Tinopal AN as a selective agent for the differentiation of phytopathogenic and saprophytic *Pseudomonas* species

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The selective toxicity of the respiratory inhibitor Tinopal AN (1,1-bis (3,*N*-5-dimethylbenzoxazol-2-yl) methine *p*-toluene sulphonate) towards phytopathogenic bacteria was investigated further and in general was confirmed using more than 160 additional strains of *Pseudomonas* spp. The mechanism(s) of the resistance shown by saprophytic fluorescent pseudomonads were studied to elucidate the differences between resistant saprophytic and sensitive phytopathogenic *Pseudomonas* species. Damage to, or partial removal of the cell wall of Tinopal AN-resistant *Pseudomonas aeruginosa*, resulted in a marked Tinopal AN-sensitivity, as judged by the ability of Tinopal AN to inhibit oxygen uptake. Furthermore, removal of part of the lipopolysaccharide (LPS) component of the outer membrane also resulted in sensitivity. Mutants of *Ps. aeruginosa* with modified outer cell walls were tested for their reactions towards Tinopal AN, and two cell wall lipopolysaccharide (LPS) mutants of *Escherichia coli* (*env* Al) and *Salmonella typhimurium* (*rfa*) were, in contrast to the wild-type strains, found to be sensitive towards Tinopal AN. The results therefore suggest that the resistance of saprophytic pseudomonads towards Tinopal AN is due (at least in part) to the selectively permeable properties of the outer membrane of the cell wall. The usefulness of Tinopal AN for screening potentially phytopathogenic strains of *Pseudomonas* was confirmed.

The fluorescent optical brightening agent, Tinopal AN, (1,1-bis (3,*N*-5-dimethyl benzoxazol-2-yl)-methine *p*-toluene sulphonate), exhibits a marked selective toxicity towards phytopathogenic bacteria (Gonzalez-Lopez *et al.* 1981). In sharp contrast closely-related saprophytic species, particularly members of the genus *Pseudomonas*, remained unaffected by relatively high concentrations of the compound. This selective toxicity was confirmed in the studies reported here, using 160 additional strains of *Pseudomonas*. Knowledge of the mechanism(s) of the resistance to Tinopal AN shown by the saprophytic species would clearly aid the differentiation of saprophytic and phytopathogenic *Pseudomonas* species, without recourse to extensive plant inoculation tests.

Previous studies showed that Tinopal AN resistance was often correlated with the ability of pseudomonads to grow anaerobically by L-arginine fermentation or nitrate respiration (Gonzalez-Lopez *et al.* 1981). Further studies, however, showed that 8/160 *Pseudomonas* strains were able to grow anaerobically on L-arginine, but not in the presence of 50 μmol/l Tinopal AN. Similarly, 10 strains were able to grow anaerobically in the presence of nitrate, but not in the presence of 20 μmol Tinopal AN. Thus the ability to obtain energy via pathways other than oxygen-dependent respiration did not always correlate with Tinopal AN resistance. This conclusion was confirmed by the finding that three dissimilatory nitrate reductase (*nar*) deficient mutants of *Ps. aeruginosa* (van Hartingsveldt *et al.* 1971) remained resistant to Tinopal AN (Phillips 1983); hence nitrate respiration alone did not confer Tinopal AN resistance.

The resistance of many Gram negative bacteria to anti-microbial drugs has often been

attributed (e.g. Nikaido, 1976; DiRienzo *et al.*, 1978; Zimmermann, 1979; Normark *et al.* 1980) to the nature of the cell wall, and more specifically, to the outer membrane, which often acts as an efficient permeability barrier to such drugs. The present studies showed that sensitivity to Tinopal AN was induced in a Tinopal AN-resistant strain of *Ps. aeruginosa* if the cell envelope layers of the cell wall were partially removed or merely damaged. Furthermore, removal of some of the lipopolysaccharide component of the outer membrane using the mild ethylenediaminetetraacetic acid (EDTA) treatment devised by Leive (1965) also elicited a marked sensitivity. Confirmation of a crucial role of the outer membrane in Tinopal AN exclusion by the resistant cells was obtained here from studies of mutants with well characterized defective outer cell wall membranes.

