The Sensitivity of *Pseudomonads* to Ethylene-diaminetetra-acetic Acid

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SUMMARY

Forty-one strains representing 21 nomen species of the genus *Pseudomonas* and 2 of the genus *Alcaligenes* were screened for sensitivity to ethylene-diaminetetra-acetic acid (EDTA). Sensitivity of organisms was assessed by determining the release of intracellular solutes and the loss of viability under the action of EDTA. The latter criterion was found to be the more useful for the differentiation of sensitive and resistant organisms. Strains of *Pseudomonas diminuta*, *P. geniculata*, *P. iodinum*, *P. maltophilia*, *P. pavonacea* and possibly *P. rubescens* were considered as resistant to EDTA. Strains of *P. alcaligenes* were clearly differentiated from strains of *Alcaligenes faecalis*, which were highly resistant to EDTA. The practical value and possible taxonomic significance of the results are discussed.

INTRODUCTION

There is growing evidence that metal cations may play important roles in maintaining the structural integrity and permeability characteristics of the surface structures of a variety of Gram-negative bacteria (Vincent & Humphrey, 1963; Strange, 1964; Brown, 1964; MacLeod, 1965; Asbell & Eagon, 1966). The various effects of ethylene-diaminetetra-acetic acid (EDTA) on these organisms probably involve binding to or extraction of such cations. Thus, EDTA is known to potentiate the action of lysozyme (Repaske, 1956, 1958) and a variety of bactericides (MacGregor & Elliker, 1958; Gray & Wilkinson, 1965*a*; Leive, 1965*a*; Brown & Richards, 1965). It has also been found to increase the death rate of starved bacteria (Strange & Dark, 1965). It is generally considered that EDTA disorganizes an outer layer of the cell wall, facilitating penetration or action of the antibacterial agent. Recent work has confirmed that EDTA causes a relatively unspecific increase in the permeability of cells of sensitive organisms (Leive, 1965*b*; Hamilton-Miller, 1965), without necessarily killing or altering the growth rate of the cells (Leive, 1965*b*; Brown & Richards, 1965).

During his investigation of lysozyme potentiation, Repaske (1956, 1958) noted that EDTA alone had appreciable lytic activity against *Pseudomonas aeruginosa*, as judged by a decrease in the turbidity of suspensions of the organism. This observation, which was not made for other organisms tested, has been confirmed by other workers (Shively & Hartsell, 1964*a, b*; Eagon & Carson, 1965). It is now clear that EDTA can cause extensive damage to the cell wall of *P. aeruginosa* (Voss, 1964; Eagon & Carson, 1965), the release of intracellular solutes (Bernheim, 1963; Gray & Wilkinson, 1965*a*), and death of the organism (MacGregor & Elliker, 1958; Gray & Wilkinson, 1965*a*; Eagon & Carson, 1965). The extent of the lytic action of EDTA is very markedly

dependent on the experimental conditions used (Repaske, 1958; Wilkinson, 1962; Shively & Hartsell, 1964*a, b*). With isolated cell walls of *P. aeruginosa*, Gray & Wilkinson (1965*b*) found that EDTA solubilized material containing phosphorus and carbohydrate, and concluded that metal cations and lipopolysaccharide are essential components of these cell walls. Similar conclusions were reached by Eagon and his co-workers (Eagon & Carson, 1965; Carson & Eagon, 1966; Asbell & Eagon, 1966).

Of a range of organisms examined by Gray & Wilkinson (1965*a*), only three species of the genus *Achromobacter* (one of which was stated to be *Alcaligenes faecalis*) displayed the exceptional sensitivity to EDTA characteristic of *Pseudomonas aeruginosa*. However, these hypersensitive organisms may differ from other Gram-negative bacteria only in degree. Thus, lysis of organisms or solubilization of wall components by EDTA has been noted for species of the genus *Salmonella* (Colobert, 1957*a, b*; Herzberg & Green, 1964). Leive (1965*c*) reported the release of lipopolysaccharide from the walls of whole organisms of several strains of *Escherichia coli* treated with EDTA. This result was not obtained with the isolated cell walls of other strains (Edwards & Noller, 1964; Gray & Wilkinson, 1965*b*). Nevertheless, it seemed possible, as suggested by Shively & Hartsell (1964*a*), that sensitivity to EDTA might be an important character in the taxonomy of the pseudomonads.

