Loss of Sensitivity to EDTA by *Pseudomonas aeruginosa* Grown under Conditions of Mg-Limitation

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SUMMARY

Pseudomonas aeruginosa was grown in batch culture in simple salts medium under conditions of Mg-limitation and varying degrees of Mg-excess. Sensitivity to EDTA was measured in terms of lysis and decrease in colony count. The greater the degree of Mg-limitation the greater was the resistance to loss of viability and lysis. Loss of viability of sensitive bacteria occurred more rapidly than lysis. This suggests that bacterial death preceded cell lysis.

INTRODUCTION

EDTA has been shown to render Gram-negative bacteria sensitive to the action of lysozyme and it has been suggested that EDTA increases the permeability of the outer layers of the cell wall, allowing lysozyme to penetrate to the underlying mucopeptide layer (Costerton *et al.* 1967). Treatment of Gram-negative organisms with EDTA has been shown to result in lysis, loss of viability and release of substances absorbing at 260 m μ (Gray & Wilkinson, 1965*a*; Neu, 1966) and to increased sensitivity to a variety of antibacterial agents (Brown & Richards, 1965; Leive, 1965). The degree of sensitivity of Gram-negative organisms has been shown by Wilkinson (1967) to vary between species. Gray & Wilkinson (1965*b*) found that this sensitivity was related to the ability of EDTA to solubilize components of isolated cell walls of the particular organism and they concluded that the lipopolysaccharide portion was probably involved. The presence of divalent cations in the cell walls of *Pseudomonas aeruginosa* has been demonstrated (Eagon, Simmons & Carson, 1965), and Asbell & Eagon (1966) suggested that EDTA acted by chelating cations involved in cross-linkages with lipopolysaccharide components of the cell wall. Costerton *et al.* (1967) suggested that Mg might be one of the cations involved. The object of the present work was to determine the sensitivity to EDTA of *P. aeruginosa* organisms grown with various Mg concentrations. To avoid changes in sensitivity due to washing procedure (Brown, 1968), unwashed cultures were used.

METHODS

Organism. *Pseudomonas aeruginosa* NCTC 6750 was used throughout this study.

Chemicals. All chemicals used were of AnalarR grade.

Cleaning procedures. All glass-ware was treated with sulphuric+chromic acid mixture, washed with tap water and finally in glass-distilled water.

Culture methods. The culture was maintained in a liquid medium without added Mg

which consisted of: 0.001 M-D(+)glucose, 0.01 M-(NH₄)₂HPO₄, 0.01 M-(NH₄)₂SO₄, 0.0005 M-NaCl, 0.0005 M-KCl dissolved in glass-distilled water (pH 7.3). Bacteria used in the following experiments were grown in media identical to the above, but containing graded concentrations of MgSO₄. Cultures (500 ml.) were grown in 2 l. flasks in a Mickle shaker bath (The Mickle Laboratory Engineering Co. Gomshall, Surrey) at 37.5°. The growth rate was initially the same in all cultures but became increasingly decreased because of Mg limitation. Cultures with the lower concentrations of Mg showed the greater degree of Mg limitation of growth. Cell division eventually ceased owing to depletion of glucose at an extinction (*E*) of about 0.180 measured at 470 mμ by using a Unicam SP 600 spectrophotometer. This corresponded to a colony count of about 3.5×10^8 bacteria/ml. Unwashed cultures in this condition were used for all studies with EDTA and were taken for treatment when the *E*₄₇₀ value had remained constant for 1 hr. The growth medium was at pH 7.3 at inoculation and decreased to 7.2 by the time cell division ceased. This pH value was unaltered by the EDTA treatments used.

Treatment of bacteria with EDTA. Samples (93 ml.) of unwashed culture at 37.5° were added to 7 ml. volumes of EDTA solutions of various concentrations in 250 ml. flasks in a water bath at 37.5°. The EDTA treated cultures (pH 7.2) were maintained at 37.5° throughout the experimental period.