Materials and Methods

ORGANISMS AND GROWTH CONDITIONS

The majority of the 160 *Pseudomonas* strains used here were from the Rhodes Collection (Rhodes 1959, 1961), and included representatives from the Dowson collection of named phytopathogenic strains. *Pseudomonas aeruginosa* PAC 1 (NCIB 10848), strain PAC 1R and the cell wall mutants PAC 556, 605, 609 and 610 (Meadow & Wells, 1978) were a generous gift from Dr P.M. Meadow, Department of Biochemistry, University College London, UK. However, because there are more well characterized cell wall mutants of enterobacteria available, compared with pseudomonads, the following strains were also studied: *Escherichia coli* strains D21 (wild type) and D22 (*env* A1) were kindly donated by Dr G. Fletcher, Department of Microbiology, The University of Texas, Austin, Texas 78712 and by Dr S. Normark, Department of Microbiology, University of Umeå, Umeå 6, Sweden. *Escherichia coli* strain D22 is an outer membrane mutant strain containing less lipopolysaccharide than the wild type strain D21 (Grundström *et al.* 1980). *Salmonella typhimurium* strains TA 2167 (wild type), TA 2169 (Rc), TA 2170 (Rd2) and Ta 2168 (Re) were generous gifts from Professor H. Nikaido, Department of Bacteriology and Immunology, University of California, Berkeley, California 94720 and Dr G. F-L. Ames, Department of

Biochemistry, University of California, Berkeley, California 94720. These were lipopolysaccharide mutant strains containing shorter polysaccharide chains than the wild type, and they showed a greater sensitivity to certain antibiotics (Ames *et al.* 1974).

PHYTOPATHOGENICITY TESTS

The laboratory methods of Lelliott *et al.* (1966), viz the 'Lopat' tests, devised to detect phytopathogenic strains of *Pseudomonas*, were used as specified, except that soft-rotting ability was assessed on slopes of sterile potato tissue after 14 days incubation at 25°C, and lipolysis was detected on Night Blue margarine agar (Rhodes 1959).

SENSITIVITY TO TINOPAL AN

The general screening technique described by Gonzalez-Lopez *et al.* (1981) was used to detect the growth sensitivity of strains to Tinopal AN. Sterile filter paper strips (2 × 0·5 cm, Whatman No. 1) were soaked in a sterile ethanolic solution containing 1 mmol/l or 10 mmol/l of Tinopal AN and dried for 2 h at 55°C. Strips were placed on dried nutrient agar plates previously streak-inoculated with young nutrient broth cultures of the test organisms. After 48 h at 25°C, the width of the zone of inhibited growth was measured from the long edge of the strip.

Previous studies indicated that the respiration of sensitive cells was markedly inhibited by Tinopal AN (Phillips & Kell 1981), whereas that of Tinopal AN-resistant strains was unaffected. Oxygen uptake was therefore used as a convenient technique for screening sensitivity to Tinopal AN. Respiration by cell suspensions was monitored using a Clark-type oxygen electrode at 30°C (Kell *et al.* 1978).

REMOVAL OF EXOPOLYSACCHARIDE

Hyaluronidase treatment for the removal of exo-polysaccharide was based on the method of Warren & Gray (1954). *Pseudomonas aeruginosa* 24/2 (Rhodes) was cultured aerobically at 37°C in 1% (w/v) glucose nutrient broth (GNB) which contained (g/l): peptone, 5·0; Lab Lemco (Oxoid), 5·0; NaCl, 5·0; glucose, 10·0 at pH 7·2. Cells from a late-exponential culture (about 10^8

cells/ml) were washed once in 0·1 mol/l sodium phosphate buffer at ph 7·0 and resuspended in the same buffer (20 mg dry wt cells/ml) on ice. Samples of this stock suspension were added to 10 ml of 0·1 mol/l sodium phosphate buffer pH 7·0 containing 1·25% (w/v) glucose to give a cell concentration of 3·5 mg dry wt/ml hyaluronidase. Cells were incubated aerobically in an orbital shaker for 6 h at 37°C, and cell samples (2 mg dry wt) were assayed at 30°C for sensitivity to Tinopal AN, using the oxygen electrode as described, in 3 ml of 0·1 mol/l sodium phosphate buffer, pH 7·3 containing 0·1% (w/v) glucose.

