SHORT ARTICLES

PROLONGED SURVIVAL OF *UREAPLASMA UREALYTICUM* IN LIQUID CULTURE

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BROTH CULTURES of *Ureaplasma* begin to lose viability rapidly after growth for only 16 h (Ford, 1962), apparently due to accumulation of ammonia produced by the breakdown of urea (Shepard and Lunceford, 1967; Ford and MacDonald, 1967); this makes the harvesting and study of the cells difficult. Viability has been preserved over longer periods by continuous culture methods (Brighton *et al.*, 1967) in which replenishment with fresh medium controls the concentration of ammonia, and by passing a current of air across shallow, 1-mm-deep, cultures to remove ammonia (Hendley and Allred, 1972). Another method is to enclose the culture in a dialysis sac surrounded by fresh medium, thereby allowing exchange diffusion of ammonia and fresh nutrients (Masover and Hayflick, 1974). The present paper describes the use of an ion-exchange resin to exchange ammonium ions for sodium ions.

MATERIALS AND METHODS

Organisms. *U. urealyticum*, strain Tc, a vaginal isolate, was used for the preliminary studies, and the later work was done with a freeze-dried culture of *U. urealyticum*, no. ATCC27618.

Media. The liquid medium consisted of distilled water, 55 ml; horse serum (Wellcome No. 3), 20 ml; 25% (w/v) extract of bakers' yeast containing 18% (w/v) Tryptone Soya Broth (Oxoid), 10 ml; 10% (w/v) solution of urea (BDH Analar), 10 ml; 0.4% (w/v) phenol red, 0.5 ml; 1% (w/v) thallium acetate, 1 ml; and penicillin (10 000 units per ml), 2 ml. The medium was adjusted to pH 6.5, with a pH meter, by adding N HCl (prepared by diluting 1 ml of BDH Analar HCl in 10 ml of distilled water). Subsequent adjustments of pH during culture were done by eye.

The formula of the phosphate-buffered agar medium used to titrate numbers of viable organisms in broth cultures has been previously described (Windsor, Edward and Trigwell, 1975), but Fungizone (Squibb), 2.5 µg per ml, was substituted for the thallium acetate.

Both agar and liquid cultures were incubated at 36°C and, in the case of the agar cultures, in an atmosphere of 95% nitrogen and 5% CO₂.

Preparation of ion-exchange resins. Zerolit 225 H⁺ form was converted to the Na⁺ form as follows: 500 g of Zerolit beads were treated with 1000 ml of a 10% (w/v) solution of NaCl (Analar) in distilled water for 24 h, washed in running tap water until testing with phenol red showed an alkaline reaction, again treated with 1500 ml of 10% NaCl solution, and finally washed to remove NaCl by stirring for 1 h in each of three changes of 1500 ml of distilled water. The Ca⁺⁺ form of Zerolit 225 was prepared in the same way but with 1500 ml of a 10% (w/v) solution of CaCl₂ (Analar) instead of NaCl for the second treatment. The washed resins were dried at 37°C and autoclaved at 121°C for 30 min.

Viability titrations. Samples were removed from broth cultures at intervals and 10-fold dilutions were made in Tryptone Soya Broth. These were inoculated, in 0.02 ml amounts,

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FIG. 1.

FIG. 2.

FIG. 1.—Effect of different amounts of Zerolit on survival of *Ureaplasma urealyticum* in liquid culture. Control culture without Zerolit, $\triangle$ — $\triangle$ — $\triangle$. Cultures containing Zerolit: 2.5 g, $\bullet$ — $\bullet$; 5 g, $\blacktriangledown$ — $\blacktriangledown$; 7.5 g, $\blacktriangle$ — $\blacktriangle$; 10 g, $\blacksquare$ — $\blacksquare$.

FIG. 2.—Effect of Zerolit (10 g) and 4-h adjustments of pH to 6.5 with N HCl on survival of *U. urealyticum*. No Zerolit or pH adjustment, $\blacktriangledown$; no Zerolit but pH adjusted, $\blacktriangle$; Zerolit and pH adjusted, $\blacksquare$; Zerolit without pH adjustment, $\square$ — $\square$ († the accuracy of the 24-h count is uncertain). Amount of N HCl required to adjust pH of cultures during the experiment: $\circ$ — $\circ$, culture without Zerolit; $\bullet$ — $\bullet$, culture with Zerolit.

on to agar medium. After incubation for at least 3 days, colonies were counted and viability expressed as log 10 colony-forming units (c.f.u.) per ml.

