ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1991, p. 341–344
0066-4804/91/020341-04$02.00/0

Vol. 35, No. 2

Damage to the Cytoplasmic Membrane and Cell Death Caused by Dodine (Dodecylguanidine Monoacetate) in *Pseudomonas syringae* ATCC 12271

JOÃO P. S. CABRAL

Instituto Nacional de Investigação Científica, Centro de Citologia Experimental da Universidade do Porto, Rua do Campo Alegre, 823, 4100 Porto, Portugal

Received 27 September 1990/Accepted 13 November 1990

Treatment of *Pseudomonas syringae* cells with low concentrations of the fungicide dodecylguanidine monoacetate (dodine) resulted in cell death and leakage of K^+, UV-absorbing materials, and ribose-containing molecules. The results suggest that dodine causes gross and extensive damage to the cytoplasmic membrane, which is probably implicated in the death of cells.

Pseudomonas syringae van Hall is one of the most important plant pathogens, having a very wide host range and causing spots, necrosis of leaves, twigs, and fruits, and stem cankers (1).

Dodine (dodecylguanidine monoacetate) was introduced in 1956 by the American Cyanamid Company (Princeton, N.J.) as a protective fungicide (30). It is an effective fungicide against apple scab caused by *Venturia* spp. (23–25) and cherry leaf spot caused by *Coccomyces hiemalis* (10), and it has also been used with success in controlling several bacterial plant diseases (9, 21). Dodine is a soluble amphiphile (surfactant) consisting of a hydrophobic apolar group (C_{12} hydrocarbon chain) and a positively charged hydrophilic polar head group (guanidine).

In vitro, dodine severely affects the metabolism of fungal cells. Low concentrations of dodine inhibit growth (2, 8, 26, 31), respiration on glucose and acetate (2, 26), and active transport of ^{32}P, [^{14}C]glucose, [^{14}C]acetate, and L-[^{14}C] phenylalanine (2, 19, 26).

The literature on the action of dodine in bacterial cells is very scarce. It has been reported that dodine inhibits growth and O_2 uptake in *Rhizobium* spp. (13, 29) and the nitrogenase activity of several soil bacteria (18).

The development of an effective chemical control of bacterial plant diseases demands an understanding of the mechanisms of action of active chemical agents. The aim of this study was to evaluate the bactericidal and membrane-damaging activities of dodine in *P. syringae* in order to contribute to a better understanding of the mode of action of this fungicide in bacterial cells.

MATERIALS AND METHODS

Chemicals. Dodine (molecular weight, 287) (analytical grade; solubility in water, 0.063% [wt/vol], according to the manufacturer) was a gift from American Cyanamid Company. Other chemicals were reagent grade.

Culture and cell suspensions. *P. syringae* ATCC 12271 was grown in a semisynthetic medium as previously described (3, 5). Cells were suspended in distilled water (pH 6.5) or 10 mM Na^+-dimethylglutaric acid (DMGA) buffer (10 mM DMGA, pH adjusted to 6.5 with NaOH) to a final concentration of approximately 0.28 mg (dry weight) of cells ml^{-1} (approximately 10^9 CFU ml^{-1}).

Biochemical determinations. Cell suspensions were treated with dodine concentrations ranging from 5 to 75 μM at room temperature (22 to 23°C). At intervals, samples were withdrawn and filtered through a 0.45-μm-pore filter (Sartorius). Biochemical determinations were carried out with the filtrates. K^+ was assayed by using flame photometry, UV-absorbing materials were assayed by measuring the A_{260}, and ribose-containing molecules were assayed by using the orcinol reaction with RNA as the standard, as previously described (4). The total concentration of pool metabolites in untreated suspensions in Na^+-DMGA buffer was determined by extraction with cold perchloric acid, as previously described (4).