PREPARATION OF WHOLE CELL AND OSMOPLAST SUSPENSIONS

To obtain whole cell and osmoplast suspensions, *Ps. aeruginosa* 24/2 (Rhodes) was cultured aerobically in 1% glucose nutrient broth, at 25°C. Cells from a mid-exponential culture (2×10^7 cells/ml) were washed once in 0·1 mol/l sodium phosphate buffer at pH 7·3. Half of the suspension (control whole cells) was washed once more in the same buffer and resuspended to give a cell concentration of 9 mg dry wt/ml. Preparation of osmoplasts (Poindron *et al.* 1977) from the other half of the above suspension was based on lysozyme treatment alone because EDTA is toxic to *Ps. aeruginosa* (Eagon & Carson 1965; Roberts *et al.* 1970). To plasmolyse the cells, suspensions were washed three times in 0·5 mol/l NaCl at 4°C, resuspended in 0·5 mol/l sucrose at 25°C to give about 10^9 cells/ml, and shaken gently for 1 h at 25°C on an orbital shaker. Harvested cells were resuspended in 0·05 mol/l Tris-HCl at pH 8·0 containing 0·5 mol/l sucrose, at a concentration of about 5×10^8 cells/ml. Lysozyme was added to give a final concentration of 100 µg/ml and the suspension was incubated at 25°C until turbidimetric estimations indicated that samples could be lysed by osmotic shock.

RELEASE OF LIPOPOLYSACCHARIDE FROM OUTER MEMBRANES

The mild EDTA treatment described by Leive (1965) and by Muschel & Gustafson (1968) was used to release outer cell wall lipopolysaccharide (LPS) from overnight cultures which were grown aerobically in GNB at 30°C. Cells were

harvested and washed once in 0·12 mol/l Tris-HCl, pH 8·0 at 4°C. To half the pellet, sufficient cold 0·2 mmol/l EDTA in 0·12 mol/l Tris-HCl, pH 8·0, was added to dilute the cells to a concentration of 1·6 mg dry wt/ml. To the other half of the pellet, 0·12 mol/l Tris-HCl alone was added as a control. After 2 min at 20°C, the suspension was diluted sixfold with the same buffer at 4°C, thus halting the EDTA treatment. Harvested cells were washed twice in 0·12 mol/l Tris-HCl buffer at pH 8·0 and 4°C and resuspended in the same buffer at a concentration of about 20 mg dry wt cells/ml.

CHEMICALS

Tinopal AN was kindly donated by Dr A.M. Paton, Division of Agricultural Bacteriology, School of Agriculture, University of Aberdeen, Scotland. Hyaluronidase (Type 1) and lysozyme (Grade I) were obtained from Sigma Chemical Co., Dorset.

Results

SELECTIVE TOXICITY OF TINOPAL AN TOWARDS PHYTOPATHOGENIC *Pseudomonas* SPECIES

When over 160 strains of *Pseudomonas* spp. of unknown phytopathogenic potential were tested for their growth sensitivity towards Tinopal AN, 24 were found to be sensitive: 10 of these were phytopathogenic strains from the Dowson collection, and 13 were from various plant, soil or water sources (see Table 1), including brown spot lesions on *Primula* leaves (isolate 18/3) and shotholes on leaves of *Ilex* sp. Almost all these Tinopal-sensitive strains exhibited the typical biochemical reactions of phytopathogens, as described by Lelliott *et al.* (1966); this is clearly evident in Table 1, where, for example, *Ps. syringae* pv. *tabaci* and *Ps. syringae* pv. *pisi* accord with their Group Ia, and *Ps. syringae* pv. *delphinii* is characteristic of the Group Ib of Lelliott *et al.* (1966). *Pseudomonas tolaasii* d69 was markedly sensitive to Tinopal AN; this has been described as a casual pathogen, and although the tobacco necrosis test was indeed negative, the overall properties were in agreement with assignation to the Ib group of phytopathogens.

