PRODUCTION OF ENZYMES AND TOXINS BY HOSPITAL STRAINS OF *PSEUDOMONAS AERUGINOSA* IN RELATION TO SEROTYPE AND PHAGE-TYPING PATTERN

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LITTLE is known about the way in which *Pseudomonas aeruginosa* exerts its damaging effects on the host. The endotoxin of this organism has a rather low toxicity (Homma, 1968), but various extracellular proteins are formed that are toxic not only to man and animals but also to insects, tissue-culture cells, plants, and some Gram-positive cocci (Caselitz, 1966; Heckly, 1970). Liu (1966) showed that *P. aeruginosa* produced both a lecithinase and a protease that were toxic and produced necrosis in various organs after intravenous injection. Recently, Meinke *et al.* (1970) reported that a partially purified elastase was lethal to mice when injected by several routes. Other cell-associated and extracellular proteins with no known enzymatic activities are toxic in animal experiments (Berk, 1964; Liu; Homma; Bartell, Orr and Garcia, 1968). A factor that inhibits mouse-liver mitochondrial respiration was recently described (Armstrong, Stewart-Tull and Roberts, 1971).

Wahba and Darrell (1965) found that atypical strains of *P. aeruginosa*, many of which were later identified as *P. fluorescens*, did not produce extracellular protease, and that marked collagenase activity was found only in strains of *P. aeruginosa*. Brown and Foster (1970) later confirmed that only strains of *P. aeruginosa* formed a protease detectable on a milk medium. Chakrabarty, Adhya and Pramanik (1970) detected lipolytic activity on Tween-80 agar in eight of 20 strains of *P. aeruginosa*. From earlier studies on microbial lipases and esterases it is obvious that *Staphylococcus aureus*, *P. aeruginosa* and other species might produce more than one enzyme of this class (Sierra, 1957; Lawrence, 1967; Sagers and Stewart, 1968). The egg-yolk reaction might be due to a lipase, as in *Staph. aureus* (Lundbeck and Tirunarayanan, 1966), or to a lecithinase as in several species of *Clostridium* and *Bacillus*.

We do not know of any systematic examination of *P. aeruginosa* strains isolated from human infections and healthy carriers for the production of toxins and enzymes. The aim of the present investigation was to find out the frequency of the formation of these various proteins and try to correlate the observations with the phage and serotypes of these strains.

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MATERIALS AND METHODS

Origin of strains. One hundred and forty-eight strains of *P. aeruginosa* had been isolated from non-bacteraemic infections and healthy carriers (group I) in the Karolinska Hospital, Stockholm, during a period of 2 yr (1969–70). Fifty strains of *P. aeruginosa* were isolated from blood or cerebrospinal fluid of patients with bacteraemia (group II) in regional hospitals in central Sweden during a period of 3 yr (1968–70). The strains were identified as *P. aeruginosa* according to Hugh (1970), and were kept on agar slants at 4°C and in broth at –20°C.

Phage typing was performed with a standard set of 22 *P. aeruginosa* bacteriophages as recently described (Sjöberg and Lindberg, 1968).

Serotyping. Antisera were produced in New Zealand white rabbits (2–3 kg) against 14 different type strains, including representatives of the 12 serotypes described by Habs (1957). Typing was made by agglutination, and tests were read after incubation for 2 hr and again overnight at 50°C (L. Sjöberg and A. A. Lindberg, to be published).

Agar-plate methods. Unless stated otherwise, the basic medium consisted of Bacto Nutrient Agar (Difco, B1) in all kinds of plates described below. The ability to produce the various factors was determined from positive reactions on the different agar plates, i.e., as zones of activity for more than 2 mm around the streaks after growth at 37°C for 24 or 48 hr.

Haemolysin production was detected on plates containing Bacto Blood Agar Base (Difco, B45) and erythrocytes from rabbit, sheep, man, horse and ox (5 per cent. final concentration, v/v) washed once with saline.

The production of protease was studied on agar containing 15 per cent. (v/v) sterile skimmed milk (Brown and Foster). Elastase was assayed on elastin agar plates (Sbarra *et al.*, 1960).

The egg-yolk reaction was determined on egg-yolk agar prepared according to Lundbeck and Tirunarayanan. Since a positive reaction can be produced both by a lipase and a lecithinase, special media were also used to differentiate between these two enzyme activities. Tributyrin agar was prepared after sonic treatment of the substrate according to Hugo and Beveridge (1962). Lecithin agar contained 5 g of lecithin per l of agar medium (T. Wadström, P. Allestam and R. Möllby, to be published). Esterase activity was detected on agar containing indoxyl butyrate (Muftic, 1954; Holt, 1971).