Lethality studies. (i) Cell viability. Samples were withdrawn from treated suspensions and serially diluted in distilled water prior to plating on nutrient agar (Difco) plates (four replicates). For each sample (0.05 to 0.1 ml), the total volume of diluent was 20 ml, and the total time for dilution and plating was approximately 3 min. With this method, the concentration of dodine in the diluted sample was negligible (<0.3 μM) and did not influence the assay of viability. The colonies were counted after 3 days at room temperature.

(ii) Determination of the lethal times for 50 and 90% killing. In a quantal phenomenon, such as cell death caused by the action of a chemical agent, the mortality is generally a sigmoidal function of the dose or time. This curve can be converted into a straight line by the probit transformation (12, 14). The values of the parameters of the formula probit of the percentage of dead cells versus $\log_{10}$ time were determined by iterative weighted-regression analysis according to the maximum-likelihood method (12). An original computer program in Fortran IV (7) was adapted to Mallard Basic (Amsoft, Brentwood, England) and was run in an Amstrad PCW 8256 computer working in single precision. The values of the lethal times for each dodine concentration were calculated from the regression equation.

RESULTS

The bactericidal and membrane-damaging activities of dodine in *P. syringae* were assessed in cells suspended in distilled water or Na^+-DMGA buffer. In these suspending media, control (untreated) cells leaked small amounts of K^+, UV-absorbing materials, and ribose-containing molecules (Fig. 1 and 2). After 4 h of incubation, cells in both suspending media remained fully viable. These results indi-

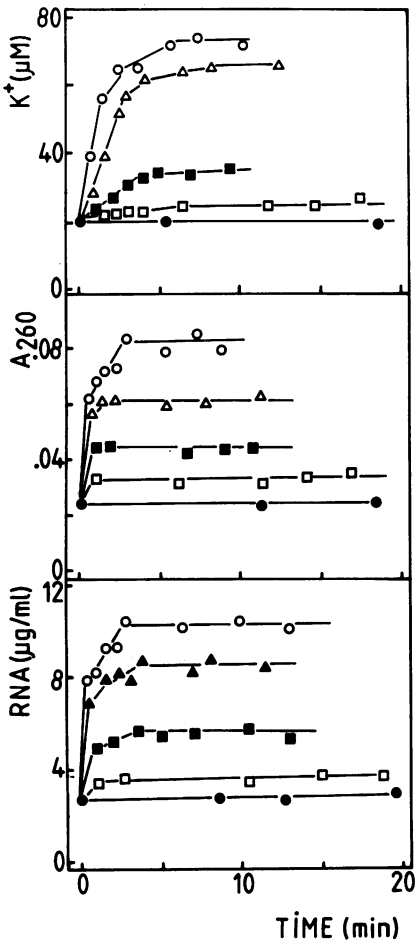

FIG. 1. Time course of leakage of potassium, UV-absorbing materials, and ribose-containing molecules in *P. syringae* cells. The cells were suspended in 10 mM Na^+-DMGA buffer (pH 6.5) and treated with 10 (□), 20 (■), 30 (△), 40 (▲), and 50 (○) μM dodine. Values for controls (●) are also shown. Biochemical determinations were carried out with the supernatants. K^+, UV-absorbing materials, and ribose-containing molecules were assayed as described in the text. The results are from a representative experiment.