When, to test the correlation between phytopathogenicity on the basis of the results of the

Table 1. Phytopathogenicity testing of Tinopal AN-sensitive and Tinopal AN-resistant strains of *Pseudomonas* spp. using biochemical characteristics, (LOPAT) tests (Lelliot *et al.* 1966)

Strain	Source	Zone of growth inhibition to 1 mmol/l Tinopal AN (mm)	LOPAT test (7 days/25°C)					Subsidiary test (7 days/25°C)				Lelliot et al. Group
			Levan from sucrose	Oxidase (1 day/25°C)	Potato rot	Arginine dihydrolase	Tobacco necrosis	2-keto-gluconate from gluconate	Lipase	Nitrate reduction	Acid from sucrose	
Tinopal AN-resistant												
8[7]	Medical	0	+	+	−	+	−	+	+	−	−	V
26[6]	Dairy	0	−	+	−	+	−	+	+	−	−	V
28[5]	Water	0	+	+	−	+	−	+	+	−	−	V
31[1]	Plant	0	−	+	−	+	−	+	−	−	−	V
KB1	Stock	0	−	+	−	+	−	+	−	−	−	V
Pseudomonas syringae pv.												
phaseolicola d52	W.J. Dowson	0	+	+	−	+	−	+	+	−	−	V
viridiflava d53	W.J. Dowson	0	+	−	−	+	−	+	+	+	−	V
viridilivida d152	W.J. Dowson	0	−	+	−	+	−	+	−	+	−	V
syringae pv. *barkeri* d244	W.J. Dowson	0	−	+	−	+	−	+	−	−	−	V
cruciviae d251	W.J. Dowson	0	−	−	−	+	−	−	+	−	−	V
angulata d263	W.J. Dowson	0	+	−	−	+	−	+	+	−	−	V
marginalis d289	W.J. Dowson	0	+	+	+	+	−	+	+	+	−	IVa
Tinopal AN-sensitive												
3	Water	7·5	−	+	(+)	+	−	+	+	+	−	IVb
5[10]	Water	7·0	+	+	+	+	−	+	+	+	−	IVa
8[11]	Medical	4·5	−	+	(+)	+	−	+	+	−	−	IVb

8[12]	Medical	4·5	−	+	−	+	−	+	−	−	−	V
15[1]	Water	5·0	+	+	−	+	−	−	−	−	−	IV
18[3]	Plant (*Primula* sp.)	14·0	−	+	−	−	+	−	−	−	−	III
23[1]	Plant (*Ilex* sp.)	12·5	−	−	−	−	+	−	−	−	−	II or III
26[1]	Dairy	3·0	−	+	+	+	−	+	+	+	−	IVb
44	Water	6·5	−	−	−	+	−	+	−	+	−	V
52[2]	Water	4·5	+	+	−	+	−	+	+	+	−	V
52[7]	Water	9·5	+	+	−	+	−	+	−	−	−	IV or V
52[8]	Water	10·5	−	+	−	+	−	+	+	−	−	V
53[2]	Water	4·0	+	+	−	−	+	+	−	+	−	III
Pseudomonas syringae pv.												
savastanoi d64	W.J. Dowson	17·0	−	−	−	−	+	−	−	−	−	Ib or II
tolaasii d69	W.J. Dowson	19·0	−	−	−	−	−	−	−	−	−	Ib
syringae pv. *delphinii* d72	W.J. Dowson	19·0	−	−	−	−	+	+	−	−	−	Ib
syringae pv. *tabaci* d221	W.J. Dowson	16·0	+	−	−	−	−	−	−	−	+	Ia
syringae pv. *lachrymans* d236	W.J. Dowson	19·0	−	−	−	−	+	−	−	−	+	Ib
syringae pv. *atrofaciens* d428	W.J. Dowson	4·0	+	+	−	−	+	−	−	−	+	Ia or III
syringae pv. *aptata* d260	W.J. Dowson	20·0	−	−	−	−	+	−	−	−	−	Ib or II
syringae pv. *morsprunonum* d270	W.J. Dowson	15·0	+	−	−	−	+	−	+	−	−	Ia or II
syringae pv. *syringae* d281	W.J. Dowson	15·0	+	−	−	−	+	+	−	−	−	Ia
syringae pv. *pisi* d300	W.J. Dowson	11·0	+	−	−	−	+	−	−	−	+	Ia

+ , positive result; − , negative result; (+) weakly positive.