Measurement of lysis. Samples (3 ml.) of EDTA-treated cultures were taken and the extinction of the sample measured at 470 mμ (*E*₄₇₀).

Colony count estimations. Samples (1 ml.) of EDTA-treated bacteria were removed and after inactivation of the EDTA by dilution in nutrient broth (Oxoid) to which CaCl₂ was added, 0.5 ml. volumes were spread on surface-dried nutrient agar plates. Colonies were counted after incubation for 36 hr at 37.5°.

RESULTS

Preliminary experiments indicated a definite Mg requirement for growth by *Pseudomonas aeruginosa*. This accords with the findings of other workers (Webb, 1949) and of Tempest, Hunter & Sykes (1965) who showed Mg to be an essential component of the ribosomes of *Aerobacter aerogenes*. Figure 1 illustrates representative results of growing pseudomonads in the basal medium with the addition of graded amounts of Mg. The lag period was inversely proportional to the Mg concentration originally in the medium. Cell division did not cease abruptly as Mg became limiting, but progressively slowed. This finding was in agreement with that of Tempest *et al.* (1965). Eventually cell division ceased in all cultures owing to depletion of glucose, but at the lower Mg concentrations the final extinction value was lower than that attained by cultures containing higher concentrations of Mg. Presumably during the period of slower growth (depending on Mg concentration) glucose was depleted by cell respiration. Mg concentrations were selected such that, although at the lowest concentrations Mg limitation of growth had occurred, the final *E*₄₇₀ value was close to the maximum possible for the glucose concentration (0.001 M in every case).

Effect of Mg-limitation on lysis of Pseudomonas aeruginosa by EDTA

Suspensions of bacteria were taken for EDTA treatment when the *E*₄₇₀ value had remained constant for 1 hr. Lysis of bacteria grown in the basal medium with the

addition of Mg 1 $\mu\text{g./ml.}$ resulted after treatment with various EDTA concentrations (Fig. 2). This procedure was repeated with bacteria grown in a range of Mg concentrations and the decrease in E_{470} value after 180 min. taken as a measure of sensitivity.

Figure 3 illustrates the effectiveness of several EDTA concentrations in causing lysis, measured for bacteria grown in 0.05, 0.2, 0.5, 1.0, 2.0 and 4.0 $\mu\text{g. Mg/ml.}$ Two distinct

Fig. 1. Effect of magnesium content of medium on growth of *Pseudomonas aeruginosa*. Mg concentrations ($\mu\text{g./ml.}$) were: $\circ$, 0.05; $\blacktriangle$, 0.1; $\diamond$, 0.45; ∇ , 1.

Fig. 2. Effect of EDTA concentration on lysis of *Pseudomonas aeruginosa* grown in medium with 1 $\mu\text{g. Mg/ml.}$ Bacteria not Mg-limited. EDTA concentrations ($\mu\text{g./ml.}$) were—(a): $\times$, zero; $\circ$, 10; $\triangle$, 20; $\square$, 25; $\bullet$, 37.5; and (b): $\times$, 50; $\circ$, 100; $\triangle$, 375; $\square$, 1000.

effects were observable. First, as the EDTA concentration was increased the rate of lysis increased to a maximum for bacteria grown in any one Mg concentration. The EDTA concentration required to produce a maximum effect was greater, the higher

Fig. 3. Effect of EDTA concentration on rate of lysis of *Pseudomonas aeruginosa* grown in media with different Mg concentrations. Mg concentrations ($\mu\text{g./ml.}$) were—(a): $\circ$, 0.5; Δ , 0.2; $\times$, 0.05 and (b): $\times$, 4; $\circ$, 2; Δ , 1.

Fig. 4

Fig. 5

Fig. 4. Maximum rate of lysis by EDTA of *Pseudomonas aeruginosa* grown in media with different Mg concentrations. Maximum rate of lysis at any one Mg concentration can be deduced from Fig. 3.