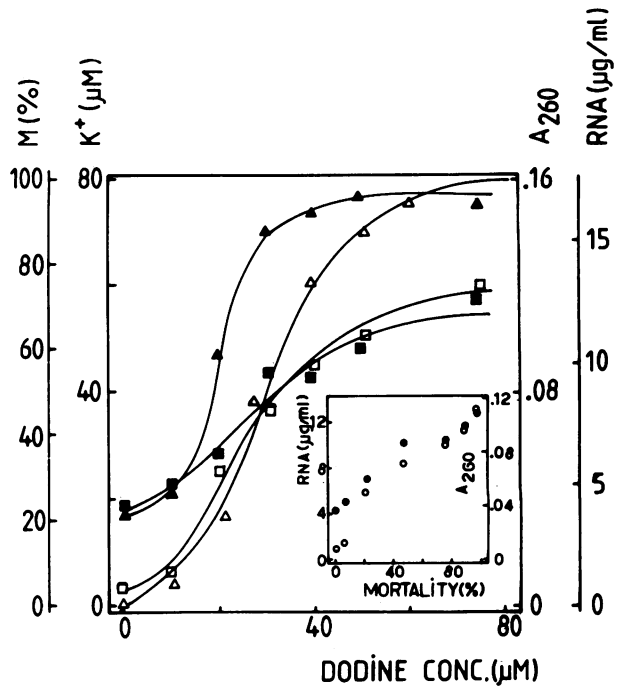

FIG. 2. Mortality (△) and leakage of potassium (▲), UV-absorbing materials (■), and ribose-containing molecules (□) in *P. syringae* cells suspended in distilled water (pH 6.5) and treated with dodine for 15 min. The results of cell viability are from two experiments. Other results are from representative experiments. (Inset) Relationship between the amounts of released UV-absorbing materials (●) and ribose-containing molecules (○) and the percentage of cells killed in *P. syringae* cells suspended in distilled water and treated with dodine for 15 min. Biochemical determinations were carried out with the supernatants. K^+, UV-absorbing materials, and ribose-containing molecules were assayed as described in the text. Cell viability was assessed by plating on nutrient agar.

cate that suspension of *P. syringae* cells in distilled water or Na^+-DMGA buffer did not cause appreciable cell damage. The extent of metabolite release in these media was similar to that in Na^+-PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer, a medium previously used (3–5) to study the action of cupric ions on *P. syringae* cells.

Treatment of *P. syringae* cells suspended in distilled water or Na^+-DMGA buffer with low concentrations of dodine resulted in a fast leakage of K^+, UV-absorbing materials, and ribose-containing molecules (Fig. 1). In both suspending media, after 15 min of dodine treatment, an increase in dodine concentration resulted in an increase in the amounts of leaked K^+, UV-absorbing materials, and ribose-containing molecules (Fig. 2). Comparable levels of release of these metabolites were observed in cells suspended in distilled water and in Na^+-DMGA buffer. In both suspending media, a pronounced efflux of K^+, UV-absorbing materials, and ribose-containing molecules was observed with dodine concentrations higher than 30 to 40 μM. With times of exposure to this surfactant of up to 15 min, in the range of concentrations used, no significant number of lysed cells was observed.

Whereas the process of potassium leakage was essentially completed in less than 20 min after the addition of dodine, the efflux of UV-absorbing materials and ribose-containing molecules continued for at least 8 h. This secondary release of metabolites was a linear function of time. Within the range of 10 to 60 μM dodine, an increase in the surfactant concentration resulted in an increase in the rate of release of UV-absorbing materials and ribose-containing molecules.

The effect of the fungicide on the viability of *P. syringae* cells suspended in distilled water and treated with dodine concentrations in the range of 10 to 60 μM was evaluated. It was found that treatment of the cells with dodine concentrations higher than 40 μM resulted in a rapid and pronounced decrease in cell viability (Table 1). In *P. syringae* cells suspended in distilled water and treated with 10 to 60 μM dodine for 15 min, the amounts of leaked UV-absorbing materials and ribose-containing molecules were almost directly proportional to the percentage of dead cells (Fig. 2, inset).

DISCUSSION

Untreated *P. syringae* cells suspended in distilled water or Na^+-DMGA buffer leaked small amounts of K^+, UV-absorbing materials, and ribose-containing molecules and re-

TABLE 1. Effect of dodine concentration on *P. syringae* cell viability[a]

Dodine concn (μM)	LT (min)[b]	
	50%	90%
10	2.8×10^3	—[c]
20	3.0×10^3	—
30	2.8×10^1	2.5×10^5
50	1.8×10^{-1}	2.2×10^1
60	1.5×10^{-1}	4.2×10^0

[a] Cells were suspended in distilled water at an initial concentration of approximately 10^9 CFU ml^{-1}. Cell viability was assessed by plating on nutrient agar. The values of the parameters of the formula probit of the percentage of dead cells versus $\log_{10}$ time were determined by iterative weighted-regression analysis (12). The LTs for each dodine concentration were calculated from the regression equation.

