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Production by Various *Pseudomonas* Species of a Factor Modifying the Enterobacterial Common Antigen (41689)

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Abstract. The enterobacterial common antigen (ECA) is a common determinant shared by almost all members of the family Enterobacteriaceae. The antigen modifies erythrocytes for agglutination by ECA antibodies. Previously it was reported that *Pseudomonas aeruginosa* produces a factor (PF) which destroys the erythrocyte-modifying capacity of ECA. The present investigation was undertaken to determine whether other species of this genus also produce PF. The passive bacterial hemagglutination and hemagglutination inhibition tests were used. It was observed that 47 strains belonging to 8 species of the genus *Pseudomonas* produce this factor and 34 strains representing 12 other species do not. Multiple strains of a given species gave concordant results. Mucoid variants of *P. aeruginosa* produced more of this factor than did nonmucoid isolates recovered from the identical sputum specimens from patients with cystic fibrosis. ECA treated with PF no longer modifies erythrocytes for agglutination by ECA antibodies and exerts less antibody-neutralizing capacity than untreated antigen.

The enterobacterial common antigen (ECA), first described by Kunin *et al.* (1, 2) and recently reviewed by Makela and Mayer (3) and by Mayer and Schmidt (4), is a common antigenic determinant shared by almost all members of the family Enterobacteriaceae. ECA has been isolated and chemically characterized (5, 6) and its presence on the bacterial surface documented (7). The antigen is of particular interest from an immunological point of view, as it exists in both an immunogenic and a nonimmunogenic form. The latter is produced by smooth strains of Enterobacteriaceae and the former by certain mutants (8–10). As reported recently, determination of ECA production serves as a valuable aid to classification (11).

Following a chance observation, it was shown that *Pseudomonas aeruginosa* produces a factor (PF) which destroys the nonimmunogenic form of ECA without affecting the simultaneously present O antigen (12). The factor also alters the ethanol-soluble ECA after its separation from the ethanol-insoluble lipopolysaccharide (LPS). PF is heat-labile, being destroyed at 100°C in 10 min. It is produced also by a psychrophilic *Pseudomonas* strain (13). PF has been identified as a lipase (14). It was of interest to learn whether PF is produced by other members of the genus *Pseudomonas* and to determine whether mucoid and nonmucoid strains of *P. aeruginosa* often present simultaneously in the respiratory tract of patients with cystic fibrosis (15) differ in its production.

Materials and Methods. A total of 81 strains representing 20 species of the genus *Pseudomonas* were studied for production of PF. The strains were kindly supplied by Dr. G. L. Gilardi, The Hospital for Joint Diseases and Medical Center, New York, by Dr. M. Shayegani, Division of Laboratories and Research, New York State Department of Health, Albany, and by Dr. John M. Pickett, University of California, Los Angeles. Twelve mucoid and 6 nonmucoid strains of *P. aeruginosa* were freshly isolated from patients at the Erie County Medical Center and Children's Hospital, Buffalo, New York. Among these strains were 4 pairs of both types recovered from the identical sputum specimens from four patients with cystic fibrosis. Information on all strains is given in Table II.

For the preparation of PF, the previously described method was used (12, 13). Briefly, the strains were grown on nutrient agar at 37°C for 72 hr. The resulting growth was sus-

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TABLE I. EFFECT OF *Pseudomonas aeruginosa* AND *P. stutzeri* ON ETHANOL-SOLUBLE ECA FROM *S. typhimurium* AS DETERMINED BY HEMAGGLUTINATION

ECA antiserum (*E. coli* 014) dilutions	PF from						
	P. aeruginosa			*P. stutzeri*			
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	0
1:40	0[a]	0	3	3	3	3	3
1:80	0	0	3	3	3	3	3
1:160	0	0	2	2	3	2	2
1:320	0	0	2	2	2	1	2
1:640	0	0	1	1	1	1	1
1:1280	0	0	1	1	1	1	1
1:2500	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0

Note. Abbreviations used: PF, *Pseudomonas* factor; ECA, enterobacterial common antigen.
[a] Data expressed as absent hemagglutination (0) or various degrees (1 to 3) of hemagglutination.

pended in phosphate-buffered saline (pH 7.3, 5 ml/plate). The suspension was centrifuged at 2350*g* for 30 min and the resulting supernatant fluid was stored in aliquots of 1 ml each at −20°C.