RESULTS

Initial studies showed that Zerolit 225 (Na^+ form) prolonged the survival of *U. urealyticum* in broth cultures only if the pH was maintained at 6.5 by adding N HCl every 4 h. The Ca^{++} form of Zerolit 225 gave similar results, although it produced a white precipitate (which contained 14–18% Ca^{++}) during broth culture and required less acid for pH adjustment. After use and washing with distilled water, both forms of the resin released ammonia in 0.1N-HCl or in 10% NaCl solution; the free ammonia was detected with Nessler's reagent.

The effect of different concentrations of resin was then examined. The Na^+ form was added, in 2.5-, 5.0-, 7.5- and 10-g amounts, to 20 ml of medium in 110-ml screw-capped, "medical flat" bottles; medium without Zerolit was included as a control. Of an actively growing culture of *U. urealyticum* no. ATCC27618, 0.2 ml was inoculated into each bottle, and the pH was adjusted to approximately 6.5 with HCl every 4 h during incubation. At 12-h intervals, samples were removed and counts were performed (fig. 1). In the control culture, without Zerolit, viable organisms were present at the minimum dilution tested, 1 in 100, at 36 h but not at 48 h. On the other hand, in the presence of Zerolit the survival time increased, reaching 72 h with 10 g. In every case, about 3 ml of HCl were needed to control the pH during the incubation period. A further control culture (not shown in fig. 1), which did not contain Zerolit and was not adjusted with HCl, achieved a viable count of 10^6 c.f.u. per ml at 12 h, but at 24 h no viable organisms were detected in a sample diluted 100-fold.

As before, when the Zerolit beads from the various cultures were thoroughly washed and suspended in 10% NaCl solution, a heavy brown precipitate formed immediately on adding Nessler's reagent.

Although 10 g of Zerolit absorbed almost the entire 20 ml of liquid medium, it allowed longest survival of *U. urealyticum* and was therefore used for a further growth-curve experiment. Four 20-ml cultures were set up, as above, two containing Zerolit and two without. Only one of the cultures containing Zerolit and one of those without were adjusted every 4 h with HCl; the others were left untouched. At 12-h intervals samples were removed and counts were performed, on three plates per dilution (fig. 2). Viable organisms were detected for only 12 h in the untouched culture without Zerolit, and for only 24 h in both the untouched culture with Zerolit and the pH-adjusted culture without Zerolit. By contrast, the combination of Zerolit and pH adjustment led to survival of organisms for 72 h. The amounts of N HCl required to adjust the pH during incubation were very similar for the cultures with and without Zerolit, even during the later stages when most of the organisms in the culture without Zerolit were no longer capable of forming colonies on subculture.

DISCUSSION

The adsorption and fixation of ammonium ions from *U. urealyticum* cultures by Zerolit were demonstrated by the subsequent release of the ions in NaCl solution, and the ion-exchange reaction of the sodium form can be expressed as $R^-Na^+ + NH_4^+ + OH^- \rightleftharpoons R^-NH_4^+ + Na^+ + OH^-$, and of the calcium form as $(R^-)_2Ca^{++} + 2NH_4^+ + 2OH^- \rightleftharpoons 2(R^-NH_4^+) + Ca(OH)_2$. Thus, the ammonium-ion concentration is controlled but not the alkalinity.

The ability to extend the growth cycle by pH adjustment alone suggests that the rapid death of ureaplasmas in conventional cultures is due to alkalinity rather than to ammonium-ion accumulation. However, this is limited presumably by the accumulation of ammonium ions to a toxic level at acid pH, as prolonged survival is dependent on the presence of Zerolit. A previous attempt to use an ion-exchange resin for culture of ureaplasmas was unsuccessful (Ford and MacDonald, 1967).

It is hoped to extend this technique to large volume cultures.

SUMMARY

The growth cycle of *U. urealyticum* cultures was extended by continual pH adjustment in the presence of an ion-exchange resin.

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