacterial growth could be estimated by comparing the inhibition zones produced when the discs are transferred to plates at set time intervals.

By defining the parameters of type and depth of media, concentration and distribution of inoculum, and preparation of the metal-impregnated discs, reproducible results can be achieved in repeated comparative tests. Nevertheless, there remains the inherent variability of any bio-assay test so that to quote and rely on the absolute minimum inhibitory concentrations which have been determined is optimistic and unjustified. Instead it is suggested that a semiquantitative assessment of the results be made in terms of moderately sensitive, sensitive and very sensitive. Zone sizes equivalent to these terms are up to 2.5 mm 2.5–11.0 mm and >11.0 mm respectively; resistant bacteria are those which grow right up to the edge of the disc. Zone sizes are particularly useful when the effects of a given metal are being assessed in a comparative study using several bacterial species. Greater care must be taken when comparing the effects of different metals on a single bacterial species.

For the purpose of this study, only one disc was used per plate but it is quite possible to place four discs containing different metal solutions on a single plate. The method lends itself to comparative investigations concerning the effects of a wide range of metals on an equally wide range of microorganisms. Results are obtained within 36 h and the plotted diffusion gradients can be used to quantify the metal concentrations to which the bacteria are sensitive.

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