'Lopat' tests of Lelliott *et al.* (1966), a few Tinopal AN-resistant strains of the Rhodes collection were randomly selected, they indeed possessed (overall) the properties characteristic of the *Pseudomonas* Group V saprophytes (see Table 1). Six out of the seven unexpectedly Tinopal AN-resistant 'phytopathogens' from the Dowson collection were in fact also shown here to be organisms typical of the Group V saprophytes (Table 1).

EFFECT OF TINOPAL AN ON
HYALURONIDASE-TREATED CELLS OF
Pseudomonas aeruginosa

Hyaluronidase treatment had no effect on the resistance of *Ps. aeruginosa* to Tinopal AN (Fig. 1). Both treated and untreated cells maintained
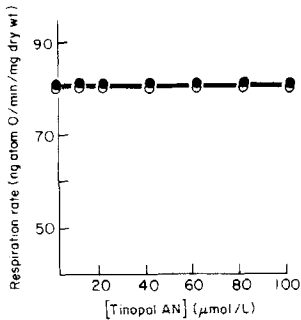
Fig. 1. The effect of Tinopal AN on the respiration of hyaluronidase-treated cells of *Pseudomonas aeruginosa* 24/2. Two mg dry wt cell suspensions were ●, treated with hyaluronidase or ○, untreated; respiration was monitored in the presence of Tinopal AN in 0·1 mol/l phosphate buffer pH 7·3, plus 1% glucose, using a Clark-type oxygen electrode at 30°C.

Tinopal AN resistance, as judged by oxygen uptake measurements in the presence of concentrations of Tinopal AN up to 100 μmol/l, suggesting that the resistance of *Ps. aeruginosa* was not due to exclusion of the compound by the hyaluronidase-sensitive   exo-polysaccharide layer.

SENSITIVITY OF OSMOPLASTS OF
Pseudomonas aeruginosa TO TINOPAL AN

The method used for the partial removal of cell walls resulted in the formation of osmoplast preparations of *Ps. aeruginosa* which were

osmotically unstable, but still rod-shaped (although non-motile), as observed by phase contrast microscopy. Respiration of untreated whole cell suspensions was unaffected by the addition of concentrations of Tinopal AN up to 100 μmol/l (Fig. 2). However, the respiration of
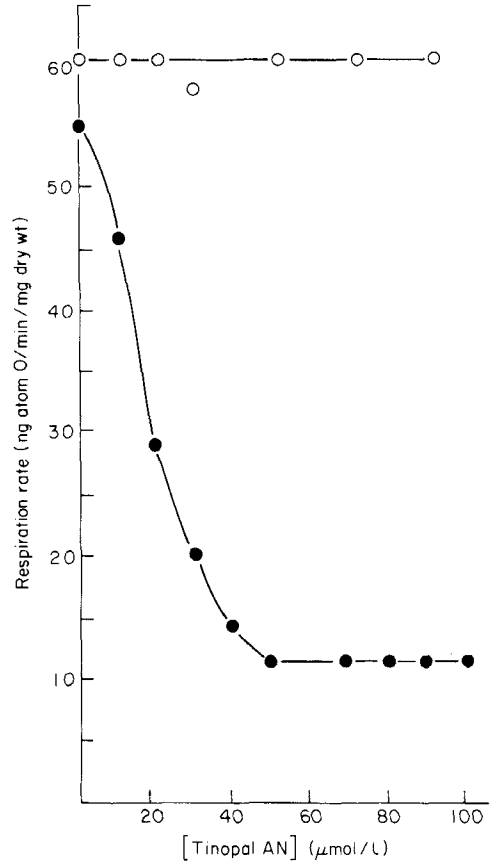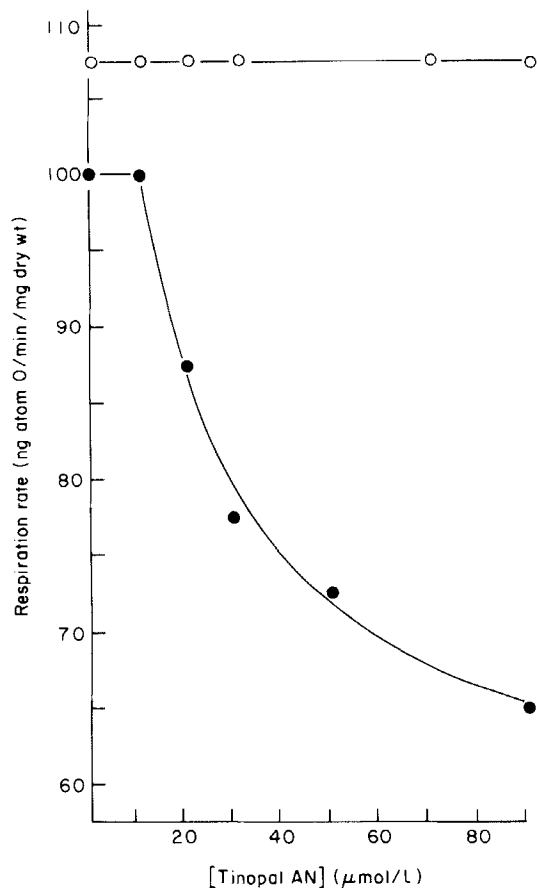

