

CHAPTER 42

**Regrowth of *Pseudomonas aeruginosa* Following Treatment with a Formaldehyde Condensate Biocide**

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Treatment of cultures of *Pseudomonas aeruginosa* with a formaldehyde condensate biocide (hexahydro-1,3,5-tris ethyl-s-triazine) resulted in the phenomenon of regrowth. Regrowth is a sudden increase in colony-forming units following a period of undetected viability (eclipse period). The time of treatment required to reach undetectable survivors (<0.03 colony-forming units per ml) and the length of the eclipse period were dependent upon the initial cell density, the manner in which the culture was prepared, and the concentration of biocide used. Regrowth occurs in trypticase soy broth but not in mineral salts medium, and preliminary experiments suggest that the organisms responsible for regrowth exist in an undetectable form rather than undetectable numbers; that is, the survivors may be injured cells temporarily protected from the biocide within multicellular aggregates. These injured cells are incapable of colony formation until biocide inactivation and damage repair have occurred.

INTRODUCTION

Formaldehyde condensates are one important group of antimicrobial compounds. There is a great chemical variety among the formaldehyde condensate biocides, and they are used widely in industrial contamination control. Their antimicrobial activity presumably results from formaldehyde release (Rossmore 1983). Their ultimate shortcoming, common to formaldehyde itself, is poor biocidal activity against fungi (Paulus 1976; DeMare et al. 1972; Rossmore and Holtzman 1974; Rossmore 1979). The mode of action of formaldehyde condensate compounds has not been defined clearly, although a series of reports (DeMare et al. 1972; Bennett 1973; Scott and Wolf 1962) suggest that low levels of formaldehyde act by blocking methionine biosynthesis via cyclization with homocysteine. A series of studies is being conducted in our laboratory to determine the factors that affect neutralization, the methods used to evaluate their effectiveness, and the possible mode of action of these compounds on microorganisms isolated from industrial systems.

This report describes the results of studies evaluating the action of one formaldehyde condensate biocide (hexahydro-1,3,5-tris ethyl-s-triazine, commercially known as Vanicide TH) against a common industrial contaminant, *Pseudomonas aeruginosa*. The unexpected observation that regrowth of this bacterium occurs after long periods of undetected viability (<0.03 colony-forming units [cfu]/ml) is described. The phenomenon of "regrowth" of *Ps. aeruginosa* following treatment with  $\beta$ -lactam antibiotics has been reported (Gwynn et al. 1981). This regrowth, however, was a recovery in population densities from low but detectable levels ( $10^3$ - $10^4$  cfu/ml) rather than recovery from undetectable levels as reported here.

## MATERIALS AND METHODS

*Media and Culture Preparation*

An isolate of *Ps. aeruginosa* obtained from contaminated metal-working fluid was used for these experiments. Cultures were maintained on trypticase soy agar (TSA) or Mineral Salt Base agar (Palleroni and Duodoroff 1972) containing 0.1% pyruvate as a carbon source (MSB-Py). Cultures were grown in trypticase soy broth (TSB) or MSB-Py broth at 30 C overnight. To insure that the cultures were growing exponentially, overnight cultures were transferred to fresh media for 3–5 h prior to use. Fresh, exponential cultures at  $1-3 \times 10^7$  cfu/ml were used in all experiments.

*Biocide Treatment*

A 10% stock solution of Vancide TH (hexahydro-1,3,5-tris ethyl-s-triazine) in distilled water was used (% active ingredient, w/v). The biocide was added to duplicate 250-ml flasks containing fresh culture to a final volume of 100 ml incubated in a rotary shaker at 250 rpm at 30 C. The effect of the biocide was evaluated by standard plate counts (pour-plate method), and the plates were observed for colony formation for up to 7 d to determine if delayed growth occurred.

For some experiments, volumes of the treated culture greater than 1 ml were evaluated for microbial survival. The treated culture was concentrated 15-fold by centrifugation ( $27,000 \times g$  for 15 min) prior to the standard plate count. Alternatively, a volume of 30 ml was filtered through an 0.45  $\mu$ m membrane filter (Gelman), rinsed with TSB, and incubated on an absorbent pad containing TSB. The filter was flooded with oxidase reagent (tetramethyl-*p*-phenylene-diamine dihydrochloride) after 48 h incubation and evaluated for oxidase positive cfu.