This paper presents results obtained with a range of pseudomonads and some other organisms, during a general survey of Gram-negative bacteria for sensitivity to EDTA. Since the completion of the work, Wolin (1966) has reported a lytic action by EDTA on *Vibrio succinogenes*.

METHODS

In their survey of pseudomonads, Shively & Hartsell (1964*a*) measured the sensitivity of an organism to EDTA by the change produced in the turbidity of a suspension of the organisms. As such changes might result from simple osmotic effects (Bernheim, 1963) as well as from gross lysis, more direct methods of determining EDTA sensitivity were preferred in the present study. The general techniques used for measuring the bactericidal action of EDTA and the release of intracellular solutes from the bacteria were based on those used by Gray & Wilkinson (1965*a*).

Organisms. Most organisms were freshly obtained from the National Collection of Type Cultures (NCTC) or the National Collection of Industrial Bacteria (NCIB). A strain of *Pseudomonas aeruginosa* coded FB was obtained from Dr F. Bernheim, and the organism coded BR 1/2 from the Bacteriological Laboratory of Reckitt & Sons Ltd. (Dansom Lane, Hull). Individual strains are listed in Tables 1-3.

Cultural conditions. For the preparation of thick suspensions of bacteria, the organisms were grown for 24 hr on nutrient agar (Oxoid). Slopes were inoculated from test-tube cultures (24 hr) of the organisms in nutrient broth no. 2 (Oxoid). Strains of *Pseudomonas aeruginosa*, *P. alcaligenes*, *P. maltophilia*, *P. ovalis*, *P. iodinum*, *Alcaligenes faecalis*, *A. odorans* and an *Achromobacter* species were grown at 37°; all other organisms were grown at 25°.

Test procedure. Organisms were washed from the slopes with 0.2 M-borate buffer (pH 7.1) and the suspension was filtered through a glass sinter (no. 1 porosity). The bacteria were collected by centrifugation at 20°, washed twice, and finally dispersed as a thick suspension in the buffer. Bacterial dry weights were determined by drying samples (2 ml.) of suspension and of buffer overnight at 110°, and were within the range

4–18 mg./ml. Tests showed that variations in bacterial concentration within this range had only slight effect on the subsequent action of EDTA.

Samples (2 ml.) of bacterial suspensions were added to volumes (20 ml.) of each of the following solutions kept at 20°: (a) 0.0034 M-EDTA in the borate buffer; (b) borate buffer; (c) 0.0034 M-EDTA in borate buffer containing sucrose (0.5 M); (d) borate buffer containing sucrose (0.5 M). After 1 hr, samples (1 ml.) of suspensions from (a) and (b) were taken for viability determinations, then all suspensions were centrifuged (12,000 g, 5 min.). The supernatant fluids were decanted and clarified by further centrifugation (3,500 g, 30 min.). Supernatant fluids were similarly prepared from bacteria suspended in borate buffer as in (b), but heated for 10 min. at 100°. Repeat experiments, which gave satisfactory results, were done with most organisms. A similar series of tests (excluding sucrose-containing solutions) in which the borate buffer (pH 7.1) was replaced in all solutions and suspensions by 0.05 M-borate buffer (pH 9.2) was done with selected organisms.

Viability determinations. The number of viable organisms in a suspension was determined after serial dilution (usually 10^4 – 10^7 times) of the samples in 1% (w/v) aqueous NaCl. Volumes (0.5 ml.) were placed dropwise on plates of nutrient agar which had been dried for 2 hr at 60°. Colonies were counted after incubation for 24–48 hr at the original growth temperature for the organism.