[b] LT, Lethal time; 50% and 90%, lethal time for killing of 50 and 90% of the initial population, respectively.

[c] —, Number too big; no biological significance.

mained fully viable, indicating that these suspending media did not cause significant cell stress. It has been reported that some *Rhizobium* strains remained viable and able to nodulate after being stored in water, at room temperature, for 1 year or longer (6). Other authors showed that after 20 or 24 years of storage in distilled water at 10°C, the great majority of the isolates of *Agrobacterium tumefaciens* and fluorescent *Pseudomonas* spp. were still alive, and almost all the isolates of *P. syringae* subsp. *syringae* maintained their ability to produce the toxin syringomycin and were pathogenic to bean seedlings (17). It seems, therefore, that distilled water, although it does not provide a buffered environment, can be used to suspend bacteria (at least certain species) without causing important stress and damage.

Low concentrations of dodine induced rapid leakage of K^+, UV-absorbing materials, and ribose-containing molecules from *P. syringae* cells, indicating damage to the cytoplasmic membrane. It has been reported that dodine can disrupt the selective permeability of the cytoplasmic membrane of eucaryotic cells. In fungal cells, dodine induces the loss of ^{32}P (2, 26), P_i (2), amino acids (2), and UV-absorbing materials (2, 26, 27) and increases the permeability of the cytoplasmic membrane to externally added Ni^{2+} (19). In plant cells, dodine induces an efflux of betacyanin and total ions (28). Other cationic amphiphiles (namely, cetyltrimethylammonium bromide, chlorhexidine, and polymyxins) have been reported to cause severe damage to the cytoplasmic membrane in several bacterial species (11, 16, 20, 22).

Treatment of *Escherichia coli* cells with dodecyldiethanolamine causes a rapid leakage of K^+, UV-absorbing materials, ribose- and deoxyribose-containing molecules, and P_i, corresponding to the release of low-molecular-mass metabolites (pool metabolites) (18a). The concentrations of pool metabolites in control (untreated) cell suspensions of *P. syringae* in Na^+-DMGA buffer were determined as described in Materials and Methods and found to be 80 μM K^+, an A_{260} of 0.16, and 16 μg of RNA ml^{-1}. If the rapid initial release of K^+, UV-absorbing materials, and ribose-containing molecules from *P. syringae* cells treated with dodine corresponds to the leakage of pool metabolites, then the results presented in this study indicate that treatment of *P. syringae* cells with dodine concentrations in the range of 40 to 75 μM for 15 min resulted in the release of the great majority of the intracellular K^+ ions and of a significant fraction of the pool of UV-absorbing materials and ribose-containing molecules. These results, therefore, suggest that the damage to the cytoplasmic membrane of *P. syringae* cells caused by dodine concentrations higher than 40 μM was gross and extensive.

It has been reported that leakage of UV-absorbing materials and of ribose-containing molecules induced by cationic amphiphiles in bacteria is usually a biphasic process: after a rapid efflux caused by the loss of the selective permeability of the cytoplasmic membrane, there is a slow leakage at an approximately constant rate (15, 16, 20). In the present study, a similar secondary release of UV-absorbing materials and ribose-containing molecules in *P. syringae* cells treated with dodine was observed. In *Pseudomonas aeruginosa* cells treated with polymyxin, the secondary leakage of UV-absorbing materials and ribose-containing molecules is a consequence of an enzymic degradation of RNA (20). In the present study, it was shown that *P. syringae* cells treated with dodine release UV-absorbing materials and ribose-containing molecules at a constant rate and that the amount of leaked metabolites usually exceeds the pool concentration. These results suggest that the secondary release of UV-absorbing materials and ribose-containing molecules in *P. syringae* cells treated with dodine is probably also a direct consequence of an enzymic degradation of RNA. Further studies are needed to confirm this hypothesis.