*Evaluation of the Effect of Cell Aggregation*

To evaluate the effect of cell aggregation on the observed regrowth, 100 ml of exponential culture (about  $10^8$  cfu/ml) were vigorously vortexed at 4 C and centrifuged at  $10,000 \times g$  for 30 min. The cell pellet was resuspended in fresh TSB and the vortex/centrifugation process repeated. This washed-vortexed cell fraction then was compared to an identical 100-ml untreated culture. The objective of this treatment was to minimize the number of cell aggregates in the culture and to remove loosely adhering extracellular polysaccharides.

## RESULTS

*Effect of Biocide on Pseudomonas aeruginosa in TSB*

Preliminary experiments using shake-tube cultures in TSB confirmed that Vancide TH was an effective biocide against *Ps. aeruginosa*. The minimum inhibitory concentration (MIC) was 300–400 ppm. Observations of visible turbidity in shake tubes showed that 200–300 ppm Vancide TH significantly delayed the time required for growth to occur, and 400 ppm appeared to totally inhibit growth (no visible turbidity in 90 h compared to 4 h for the untreated control).

The effect of various concentrations of Vancide TH on viable cell counts in 250-ml shake flasks is shown in Fig. 1. As expected, there is an initial rapid decrease in viable cell numbers with increasing biocide concentration. At intermediate biocide

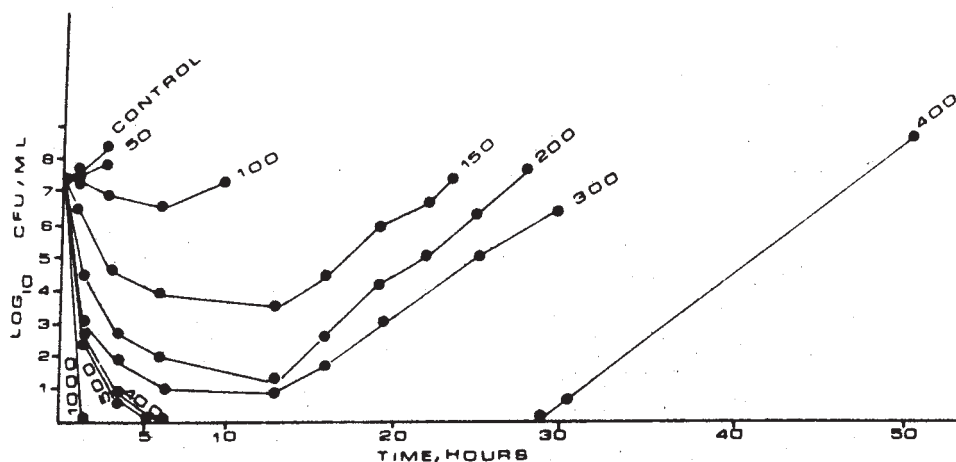


FIG. 1. Treatment of *Pseudomonas aeruginosa* with Vancide TH in TSB. The ppm Vancide TH used is shown.

concentrations (100 to 300 ppm), this initial decrease to low but detectable cell numbers was followed by a lag and subsequent recovery. At a biocide concentration of 400 ppm, there were no detectable survivors in 1 ml of culture suspension after 6 h of treatment. This apparent total kill, however, was followed by regrowth after a total treatment time of approximately 30 h. In some experiments, this "lag" was extended for up to 7 d before regrowth was observed. The time delay before regrowth occurs and the maximum concentration of biocide that results in regrowth are dependent upon the concentration of the initial microbial inoculum, that is, higher initial cell density reduces the lag preceding regrowth and increases the concentration of biocide required to prevent regrowth.

The observation of  $<1$  cfu/ml during the lag preceding regrowth could be due to residual biocide transfer. Since no attempt was made to neutralize biocide in the media used for the standard plate counts, 1 ml of the culture suspension contained an approximate 1:20 dilution of the initial biocide concentration (approximately 20-ml volume of pour-plate medium). Although it is unlikely that such levels of residual biocide could inhibit colony formation, two techniques were used to check this possibility. (1) During the lag period that precedes regrowth, centrifugation of 30 ml of culture suspension followed by resuspension of the cell pellet in 2 ml of fresh TSB or (2) membrane filtration of 30 ml of culture suspension both resulted in undetectable cfu (therefore  $<1$  cfu/30 ml) (Fig. 2).