Release of intracellular solutes. The extinction at 260 m μ of a supernatant fluid (corrected for test solutes) was used as a measure of the leakage of intracellular solutes during the test period. Extinctions were measured in 1 cm. silica cells in a Unicam SP. 500 spectrophotometer. Concentrations of solutes released were calculated as percentages of those from heated (100°) organisms.

RESULTS

The results of tests on 10 strains of *Pseudomonas aeruginosa* using EDTA at pH 7.1 (Table 1) confirmed the existing impression that sensitivity to EDTA is a characteristic property of the organism. In spite of the heterogeneity of bacteria grown in surface culture and the desensitizing effect of the borate buffer (Gray & Wilkinson, 1965a), EDTA consistently produced a leakage of material absorbing at 260 m μ and a substantial decrease in viability. Although the leakage of such solutes was usually about 30% of that from heated bacteria, from 2 strains it was nearly 60%. As both types of result have previously been obtained in similar tests with strain NCTC 1999 (Gray & Wilkinson, 1965a), it is doubtful whether strains giving the greater leakage are intrinsically more sensitive to EDTA. Also, because of rather high leakages into buffer alone in some instances, the increase in leakage produced by EDTA could not be relied upon as the sole index of sensitivity, as seemed possible from the earlier more limited study. For this reason, the decrease in viability caused by EDTA was introduced into the screening procedure as a second index. By using the latter index, the most sensitive strain of *P. aeruginosa* was strain FB (Bernheim, 1963), which was also the only one which did not produce either a visible or a fluorescent pigment.

In the presence of sucrose (0.5 M), the leakage induced by EDTA was substantially decreased (40–87%). Protection of the bacteria by sucrose, even when incomplete, could be taken as evidence that the action of EDTA was against the cell wall rather than the protoplast membrane. The ability of non-penetrating solutes, including NaCl

and sucrose, to inhibit lysis of pseudomonads treated with EDTA and lysozyme has previously been shown to depend on the species (Shively & Hartsell, 1964*b*).

In addition to *Pseudomonas aeruginosa*, it has been reported that *Alcaligenes faecalis* is also hypersensitive to EDTA (Gray & Wilkinson, 1965*a, b*). However, tests on 4 strains of this organism and 2 strains of *A. odorans* (Table 2) showed them to be highly resistant to EDTA. The resistance was maintained in similar tests at pH 9.2.

Table 1. *Sensitivity to EDTA of Pseudomonas aeruginosa*

Organisms were treated for 1 hr at 20° with 0.0031 M-EDTA in borate buffer pH 7.1 (T), buffer alone (B), and similar solutions containing 0.5 M-sucrose (T/S and B/S).

Strain	Suspension				Decrease in viability (%)†
	T	B	T/S	B/S	
	Release of solutes absorbing at 260 mμ*				
NCTC 1999	26.4	2.5	8.6	2.2	75
NCTC 6750	37.1	2.9	20.7	10.4	95
NCTC 7244	32.0	7.2	21.1	12.5	99+
NCTC 8060	56.2	6.3	15.9	6.7	84
NCTC 8203	20.7	4.1	8.6	4.4	60
NCTC 8505	57.3	13.6	13.9	6.8	86
NCTC 10332	36.0	3.3	12.8	5.1	74
NCIB 8626	33.6	4.8	21.8	5.7	89
FB	22.3	1.8	5.1	2.2	99+
NCTC 5083 var. <i>erythrogenes</i>	42.8	12.3	29.9	11.5	93

* Expressed as a percentage of solutes from organisms heated 10 min. at 100°.

† Organisms in T, relative to those in B.

Table 2. *Sensitivity to EDTA of Alcaligenes species*

Organisms were treated for 1 hr at 20° with 0.0031 M-EDTA in borate buffer pH 7.1 (T), buffer alone (B), and similar solutions containing 0.5 M-sucrose (T/S and B/S).