DNAase activity was determined on DNAse Test Agar (BBL) according to DiSalvo (1958) and RNAase activity on RNA agar plates as described by Miller, Sandine and Elliker (1971). After 48 hr at 37°C the plates were flooded with 1.0N-hydrochloric acid and positive cultures showed a clear zone around the streak.

Bacteriolytic activity was determined on agar plates containing heat-killed *Micrococcus lysodeikticus*, strain NCTC2665 (Hawiger, 1968), or *Staph. aureus*, strain Copenhagen (Schindler and Schuhardt, 1964; Wadström and Hisatsune, 1970). Coagulase was assayed on reconstituted rabbit plasma (Bacto-coagulase Plasma, Difco) as prescribed by the manufacturer.

Hyaluronate lyase (hyaluronidase) activity was assayed on a medium containing umbilical cord hyaluronic acid (Smith and Willett, 1968) with a strongly positive *Staph. aureus* strain (strain M18; Wadström and Hisatsune) as a control. Because this procedure is not very sensitive, enzyme activity was also assayed on 30 strains of group I and II by a 100-times more sensitive viscosimetric method (Söder and Nord, 1969) after cultivation in liquid CCY₁ medium.

Statistical analysis. The significance of a difference in the frequency of the production of the various factors between group I and II was tested by the χ^2 test (Snedecor and Cochran, 1967).

Chemicals. Casein hydrolysate, yeast extract, and the agar bases were purchased from Difco. Elastin powder from bovine neck ligament, hyaluronic acid from umbilical cord, grade III, ribonucleic acid from yeast, grade II, and chondroitin sulphate, grade II, were obtained from Sigma Chemical Company, St Louis, Mo., USA. Indoxyl butyrate was purchased from Koch-Light, Colnbrook, Bucks, England.

RESULTS

Classification of strains. The origin of the strains from non-bacteraemic infections and healthy carriers (group I) and from patients with bacteraemia (group II) is shown in table I. More than 100 different phage-typing patterns were observed among the 198 strains in groups I and II. All strains in group II

TABLE I
Sources and serotypes of the strains of Pseudomonas aeruginosa

Group	Source	Number of strains from the stated sources of serotype																Total
		1	2	3	4	5	6	7	8	9	10	11	12	13	poly- agglutinable*	NT		
I†	Respiratory tract	1	1	1	0	1	10	0	1	0	3	3	0	2	0	2	25	
	Wound	4	3	3	3	0	8	1	1	1	2	2	2	2	2	1	35	
	Urine	0	5	4	0	1	9	1	1	1	0	3	0	5	2	2	34	
	Faeces	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	3	
	Ear	3	3	5	2	4	16	0	1	0	2	2	0	3	1	1	43	
	Abdominal abscess	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	
	Unknown	1	1	0	0	2	2	0	1	0	0	0	0	0	0	0	7	
	Any	9	13	13	5	10	47	2	5	2	7	10	2	12	5	6	148	
II‡	Blood	6	4	2	2	2	13	0	1	0	1	3	0	2	1	1	38	
	Cerebrospinal fluid	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	3	
	Necropsy	0	1	0	0	0	3	0	0	0	1	2	0	0	1	1	9	
	Any	6	5	2	2	3	17	0	1	0	3	5	0	2	2	2	50	

* 2B/5C, two strains; 9/10/13, one strain; 9/13, four strains.

† From non-bacteraemic infections and healthy carriers.

‡ From patients with bacteraemia.

NT = Untypable.

were typable, and only 4 per cent. of the group I strains were untypable, with the set of 22 phages. No phage-typing pattern was predominant in either of the two groups of strains. The results of serological typing (table I) showed that about one-third of the strains belonged to Habs' type 6 and that there was no statistically significant difference in the distribution of serotypes between groups I and II ($\chi^2 = 6.7$; $P = 0.95$).

Production of enzymes and toxins. The results of the tests on all strains in groups I and II for production of various enzymes and haemolysins are given in tables II and III. More than 90 per cent. of the strains in both groups produced lipase, esterase and protease, and lysed ox and rabbit erythrocytes. Between 60 and 90 per cent. of strains in the two groups gave a positive egg-yolk reaction, produced nuclease (RNAase and DNAase), elastase, lecithinase, and staphylytic enzyme, and lysed sheep and horse erythrocytes. No

TABLE II
Occurrence of enzymes and toxins in the strains belonging to group I (non-bacteraemic infections and healthy carriers)