#### *Effect of Biocide in MSB-Py*

Evaluation of Vancide TH in MSB-Py is shown in Fig. 3. The MIC is lower in this defined medium (100–200 ppm) than in TSB (300–400 ppm), but the pattern of inhibition is similar. Treatment with 50 or 100 ppm Vancide TH results in a decrease in viability and a delay preceding recovery growth. Observations of cellular morphology during this time indicate that macroscopic aggregate formation has occurred (Fig. 4). The specific plate counts during this time may therefore be much lower than viable cell number. At higher biocide concentrations, 200 to 300 ppm, the specific plate counts are reduced to undetectable levels ( $<1$  cfu/ml), and no regrowth is observed even after 10 d of incubation. It is not apparent why regrowth occurs in TSB but not

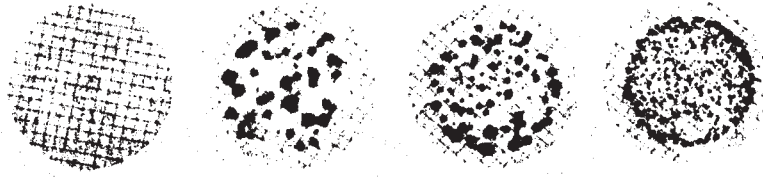


FIG. 2. Membrane filters (0.45  $\mu\text{m}$ ) developed with oxidase reagent. A culture of *Ps. aeruginosa* was treated with 400 ppm Vancide TH in TSB. A volume of 30 ml was filtered, incubated on absorbent pads containing TSB for 48 h, and then flooded with oxidase reagent to detect oxidase-positive colonies. The time of treatment (left to right) was: 7, 14, 22, and 30 h.

in MSB-Py, but preliminary experiments indicate that TSB results in significant biocide inactivation (unpubl. results).

#### Cell Aggregates and Regrowth

Regrowth occurs after biocide treatment has reduced the microbial population to undetectable levels ( $<0.03$  cfu/ml). This observation suggests the possibility that a portion of the population exists in an undetectable form rather than in undetectable numbers. An initial attempt to test this possibility involved washing the cells to remove loosely adhering extracellular polysaccharides and disruption of cell aggregates by vortexing. Vigorous vortexing of TSB cultures prior to the initiation of biocide treatment reduced the number of cell clumps and aggregates.

The results of washing and vortexing such cultures as compared to their untreated counterpart are shown in Fig. 5. During the initial period of treatment with biocide, the washed-vortexed cell cultures showed greater numbers of cfu than untreated cultures at all concentrations of biocide. Presumably, there are greater initial numbers of cfu in the treated cultures due to aggregate disruption. The culture treated with 300 ppm Vancide TH was reduced to undetectable levels after 17 h for the untreated culture (containing aggregates), and 23 h in the washed and vortexed culture (containing fewer

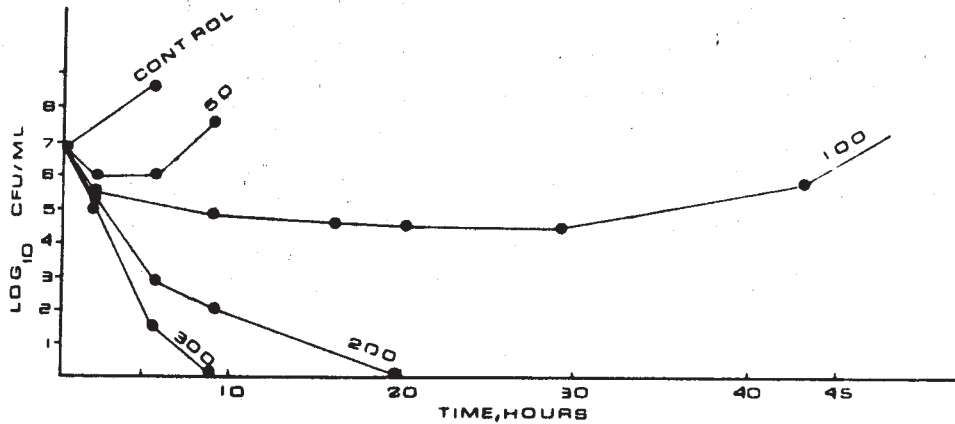


FIG. 3. Treatment of *Ps. aeruginosa* with Vancide TH in MSB-Py medium. The ppm Vancide TH used is shown.