Organism	Strain	Suspension				Decrease in viability (%)†
		T	B	T/S	B/S	
		Release of solutes absorbing at 260 mμ*				
<i>A. faecalis</i>	NCTC 415	3.5	2.0	3.4	3.1	0
<i>A. faecalis</i>	NCTC 655	3.1	1.0	2.4	3.0	0
<i>A. faecalis</i>	NCTC 8764	8.3	6.6	7.3	6.6	0
<i>A. faecalis</i>	NCIB 8156	1.7	0.9	1.9	1.7	0
<i>A. odorans</i>	NCTC 10416	1.6	0.3	1.3	1.6	0
<i>A. odorans</i> var. <i>viridans</i>	NCTC 10388	2.5	0.8	3.1	3.5	0

* Expressed as a percentage of solutes from organisms heated 10 min. at 100°.

† Organisms in T, relative to those in B.

The original strain, BR 1/2, had been identified (1960) as *A. faecalis* on the basis of its cultural and physiological properties. In apparent confirmation, tests on *A. faecalis* NCTC 8769 showed that this organism also was highly sensitive to EDTA. However, strain NCTC 8769 was reclassified in 1964 as *Pseudomonas alcaligenes*, after Ikari &

Hugh (1963). Organisms of strain BR 1/2 have now been examined under the electron microscope; this showed that their flagellation was polar and monotrichous. Hence, this organism also should probably be renamed *P. alcaligenes* and is so described in Table 3. Tests on two other strains of *P. alcaligenes* (Table 3) confirmed the high sensitivity of this organism to EDTA, although it should be noted that strains NCTC 8769 and NCIB 9390 have recently been named as members of the new species *P. pseudoalcaligenes* (Stanier, Palleroni & Doudoroff, 1966).

Table 3. *Sensitivity to EDTA of Pseudomonas species*

Organisms were treated for 1 hr at 20° with 0.0031 M-EDTA in borate buffer pH 7.1 (T), buffer alone (B), and similar solutions containing 0.5 M-sucrose (T/S and B/S).

Organism	Strain	Suspension				Decrease in viability (%)†
		T	B	T/S	B/S	
		Release of solutes absorbing at 260 mμ*				
<i>P. alcaligenes</i>	NCTC 8769	20.7	0.9	8.2	2.5	98
<i>P. alcaligenes</i>	NCTC 10367	67.6	10.1	13.8	9.8	98
<i>P. alcaligenes</i>	NCIB 9390	73.3	10.4	12.8	8.4	99+
<i>P. alcaligenes</i>	BR 1/2	17.3	0.9	5.2	1.8	99+
<i>P. aureofaciens</i>	NCIB 9030	28.3	3.5	4.5	3.8	57
<i>P. chlororaphis</i>	NCTC 7357	26.6	1.5	3.8	1.8	42
<i>P. denitrificans</i>	NCIB 8376	11.5	1.3	4.5	4.0	52
<i>P. diminuta</i>	NCTC 8545	7.4	1.4	4.8	3.9	12
<i>P. fluorescens</i>	NCTC 10038	23.5	1.5	5.1	1.7	0
<i>P. fluorescens</i>	NCIB 9494	14.7	6.0	2.1	3.6	30
<i>P. fragi</i>	NCIB 8542	26.3	2.5	4.4	2.9	46
<i>P. geniculata</i>	NCIB 9428	32.8	5.5	14.2	3.4	0
<i>P. graveolens</i>	NCTC 8067	29.0	5.9	13.7	5.1	91
<i>P. iodinum</i>	NCTC 9742	5.9	1.9	5.6	7.4	0
<i>P. maltophilia</i>	NCTC 10257	18.6	5.9	7.8	6.3	11
<i>P. maltophilia</i>	NCTC 10259	12.7	4.2	7.6	5.4	0
<i>P. maltophilia</i>	NCIB 9201	31.2	1.9	5.3	2.7	0
<i>P. mucidolens</i>	NCTC 8068	23.4	7.5	14.0	7.1	42
<i>P. ovalis</i>	NCTC 912	38.9	9.7	23.0	10.4	85
<i>P. pavonacea</i>	NCIB 9395	13.3	3.1	11.8	7.0	35
<i>P. putida</i>	NCIB 9034	25.9	1.2	9.7	2.6	23
<i>P. rubescens</i>	NCIB 8768	14.2	1.3	4.9	3.2	0
<i>P. stutzeri</i>	NCIB 9040	32.9	5.0	7.1	3.4	99+
<i>P. syncyanea</i>	NCTC 9943	29.4	5.9	11.8	8.2	99+
<i>P. synxantha</i>	NCIB 8178	20.3	3.0	6.8	3.9	53
<i>P. taetrolens</i>	NCIB 9396	41.3	8.4	21.8	8.0	95