Low concentrations of dodine were bactericidal to *P. syringae* cells. In other bacterial species, similar high bactericidal activity of dodine has been reported. The growth (expressed in cell mass) of *Rhizobium trifolii* in a liquid culture in the presence of 10- and 50-ppm dodine (approximately 35 and 175 μM) was 17 and 4%, respectively, of the control growth. Growth on the surface of a solid medium was completely arrested in the presence of 50-ppm dodine (13). Paper disks soaked in a 50-ppm dodine solution caused a detectable inhibition zone in a medium previously inoculated with *Rhizobium japonicum* (29).

Dodine concentrations that in this study induced massive leakage of potassium and low-molecular-mass compounds also caused pronounced cell death. The amount of leaked UV-absorbing materials and ribose-containing molecules was proportional to the percentage of cells killed. These results suggest that membrane damage caused by dodine was implicated in the death of *P. syringae* cells. In a study of the mode of action of cetyltrimethylammonium bromide in several bacterial species, it has also been concluded that membrane damage was directly implicated in cell death (22). In several gram-negative bacteria, it was shown that, after 20 min of treatment with polymyxin E, the amount of UV-absorbing materials (at A_{260}) released from the cells was proportional to the percentage of cells killed (11), also suggesting that damage to the cytoplasmic membrane was involved in the death of the cells.

ACKNOWLEDGMENTS

I am especially indebted to A. R. W. Smith (Thames Polytechnic, London, England) and M. T. Silva (Centro de Citologia Experimental) for guidance and helpful discussions.

This work was supported by Instituto Nacional de Investigação Científica (grants PB/1 and 83-CEN-12) and by Junta Nacional de Investigação Científica e Tecnológica (grant PMCT/CEN/67-90).

REFERENCES

1. **Bradbury, J. F.** 1986. Guide to plant pathogenic bacteria. CAB International Mycological Institute, Kew, England.
2. **Brown, I. F., and H. D. Sisler.** 1960. Mechanisms of fungitoxic action of *n*-dodecylguanidine acetate. Phytopathology **50**:830–839.