FIG. 4. Cell aggregates of *Ps. aeruginosa* treated with 50 ppm Vancide TH for 6 h in MSB-Py. The aggregates were stained with crystal violet: (a) Total aggregate at 450X magnification; (b) Edge of the aggregate, including some unattached cells at 1,000X magnification.



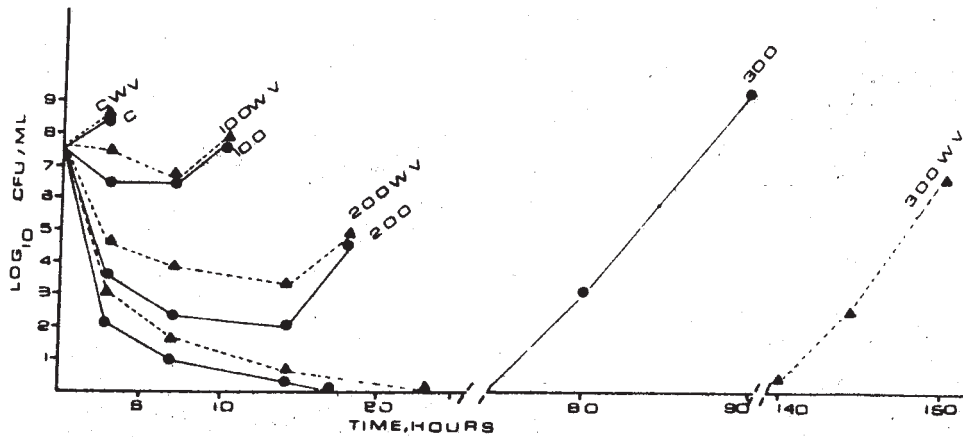


FIG. 5. Treatment of *Ps. aeruginosa* with Vancide TH in TSB. Washed/Vortexed Cells (▲—▲ WV); Untreated (●—●).

aggregates). The untreated culture showed regrowth after about 70 h of treatment, whereas in the treated culture regrowth was delayed until about 140 h (Fig. 6).

Although there is more than one interpretation of these results, a possibility is that the cells which grow after a long delay survive in multicellular aggregates. Removal of extracellular polysaccharides and aggregate disruption by the washing-vortex procedure results in a delay in regrowth.

#### DISCUSSION

Treatment of cultures of *Ps. aeruginosa* with Vancide TH in TSB resulted in the observation of regrowth (that is, a sudden increase in cfu following a long period of undetectable survivors). The efficacy of this formaldehyde condensate is cell density-dependent, suggesting that an increase in the number of "targets" increases the requirement for biocide. Vancide TH is more effective in MSB-Py than in TSB, and regrowth was not observed. This is consistent with the concept of targets for the biocide since TSB contains organic constituents that may serve as noncellular targets prior to the biocide reaching its site of biocidal activity in cells. Preliminary experiments support this idea since Vancide TH is inactivated during storage in TSB.

Marrie and Costerton (1981) observed regrowth of *Serratia marcescens* in solutions containing chlorhexidine. They also observed massive cell aggregates embedded in a fibrous polysaccharide material, but these embedded cells showed morphological abnormalities, as did the cells free in solution, and they were unable to determine if the polysaccharide matrix provided protection against the antimicrobial. Costerton and Lashen (1983) have suggested, however, that bacteria present in attached surface biofilms show an inherent biocide resistance.

The observations made here suggest that multicellular aggregates of *Ps. aeruginosa* embedded in extracellular slime (presumably polysaccharide) do provide some protection against Vancide TH and are responsible for the regrowth phenomenon. Massive cell aggregates were observed in biocide-treated cultures in TSB and MSB-Py (Fig. 4). Partial disruption of cell aggregates and removal of extracellular polysaccharides