* Expressed as a percentage of solutes from organisms heated 10 min. at 100°.

† Organisms in T, relative to those in B.

Table 3 also records the results of tests at pH 7.1 on 18 other *Pseudomonas* species; except in two cases, only single strains were tested. The results for *Pseudomonas graveolens* and *P. taetrolens*, which are different cultures of the same strain, illustrate the degree of reproducibility obtained under such circumstances. Much closer agreement was generally obtained between the results of tests on different batches of organisms grown from the same stock culture. Of the 18 species, the strains of the following exhibited sensitivity to EDTA comparable with that of *P. aeruginosa*: *P. ovalis*, *P. stutzeri*, *P. syncyanea*, *P. taetrolens*. In fact, the bactericidal activity of

EDTA against *P. stutzeri* was greater than that against any other organism so far screened. Most of the remaining organisms displayed sensitivity to EDTA on one or both of the criteria used. However, as increased permeability of cells treated with EDTA is not restricted to pseudomonads, a more clear-cut differentiation of sensitive and resistant organisms in terms of a bactericidal action by EDTA was desirable. For this reason selected organisms were re-tested at pH 9.2 under conditions known to increase the sensitivity of *P. aeruginosa* NCTC 1999. The results of these tests are given in Table 4. The strains of *P. aureofaciens*, *P. chlororaphis*, *P. denitrificans* and strain

Table 4. *Effect of increased pH value on sensitivity to EDTA of Pseudomonas species*

Organisms were treated for 1 hr at 20° with 0.0031 M-EDTA in borate buffer pH 9.2 (T) and buffer alone (B).

Organism	Strain	Suspension		Decrease in viability (%)†
		T	B	
		Release of solutes absorbing at 260 mμ*		
<i>P. aeruginosa</i>	NCTC 1999	26.9	4.0	99+
<i>P. aureofaciens</i>	NCIB 9030	18.1	3.7	99+
<i>P. chlororaphis</i>	NCTC 7357	18.1	3.9	99+
<i>P. denitrificans</i>	NCIB 8376	35.0	6.1	99+
<i>P. diminuta</i>	NCTC 8545	2.8	2.7	23
<i>P. fluorescens</i>	NCTC 10038	14.0	3.8	13
<i>P. fluorescens</i>	NCIB 9494	15.1	4.2	99
<i>P. fragi</i>	NCIB 8542	15.3	4.1	77
<i>P. geniculata</i>	NCIB 9428	11.5	11.4	0
<i>P. iodinum</i>	NCTC 9742	5.2	5.1	0
<i>P. maltophilia</i>	NCTC 10257	13.2	15.1	9
<i>P. maltophilia</i>	NCTC 10259	18.8	18.1	0
<i>P. maltophilia</i>	NCIB 9201	17.6	12.8	0
<i>P. mucidolens</i>	NCTC 8068	27.4	10.5	88
<i>P. ovalis</i>	NCTC 912	21.8	7.8	99+
<i>P. pavonacea</i>	NCIB 9395	9.6	9.3	29
<i>P. putida</i>	NCIB 9034	27.4	11.8	82
<i>P. rubescens</i>	NCIB 8768	20.7	8.6	63
<i>P. synxantha</i>	NCIB 8178	31.1	7.4	82

* Expressed as a percentage of solutes from organisms heated 10 min. at 100°.