3. **Cabral, J. P. S.** 1989. Induction of potassium efflux by cupric ions in *Pseudomonas syringae* ATCC 12271 and its correlation with cell viability. Microbios **60**:141–150.
4. **Cabral, J. P. S.** 1990. Cupric ions induce both an efflux of potassium and low molecular mass metabolites in *Pseudomonas syringae*. FEMS Microbiol. Lett. **72**:109–112.
5. **Cabral, J. P. S.** 1990. Plasmolysis induced by very low concentrations of Cu^{2+} in *Pseudomonas syringae* ATCC 12271, and its relation with cation fluxes. J. Gen. Microbiol. **136**:2481–2487.
6. **Crist, D. K., R. E. Wyza, K. K. Mills, W. D. Bauer, and W. R. Evans.** 1984. Preservation of *Rhizobium* viability and symbiotic infectivity by suspension in water. Appl. Environ. Microbiol. **47**:895–900.
7. **Davies, R. G.** 1971. Computer programming in quantitative biology. Academic Press, Inc. (London), Ltd., London.
8. **De Waard, M. A., and J. G. M. Van Nistelrooy.** 1983. Negatively correlated cross-resistance to dodine in fenarimol-resistant isolates of various fungi. Neth. J. Plant Pathol. **89**:67–73.
9. **Diener, U. L., and C. C. Carlton.** 1960. Dodine-captan combination controls bacterial spot of peach. Plant Dis. Rep. **44**:136–138.
10. **Eisensmith, S. P., and A. L. Jones.** 1981. Infection model for timing fungicide applications to control cherry leaf spot. Plant Dis. **65**:955–958.
11. **Few, A. V., and J. H. Schulman.** 1953. The absorption of polymyxin E by bacteria and bacterial cell walls and its bactericidal action. J. Gen. Microbiol. **9**:454–466.
12. **Finney, D. J.** 1952. Probit analysis. Cambridge University Press, Cambridge.
13. **Fisher, D. J.** 1976. Effects of some fungicides on *Rhizobium trifolii* and its symbiotic relationship with white clover. Pestic. Sci. **7**:10–18.
14. **Hewlett, P. S., and R. L. Plackett.** 1979. The interpretation of quantal responses in biology. Edward Arnold, London.
15. **Hugo, W. B., and M. Frier.** 1969. Mode of action of the antibacterial compound dequalinium acetate. Appl. Microbiol. **17**:118–127.
16. **Hugo, W. B., and A. R. Longworth.** 1964. Some aspects of the mode of action of chlorhexidine. J. Pharm. Pharmacol. **16**:655–662.
17. **Iacobellis, N. S., and J. E. DeVay.** 1986. Long-term storage of plant-pathogenic bacteria in sterile distilled water. Appl. Environ. Microbiol. **52**:388–389.
18. **Jagnow, G., O. Heinemeyer, and S. Draeger.** 1979. Choice of liquid, semisolid, or soil suspension media: an important factor modifying the effect of pesticides on the nitrogenase (C_2H_2) activity of *Clostridium pasteurianum*, *Azotobacter chroococcum*, and *Spirillum lipoferum* Beijerinck. Ecotoxicol. Environ. Saf. **3**:152–158.
18a. **Lambert, P. A.** 1974. Ph.D. thesis. Thames Polytechnic, London.
19. **Miller, R. W., and L. R. Barran.** 1977. The effect of ionic surface-active agents on macroconidial plasma membrane of *Fusarium sulphureum*. Can. J. Microbiol. **23**:1373–1383.
20. **Newton, B. A.** 1956. The properties and mode of action of the polymyxins. Bacteriol. Rev. **20**:14–27.
21. **Ray, S., and S. K. Addy.** 1976. Residue levels of dodine in relation to canker development in citrus trees. Indian Phytopathol. **29**:246–250.
22. **Salton, M. R. J.** 1951. The adsorption of cetyltrimethylammonium bromide by bacteria, its action in releasing cellular constituents and its bactericidal effects. J. Gen. Microbiol. **5**:391–404.
23. **Schwabe, W. F. S.** 1980. Greenhouse evaluation of fungicides for apple scab control. Phytophylactica **12**:195–197.
24. **Schwabe, W. F. S.** 1980. Curative activity of fungicides against apple scab leaf infections by *Venturia inaequalis*. Phytophylactica **12**:199–207.
25. **Schwabe, W. F. S.** 1980. Prevention of storage scab of apples. Phytophylactica **12**:209–221.
26. **Solel, Z., and M. R. Siegel.** 1984. Effect of the fungicides guazatine and dodine on growth and metabolism of *Ustilago maydis*. Z. Pflanzenkr. Pflanzenschutz **91**:273–285.
27. **Somers, E.** 1963. The uptake of dodine acetate by *Neurospora crassa*. Meded. Landbouwhogesch. Opzoekingsstn. Staat Gent **28**:580–589.
28. **Srivastava, S., and T. A. Smith.** 1982. The effect of some oligo-amines and -guanidines on membrane permeability in higher plants. Phytochemistry **21**:997–1008.
29. **Tu, C. M.** 1980. Effect of fungicides on growth of *Rhizobium japonicum in vitro*. Bull. Environ. Contam. Toxicol. **25**:364–368.
30. **Worthing, C. R. (ed.).** 1979. The pesticide manual. A world compendium. The British Crop Protection Council.
31. **Yoder, K. S., and E. J. Klos.** 1976. Tolerance to dodine in *Venturia inaequalis*. Phytopathology **66**:918–923.