Fig. 2. The inhibition of the respiration of osmoplast suspensions of *Pseudomonas aeruginosa* 24/2 by Tinopal AN. The respiration of ○, washed whole cell and of ●, osmoplast suspensions (2 mg dry wt) in the presence of Tinopal AN was monitored in 0·1 mol/l sodium phosphate buffer, pH 7·3, plus 10 mmol/l glucose (whose cells) and 0·5 mol/l sucrose plus 10 mmol/l glucose (osmoplasts), using a Clark-type oxygen electrode at 30°C.

osmoplast preparations was markedly sensitive to low concentrations of Tinopal AN: the concentration required to reduce respiration by 50% (ID_{50}) was about 20 μmol/l Tinopal AN. Additions of Tinopal AN at concentrations of more than 50 μmol/l produced no further decrease in respiration rate (Fig. 2).

SENSITIVITY OF EDTA-TREATED
Pseudomonas aeruginosa AND *Pseudomonas*
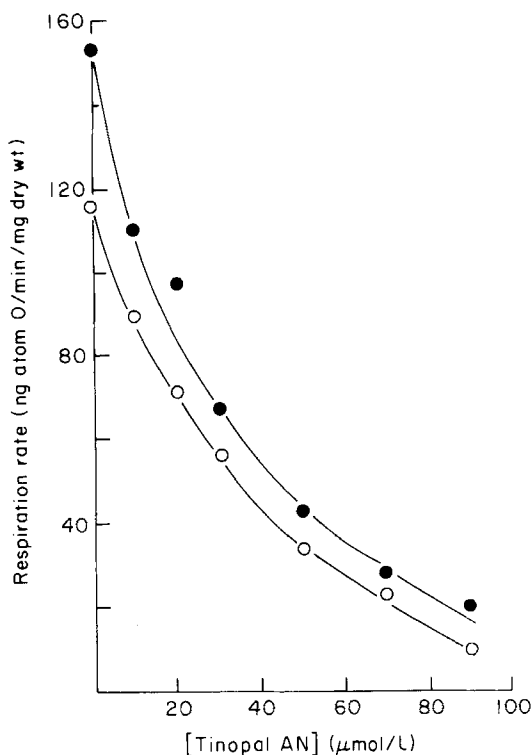*syringae* PV. *lachrymans* TO TINOPAL AN

Removal of some LSP from the outer membranes of Tinopal AN-resistant *Ps. aeruginosa* by mild EDTA treatment resulted in the cellular respiration becoming markedly sensitive to Tinopal AN (Fig. 3). Cells so treated were also

increase the sensitivity displayed by untreated cells (Fig. 4).

Fig. 4. The inhibition of the respiration of whole cells and EDTA-treated cells of *Pseudomonas syringae* pv. *lachrymans* d236 by Tinopal AN. The respiration of ○, 2 mg dry wt washed whole cells and ●, EDTA-treated cells was monitored in the presence of Tinopal AN in 0·12 mol/l Tris-HCl buffer, pH 7·3, plus 14 mmol/l glucose, using a Clark-type oxygen electrode at 30°C.