† Organisms in T, relative to those in B.

NCIB 9494 of *P. fluorescens* were then clearly seen as sensitive organisms, while the strains of *P. fragi*, *P. mucidolens*, *P. putida*, *P. synxantha* and perhaps *P. rubescens* seemed to be rather less sensitive. Since *P. fluorescens* NCIB 9494 has recently been assigned to *P. putida* biotype A (Stanier *et al.* 1966), the differing results for the 2 strains of *P. fluorescens* are less surprising. With the remaining organisms (*P. diminuta*, *P. geniculata*, *P. iodinum*, *P. maltophilia*, *P. pavonacea*) EDTA caused little or no leakage of solutes absorbing at 260 m μ , even with those strains which gave appreciable leakage at pH 7.1. These results might indicate that leakage from organisms swollen at the alkaline pH value (Shively & Hartsell, 1964*b*) masked any specific effect of EDTA on these organisms. The resistance of these organisms to EDTA is confirmed

by the fact that little or no decrease in viability was produced at either pH value (decreases in viability below 15% are considered to be of doubtful validity). With none of these resistant organisms was there evidence for any significant loss of viability in the pH 9.2 buffer alone.

DISCUSSION

It has been shown that sensitivity to EDTA is not confined to *Pseudomonas aeruginosa*, but occurs widely among both fluorescent and non-fluorescent pseudomonads and is found in representative organisms from at least 10 of the species centres proposed by Lysenko (1961). Although a proper assessment of the taxonomic value of this character must await more extensive tests on these and other Gram-negative organisms, several interesting and potentially useful correlations are already apparent.

A particularly striking example is the contrast in EDTA-sensitivity between *Pseudomonas alcaligenes* (polar, monotrichous flagellate) and *Alcaligenes faecalis* (peritrichous flagellate). These organisms have closely similar physiological properties and are normally differentiated only by the type of flagellation. High resistance to EDTA was also found for *A. odorans*, an organism which was removed from the genus *Pseudomonas* and placed in the genus *Alcaligenes* largely on the basis of its peritrichous flagellation (Málek, Radochová & Lysenko, 1963). Two *Achromobacter* strains (NCIB 8250 and an uncharacterized strain) proved to be similarly resistant to EDTA, suggesting that this property might help further in the recognition and classification of organisms in the genus *Pseudomonas* and the rather ill-defined genera *Alcaligenes* and *Achromobacter*.

Because of the lack of physiological standardization of the organisms used, the various pseudomonads tested probably cannot be arranged in a meaningful order of sensitivity to EDTA. However, *Pseudomonas aeruginosa*, *P. alcaligenes*, *P. stutzeri* and *P. synchyanea* may perhaps be differentiated from other sensitive organisms. EDTA had a potent lethal action on these organisms at pH 7.1, and the lysed organisms were centrifuged from suspension as a mucoid deposit which did not disperse on agitation. This effect was absent or much less obvious with other organisms, even when the bactericidal effect of EDTA was enhanced in tests at pH 9.2. These particularly sensitive organisms can be clearly differentiated from each other on the basis of phenotypic features (Stanier *et al.* 1966), and do not emerge as a distinctive group from Adansonian analyses nor from studies on DNA base composition (Colwell, Citarella & Ryman, 1965; De Ley, Park, Tijtgate & Van Ermengem, 1966; Mandel, 1966). However, the structure or composition of the cell wall has not been included as a character in any study of pseudomonads so far made. Although the extent of flagellation is of uncertain value in the internal differentiation of the pseudomonads (Rhodes, 1965), it is tempting to see it here as a unifying feature of *P. aeruginosa*, *P. alcaligenes*, *P. pseudoalcaligenes* and *P. stutzeri*; of the aerobic pseudomonads studied by Stanier *et al.* (1966) only these organisms were consistently monotrichous.