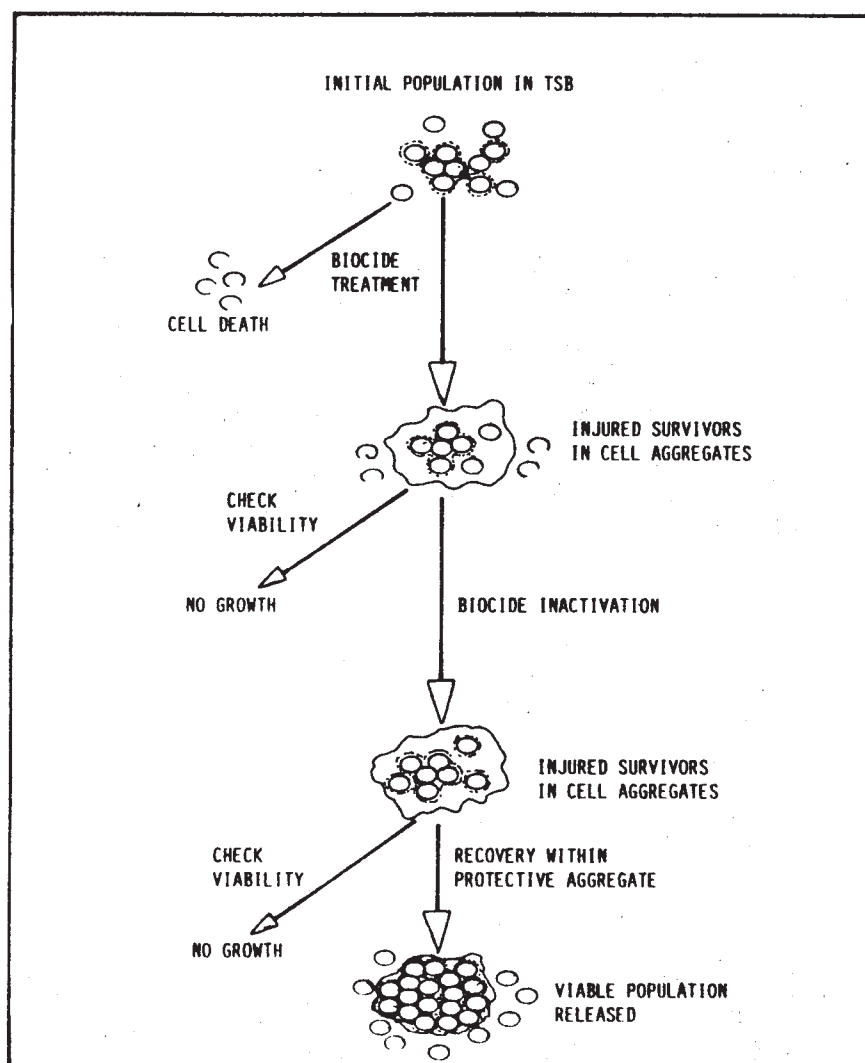


FIG. 6. Possible mechanism for the phenomenon of regrowth. (See text for details.)

in TSB prior to biocide treatment increased the eclipse period and delayed the onset of regrowth (Fig. 5).

During the eclipse period, no cfu were detectable ( $<0.03$  cfu/ml). It is possible that the survivors that later result in regrowth are simply present in numbers too low to detect ( $<1/30$  ml in a total volume of 100 ml), but it is also possible that the survivors are present in an undetectable form (Fig. 6). Injured cells embedded in a multicellular aggregate may be able to survive in the high osmotic strength environment of the aggregate yet not be able to form a colony on nutrient medium. Later, when biocide is exhausted due to interaction with available targets, either organic components of the medium or cellular debris, recovery of injured cells can occur followed by the acquisition of the ability to form colonies on nutrient medium.

Although these interpretations are speculative, they suggest questions that may lead to a better understanding of the strengths and weaknesses of formaldehyde condensate biocides. Are the targets for these biocides on the cell surface? If the primary target for Vancide TH was an intracellular target rather than the cell surface, then the effect of aggregates would not have been observed; that is, cells with "internal injuries" should be able to recover and form colonies on nutrient medium.

How do the organic components in TSB mimic cell targets and result in biocide inactivation? An understanding of the nature of the formaldehyde condensate target may prove to have practical significance in controlling contamination in industrial systems. The sudden increase in the microbial population in a treated industrial system may result from underdosing; that is, allowing the survival of injured cells in aggregates or biofilms for the time required for biocide inactivation to occur.

Further studies of the precise mode of action of formaldehyde condensate biocides and the factors that affect the regrowth phenomenon are necessary in order to provide the information needed to improve the efficient application of these compounds.

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