Fig. 3. The inhibition of the respiration of EDTA-treated cells of *Pseudomonas aeruginosa* PAC 1 by Tinopal AN. The respiration of ○, 2 mg dry wt washed whole cell and ●, EDTA-treated suspensions was monitored in the presence of Tinopal AN in 0·12 mol/l Tris-HCl buffer pH 7·3 plus 14 mmol/l glucose using a Clark-type oxygen electrode at 30°C.

more sensitive than similar untreated cells to other antibiotics such as streptomycin and novobiocin (Phillips 1983). EDTA-treatment of the Tinopal AN-sensitive phytopathogen *Ps. syringae* pv. *lachrymans* d236 did not markedly

SENSITIVITY OF SOME
LIPOPOLYSACCHARIDE MUTANTS
TO TINOPAL AN

All four wall-deficient mutants of *Ps. aeruginosa* remained resistant to 1 mmol/l Tinopal AN (Table 2), as were also most of the 'deep rough' mutants of *Salmonella typhimurium*. However, strain TA 2168, which possessed the shortest core polysaccharide chain, was moderately sensitive to the compound, exhibiting a growth sensitivity zone of 4 mm (Table 2). *Escherichia coli* strain D22, which possesses lower amounts overall of lipopolysaccharide in the outer membrane, was, unlike the Tinopal AN-resistant wild-type strain D21, sensitive to Tinopal AN,

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Table 2. The effect of Tinopal AN on outer membrane lipopolysaccharide-deficient mutants of *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhimurium*

| Strain | Zone of growth inhibition (mm) towards Tinopal AN at | | Reference to strains used |
	1 mmol/l	10 mmol/l	
Pseudomonas			
aeruginosa PAC 1, wild type	0	0	Meadow & Wells (1978)
aeruginosa PAC 1R, wild type	0	0	
aeruginosa PAC 556, LPS-deficient mutant	0	0	
aeruginosa PAC 610, LPS-deficient mutant	0	0	
aeruginosa PAC 609, LPS-deficient mutant	0	0	
aeruginosa PAC 605, LPS-deficient mutant	0	0	
Escherichia			
coli D21, wild type	0	0	Grundström *et al.* (1980)
coli D22 *env* A1, LPS-deficient	5	12	
Salmonella			
typhimurium TA 2167, wild type	0	ND	Ames *et al.* (1974)
typhimurium TA 2169, LPS-deficient mutant Rc	0	ND	
typhimurium TA 2171, LPS-deficient mutant Rc	0	ND	
typhimurium TA 2170, LPS-deficient mutant Rd	0	ND	
typhimurium TA 2168, LPS-deficient mutant Re	4	ND	

Strains were tested for sensitivity to Tinopal AN at concentrations of 1 mmol/l and 10 mmol/l using the filter strip method. ND means not determined.

exhibiting a zone of 5 mm and 12 mm of inhibited growth against 1 mmol and 10 mmol Tinopal AN respectively (Table 2).

Discussion

The correlation between phytopathogenicity and Tinopal AN-sensitivity, first reported by Gonzalez-Lopez *et al.* (1981), was particularly notable for the *Pseudomonas* spp. and was confirmed here using the 160 *Pseudomonas* strains of the Rhodes collection. Subsequent phytopathogenicity testing of the Tinopal AN-sensitive strains using the laboratory 'Lopat' tests of Lelliott *et al.* (1966) revealed the presence of three possible phytopathogens (strains 18/3, 23/1 and 53/2, see Table 1). These, and the remaining Tinopal AN-sensitive strains were clearly related (on phenotypic criteria) to the phytopathogens grouped by Rhodes (1961). In addition, the possibility that six of the named phytopathogenic strains in the Dowson collection were non-pathogenic was confirmed here by both the 'Lopat' criteria and their resistance to Tinopal AN. They clearly possessed the characteristics of Group V saprophytes (Table 1), confirming their phenotypic co-grouping with the *Ps. fluorescens* complex (Rhodes 1961). These had long been suspected to be misnamed, on the basis of other phenotypic properties (Rhodes 1959, 1961), and

so the subsequent correlation of the 'Lopat' test results with Tinopal AN resistance (saprophytes) and sensitivity (phytopathogens), as found here, was gratifying. Therefore one may reasonably conclude that within the genus *Pseudomonas* (excluding marine species, see Gonzalez-Lopez *et al.* 1981) there is a striking correlation between Tinopal AN-sensitivity and phytopathogenicity, and that this can also be revealed by classical phenotypic taxonomic methods (Rhodes 1961).