Fig. 5. Relationship between maximum rate of lysis of *Pseudomonas aeruginosa* by EDTA and Mg concentration in the medium. 375 $\mu\text{g.}$ EDTA/ml. produced maximum rate of lysis over this Mg range.

Fig. 6. Effect of EDTA 375 $\mu\text{g./ml.}$ on the colony count of *Pseudomonas aeruginosa* grown in a basal medium with different Mg concentrations. $\circ$, 0.05 $\mu\text{g.}$ Mg./ml. untreated; $\bullet$, 0.05 $\mu\text{g.}$ Mg./ml. treated with 375 $\mu\text{g./ml.}$ EDTA; $\blacktriangle$, 1 $\mu\text{g.}$ Mg/ml. untreated; $\triangle$, 1 $\mu\text{g.}$ Mg/ml. treated with 375 $\mu\text{g.}$ EDTA/ml.

the Mg concentration in the growth medium. This agrees with earlier work which showed that cations in excess of growth requirements reduced the activity of EDTA against unwashed bacteria (Brown & Richards, 1965). Secondly, over the range of Mg concentrations 0.05 to 0.5 $\mu\text{g./ml.}$ a large change in maximum rate of lysis occurred, while further increases in the Mg concentrations produced only a small increase in the maximum rate of lysis (Fig. 4). To investigate this phenomenon further, bacteria grown in Mg concentrations over the range 0.05 to 0.5 $\mu\text{g./ml.}$ were treated with 375 $\mu\text{g. EDTA/ml.}$, a concentration shown to produce maximum rate of lysis over this range of Mg concentrations. The results (Fig. 5) indicated a linear relationship between the maximum rate of lysis and the concentration of Mg in the growth medium below about 0.3 $\mu\text{g./ml.}$

Effect of EDTA on viable counts of Mg-limited cultures

Colony counts were made at intervals upon bacteria grown in (a) 1 $\mu\text{g. Mg/ml.}$, (b) 0.05 $\mu\text{g. Mg/ml.}$, which had been treated with 375 $\mu\text{g. EDTA/ml.}$ The results (Fig. 6) indicated once again that bacteria grown in the low Mg concentration were much more resistant to EDTA than bacteria produced in the high concentration. After 250 min. the colony count of the Mg-limited bacteria had decreased about 25 %, whereas the bacteria not Mg-limited had a decrease in colony count of about 40-fold. Comparison of these data with details of lysis resulting from EDTA treatment indicated that loss of viability proceeded more rapidly than lysis. This suggested that bacterial death preceded cell lysis.

DISCUSSION

It has been suggested that EDTA acts by removal of divalent cations, probably Ca or Mg, from the cell wall of *Pseudomonas aeruginosa* where these ions occupy positions of structural importance (Asbell & Eagon, 1966). The present results support this concept. Calcium was not added to the medium used in this study and any traces present would have been impurities of the Analar chemicals. In fact, the basal medium contained 0.06 $\mu\text{g. Ca/ml.}$ impurity as determined by flame photometry (Unicam SP 900). The fact that the bacteria grown in the lowest Mg concentration were almost completely resistant to the action of EDTA may have been due to a lack of such cations in the cell-wall architecture. The linear relationship between lysis and Mg concentration in the growth medium probably reflected a relationship between Mg in the medium and the wall structure of the eventual Mg-limited cells. It may also be that, as in the case of *Aerobacter aerogenes*, the organic components of the cell wall differed when the bacteria were deprived of Mg (Ellwood & Tempest, 1967). The above findings indicated that the use of EDTA in classification of pseudomonads (Wilkinson, 1967) must be approached with caution since more or less Mg in the growth medium could greatly affect the result. The effect of Mg in the growth medium may also be important where spheroplast or protoplast production involves EDTA treatment.

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