Serotype	Number of strains examined	Number (and percentage) of strains that											
		gave a positive test for						showed haemolytic activity against erythrocytes of					
		protease	elastase	DNAase	RNAase	lecithinase	egg-yolk precipitation	staphylo-lytic enzyme	rabbit	sheep	horse	ox	man
1	9	9	6	5	8	7	7	7	8	5	5	9	8
2	13	13	8	12	12	9	7	8	13	10	6	13	9
3	13	13	10	9	12	12	7	9	13	10	7	13	12
4	5	4	3	4	5	3	8	4	5	4	4	5	4
5	10	10	9	6	6	8	3	8	10	7	5	9	8
6	47	45	42	36	43	38	32	41	46	34	28	47	41
7	2	2	2	2	1	1	1	1	2	1	1	2	1
8	5	5	5	2	5	3	4	3	5	5	2	5	3
9	2	2	2	2	2	2	2	2	2	2	2	2	2
9/13 and 1/10/13	5	5	5	4	5	4	4	5	5	3	4	4	4
10	7	7	6	5	3	3	7	7	7	7	6	7	6
11	10	9	7	8	4	4	7	6	10	8	5	10	8
12	2	2	1	1	2	2	1	1	1	1	1	2	1
13	12	12	9	12	9	9	10	9	11	8	7	12	11
NT	6	6	5	5	6	3	5	5	6	6	4	6	5
Any	148	144 (97)	124 (84)	115 (78)	129 (87)	109 (74)	107 (72)	116 (78)	144 (97)	111 (75)	87 (59)	146 (99)	123 (83)

All strains were positive for lipase and esterase. All strains except one gave a clear zone on egg-yolk medium.
NT = Untypable.

TABLE III
Occurrence of enzymes and toxins in the strains belonging to group II (*bacteraemic infections*)

Serotype	Number of strains examined	Number (and percentage) of strains that											
		gave a positive test for							showed haemolytic activity against erythrocytes of				
		protease	elastase	DNAase	RNAase	lecithinase	egg-yolk precipitation	staphylo-lytic enzyme	rabbit	sheep	horse	ox	man
1	6	5	5	5	5	5	6	5	6	3	3	6	5
2	5	5	4	4	5	3	4	5	5	3	3	4	4
2B/5C	2	2	2	1	2	2	2	2	2	2	2	2	2
3	2	2	2	2	2	2	2	2	2	2	2	2	2
4	2	2	2	2	2	2	1	2	2	2	0	2	2
5	3	3	3	3	1	1	0	3	2	2	0	2	2
5	17	17	15	11	14	14	14	13	17	14	13	15	14
6	1	1	1	1	1	0	1	1	1	1	1	1	1
8	3	3	3	3	2	1	3	3	3	3	3	3	3
10	5	5	5	2	4	3	4	5	5	5	3	5	5
11	2	2	2	1	2	2	1	1	2	2	1	2	2
13	2	2	2	2	2	2	1	1	2	2	1	2	2
NT	2	2	2	2	2	1	1	1	2	2	1	2	2
Any	50	49 (98)	46 (92)	36 (72)	44 (88)	36 (72)	39 (78)	43 (86)	49 (98)	42 (84)	32 (64)	46 (92)	44 (88)

All strains were positive for lipase and esterase, and gave a clear zone on egg-yolk medium.
NT = Untypable.

significant difference in the formation of any of these factors was found between the two groups in the analysis by the χ^2 test. No strains gave a positive reaction on the plates for hyaluronate lyase or lysed cells of *M. lysodeikticus*, i.e., produced a lysozyme-like enzyme.

One strain of group 1 and 29 strains of group 2 were also grown in liquid medium, and the supernatants from cultures in the late logarithmic phase of growth were assayed for hyaluronate lyase by a sensitive viscosimetric assay, and for coagulase on rabbit plasma. None of the strains gave a positive reaction in either test.

DISCUSSION

As far as we know, attempts to correlate serotype and phage-typing pattern with the ability of different strains of *P. aeruginosa* to produce the various toxins and enzymes have not previously been reported. We did not find any difference in the characters studied between strains isolated from blood and strains isolated from other parts of the body. The strains in group II were isolated in several hospitals during a 3-yr period, and those in group I, though isolated in a single hospital, were shown by phage and serological typing to be a heterogeneous collection. In both groups we found that Habs' type 6 was the most common serotype. According to Adler and Finland (1971), immunotypes 2 and 4 in Fisher's system (probably corresponding respectively to Habs' types 11 and 1) were the types most frequently isolated from the respiratory tract. Mikkelsen (1970) reported that Habs' type 2 was the most common type in blood cultures, and Nord, Sjöberg and Wadström (1972) that more than 50 per cent. of strains isolated from oral infections belonged to Habs' type 1. Moody *et al.* (1972) found that the commonest types in cultures from the blood and pleural cavity in one hospital over a short period of time were of Fisher's immunotypes 2, 4 and 7 (probably corresponding respectively to Habs' types 11, 1 and 5). Edmonds *et al.* (1972) could find no difference between strains isolated from burns and from other sites in patients in a burns unit in the distribution of serological, bacteriophage and pyocine types.