Tinopal AN was previously shown to be a potent inhibitor of certain bacterial NADH dehydrogenases (Phillips & Kell 1981, 1982), and of RNA and thus protein synthesis (Phillips *et al.* 1984). A correlation between the resistance of *Pseudomonas* species to Tinopal AN and the potential for anaerobic growth using L-arginine or nitrate was suggested by Gonzalez-Lopez *et al.* (1981). However, further studies (Phillips 1983) showed that this correlation did not always occur (see above and Phillips 1983), and so alternative explanations were investigated experimentally here.

The resistance of many pseudomonads to a wide range of antimicrobial drugs is often attributable to the permeability barrier in the outer layers of the cell walls (Nikaido, 1976; DiRienzo *et al.* 1978; Zimmermann, 1979; Normark *et al.* 1980). Indeed, modifications in

cell wall composition with resultant changes in the permeability of drugs is a property often associated with the outer membrane of Gram negative cell walls in response to changes in growth media (Nikaido 1976; Gilbert & Brown 1978). The hypothesis that such a mechanism of resistance to Tinopal AN might occur in saprophytic pseudomonads is in full agreement with the present observations, which suggest that the exclusion of Tinopal AN by the cell wall is the primary mechanism of Tinopal AN resistance (Figs 2, 3). Furthermore, using osmoplast suspensions of *Ps. aeruginosa*, it was shown that the partial removal of, or damage to, the cell wall was accompanied by the development of a marked sensitivity to Tinopal AN, as monitored by the respiration of washed cell suspensions. Although periplasmic enzymes might be involved in a Tinopal AN resistance mechanism, this is considered unlikely since Scholes & Smith (1968) reported that lysozyme treatment alone caused no gross changes in cell wall structure, and so substantial losses of periplasmic enzymes probably did not occur under our conditions.

The mild treatment using EDTA, reported by Leive (1965) to release about 50% of the lipopolysaccharide from the outer cell wall membrane, induced a marked sensitivity towards other antibiotics (Phillips 1983), and also resulted in a sensitivity towards Tinopal AN of the respiration of normally resistant cells (Fig. 3). Similar treatment of a Tinopal AN-sensitive phytopathogen, *Ps. syringae* pv. *lachrymans*, however, did not result in an increased sensitivity to Tinopal AN (Fig. 4). Further evidence for an involvement of the outer membrane in Tinopal AN resistance was found when two lipopolysaccharide mutants were studied. *Salmonella typhimurium* strain TA 2168 is a 'deep rough' mutant (*rfa*) possessing a shorter core polysaccharide chain than the wild-type (Ames *et al.* 1974); the wild-type was resistant to Tinopal AN in the filter paper strip assay whereas the mutant strain was fairly sensitive. *Escherichia coli* strain D22 is an envelope-defective mutant (*env* A1) of the wild-type strain D21, and has lower amounts of LPS in the outer membrane (Grundström *et al.* 1980). Unlike the wild-type strain, D22 was markedly sensitive to Tinopal AN (Table 2). Clearly the relative sensitivities to Tinopal AN of these strains (and also the resistance of other outer

membrane protein (*omp*) mutants of *E. coli* and *Salm. typhimurium* which were also tested, see Phillips (1983) for full details) indicated that, as a consequence of their LPS defects, the mutant strains possessed outer membranes with altered structural arrangements and consequent changes in permeability properties. Therefore it is suggested that major differences between saprophytic and phytopathogenic pseudomonads in terms of their resistance to Tinopal AN are associated with the permeability properties of the cell walls. Further detailed studies of the molecular components of the outer cell wall membranes of different *Pseudomonas* species or mutants are much needed. In the meantime, however, the difference shown by these two groups of pseudomonads towards Tinopal AN appears to be a simple and valuable screening test for the detection of phytopathogenic *Pseudomonas* species from plant material (whether showing disease symptoms or not), soil or water.

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