The position of *Pseudomonas synchyanea* is more confused. The strain used here (NCTC 9943 = ATCC 9979) has been successively labelled *P. synchyanea*, *P. mildenbergii* and *P. convexa* (Haynes, 1961), while Stanier *et al.* (1966) propose that *P. convexa* be reduced to synonymy with *P. putida*. According to *Bergey's Manual* (1957), *P. convexa* possesses 'a polar flagellum' but electron microscopic examination of

strain 9943 showed that the organisms had multitrichous flagellation. Preliminary experiments indicate that this organism is also exceptional in being lysed by pH 9.2 buffer alone.

The organism NCTC 10038 differed from all others tested in that whereas it could be considered sensitive to EDTA on the basis of leakage produced at pH 7.1 and 9.2, and also from turbidimetric studies and the potentiation of lysozyme (Shively & Hartsell, 1964*a*), EDTA had little or no lethal action on the organisms under the conditions of the present experiments. The only two other organisms accounted by Stanier *et al.* (1966) as biotypes of *Pseudomonas fluorescens* (*P. aureofaciens*, *P. chlororaphis*) included in the present tests gave virtually identical results (Tables 3, 4). These two organisms differed significantly from the remaining biotypes in DNA base composition (Mandel, 1966). As strain NCTC 10038 has been proposed as a neotype for *P. fluorescens* (Rhodes, 1959; Stanier *et al.* 1966), the possible significance of these observations calls for further work on biotypes of this species.

It would be premature to suggest that sensitivity to EDTA should characterize all organisms qualifying for the genus *Pseudomonas*. Nevertheless, it must be noted that the inclusion in the genus of each of the organisms found here to be resistant has been questioned by other workers. From studies on DNA homology and base composition, De Ley *et al.* (1966) concluded that *P. pavonacea* and *P. rubescens* should be removed from *Pseudomonas*, while the inclusion in the genus of *P. diminuta* and *P. iodinum* was considered doubtful. The generic status of *P. diminuta* was queried by Rhodes (1958) and Thornley (1960), while *P. iodinum* is exceptional in being non-motile, oxidase-negative and Gram-variable or Gram-positive. This organism is included in the current (1964) NCIB catalogue as a coryneform organism. Although EDTA at pH 9.2 consistently caused some decrease in viable counts of *P. diminuta*, *P. pavonacea* and *P. rubescens*, the absence of EDTA-induced leakage from the first two organisms indicates that the action of EDTA on them was different from that on more sensitive organisms. The growth of *P. rubescens* on the agar slopes was very heavy and the organisms were deposited from suspension only by centrifugation at high speed: the deposit appeared to consist of two layers, suggesting that a high proportion of degenerating organisms may have been present. The strain of *P. geniculata* tested was that used by Lysenko (1961) and De Ley *et al.* (1966) and therefore does not fit the description given in *Bergey's Manual* (1957). The heterogeneity of organisms labelled *P. geniculata* was noted by Mandel (1966) who reported that some of them have been identified as *P. maltophilia*. The present strain closely resembled *P. maltophilia* NCIB 9201 in its resistance to EDTA, in its weak or negative oxidase reaction and in the type of growth on nutrient agar. *P. maltophilia* can be distinguished from the main body of pseudomonads by the oxidase test and by its production of lysine decarboxylase (De Ley, 1964). In its phenotypic features the organism is very distinctive (Stanier *et al.* 1966).

It is a danger in a simple screening procedure such as that followed here that the somewhat arbitrary cultural and test conditions used might give rise to misleading results. To verify and explore the conclusions and correlations suggested by the results of these tests, a comparative survey of the composition of the cell walls of the organisms involved is being carried out. Like the Gram reaction, sensitivity to EDTA appears to depend on the structure of the cell wall so that this property might have a similar, if less general, taxonomic value.

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