Moody *et al.* (1970) reported a relationship between immunotypes and antibiotic sensitivity in a collection of strains from hospital patients suffering from various infections. We have found no correlation between the antibiotic-sensitivity pattern and any of the parameters investigated in this study (B. Wretlind, C.-E. Nord and T. Wadström, to be published).

A high percentage of the strains in groups I and II produced most of the various extracellular proteins assayed. The results of previous reports indicate that the related genera *Pseudomonas*, *Aeromonas* and *Vibrio* are probably the only ones among the Gram-negative bacilli that are able to do so. An analysis of different species within these genera will soon be reported (B. Wretlind and T. Wadström, to be published). Examination of a large number of strains of *Escherichia coli* and other species within the Enterobacteriaceae revealed that none of the plate-assay methods gave positive results except for haemolysin and in a few cases for protease and DNAase activity (T. Wadström, unpublished data). However, the production of extracellular DNAase was recently

reported to be useful in the identification of *Serratia* (Rothberg and Swartz, 1965). Certain strains of *Proteus* also give a positive test. Strains of *Pseudomonas*, *Aeromonas*, and *Vibrio* produce bacteriolytic enzymes that lyse *Staph. aureus* ("staphylolytic enzyme"; Coles, Gilbo and Broad, 1969; Lache *et al.*, 1969), and strains of these genera also produce proteases (Moriyama, 1964; Brown and Foster, 1970; Dahle, 1971; Tanaka and Iuchi, 1971) and various haemolysins (Yanagase *et al.*, 1970; Výmola *et al.*, 1971; Wretling, Möllby and Wadström, 1971). Scharmann (1972) also recently reported that *P. aeruginosa* and *P. maltophilia* were the only two of ten pseudomonas species that produced elastase. He also postulated that this enzyme is probably identical with the fibrinolysin and might be involved in pathogenesis, since no apathogenic species was elastase positive.

A haemolysin, a lecithinase, and a partially purified elastase from *Pseudomonas* are toxic to animals and insects (Heckly, 1970). The haemolytic spectrum of the strains investigated indicate that *P. aeruginosa* might produce at least two different haemolysins, one most active on sheep and horse erythrocytes and the other most active on rabbit erythrocytes. A recent paper by Výmola *et al.* presented data from agar-gel electrophoresis indicating that three haemolytic factors are produced at least by some strains.

Further analysis of the data in tables I-III did not reveal any correlation between the different characters of the strains. No statistical difference in the qualitative production of these various metabolites was found between the two groups or between individual serotypes or phage-typing patterns. This is analogous with the observations that strains from staphylococcal septicaemia and strains from healthy carriers do not show a difference in the ability to produce various extracellular enzymes and toxins (Bülow, 1971). A more detailed analysis of the phage types and serotypes of the isolated strains will soon be reported (L. Sjöberg and A. A. Lindberg, to be published).

Kobayashi (1971) recently reported that none of the extracellular haemolysins or enzymes of *P. aeruginosa* was an important toxic factor in the mouse-virulence test, and found no difference in virulence between strains isolated from patients or healthy carriers. However, more work must be done to find out whether any of the known extracellular factors are important as virulence factors in human infections. An enterotoxin has recently been reported in a strain of *P. aeruginosa* (Kubota and Liu, 1971), and two other new factors, one which inhibits mitochondrial respiration (Armstrong *et al.*, 1971) and one which preferentially kills polymorphonuclear leucocytes (U. Scharmann, personal communication) have also been described. These findings, and the facts that acrylamide electrophoresis of extracellular proteins from *P. fluorescens* resolved 13 different protein bands (Winters and Corpe, 1971), and that several enzymes and toxins might be superficially located in the cell envelope (Heppel, 1967), emphasise the importance of further work in this field.

SUMMARY

We studied 148 strains of *Pseudomonas aeruginosa* isolated from non-bacteriaemic infections and healthy carriers (group I) and 50 strains isolated

from patients with bacteraemia (group II). More than 90 per cent. of the strains in both groups produced lipase, esterase, protease, and a haemolysin most active on ox and rabbit erythrocytes. Between 60 and 90 per cent. gave a positive egg-yolk reaction, produced nuclease (DNAase and RNAase), lecithinase, elastase, staphylytic enzyme, and another haemolysin most active on sheep and horse erythrocytes. No significant difference between the two groups in the formation of any of these factors was found. The strains of the two groups showed more than 100 different phage-typing patterns when tested with a set of 22 phages; only a few strains were untypable and no phage-typing pattern predominated. About one-third of all the strains belonged to Habs' serotype 6 and the rest of the strains were rather evenly distributed between the other serotypes without any difference between the two groups.

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