Crude, semipurified, and purified preparations of ECA were obtained in accord with previously described methods (8, 16). Briefly, supernatant fluids of heated suspensions of *Salmonella typhimurium* and *Escherichia coli* 026 served as crude preparations. The ethanol-soluble ECA was prepared from the supernatant fluids by incubation with ethanol and resuspension of the dried ethanol-soluble material in distilled water (8, 16). Purified ECA was obtained from *S. montevideo,* as described previously (6).

Antiserum against ECA was prepared in rabbits by intravenous immunization with either *E. coli* 014 or the ethanol-soluble ECA preparation from *S. typhimurium* (8, 16).

The presence of ECA was determined by means of the passive hemagglutination and hemolysis tests, described previously (10, 16). ECA, treated with either PF or buffer, was added to the sediment of washed sheep erythrocytes (2.5%). The erythrocytes were incubated at 37°C for 30 min and washed. Using ECA antiserum in twofold serial dilutions, the hemagglutination test was completed, in accord with the procedure detailed previously (8, 12). For the hemolysis tests, guinea pig complement (Cordia Laboratories) was added.

Hemagglutination inhibition (antibody neutralization) tests were carried out by absorbing ECA antiserum in serial twofold dilutions by adding equal amounts (0.2 ml) of ethanol-soluble ECA or PF-treated ECA. The mixtures were kept at 37°C for 30 min. ECA-modified erythrocytes were added and the test completed as described above.

Results. In order to determine which species of the genus *Pseudomonas* produce PF, preliminary quantitative hemagglutination tests were carried out with a few strains. The results of a representative experiment are shown in Table I. It may be seen that the strain of *P. aeruginosa,* even in dilutions up to 1:100, altered ECA so that it no longer modified erythrocytes for passive hemagglutination with ECA antiserum. In contrast, *P. stutzeri* had no such effect. It may be concluded that the latter microorganism failed to produce PF or produced less than 1% of the amount present in cultures of *P. aeruginosa.* On the basis of these results 81 strains representing 20 species of the genus *Pseudomonas* were tested. The results are shown in Table II. It is evident that 47 strains belonging to 8 species produced PF and 34 strains representing 12 other species did not. Wherever multiple strains of a given species were tested, concordant results were obtained (Table II). As expected, using the hemolytic modification of the hemagglutination test, identical results were obtained with representative strains of both PF-positive and PF-negative strains.

In view of the possibility that the negative

TABLE II. *Pseudomonas* FACTOR PRODUCTION BY VARIOUS SPECIES

PF(+)[a] *Pseudomonas* strains				PF(−)[a] *Pseudomonas* strains			
Species	Source	Number	Total	Species	Source	Number	Total
P. aeruginosa	EC	16		*P. acidovorans*	S	1	
	CH	6	22		P	2	3
P. diminuta	S	1		*P. alcaligenes*	S	1	
	P	4	5		P	3	4
P. maltophilia	ECMC	1		*P. cepacia*	G	1	
	G	1			S	1	2
	S	1					
	P	6	9	*P. fluorescens*	G	1	
					S	1	2
P. pickettii, Va-1	G	1					
				P. pseudoalcaligenes	S	1	
	P	3	4				
					P	4	5
P. pickettii, Va-2	S	1					
				P. putida	G	1	
	P	2	3				
					S	1	2
P. putrefaciens	G	1					
					G	1	
	S	1	2				
				P. stutzeri	S	1	
GP II K-2					P	3	5
(*P. multivorum*)	S	1	1				
				P. testosteroni	S	1	
G P Ve-1	S	1	1		P	3	4
				P. vesicularis	S	1	1
				G P II K-1	G	2	
				(*P. paucimobilis*)	S	1	3
				G P V b-2			
				(*P. mendocina*)	S	1	1
				G P Ve-2	G	1	
					S	1	2
Total 8			47	12			34

Note. Abbreviations used: PF, *Pseudomonas* factor; ECA, enterobacterial common antigen; EC, Erie County Medical Center; CH, Children's Hospital; S, Dr. M. Shayegani, Albany; P, Dr. J. Pickett, Los Angeles; G, Dr. G. L. Gilardi, New York.

[a] Data expressed as PF positive if the strains on repeated determinations altered ECA by inhibiting erythrocyte modification and hemagglutination by ECA antiserum and as PF negative if they failed to do so.

results obtained with some of the species may be due to the presence of an inhibitor, the following experiments were carried out. Supernatant fluids of suspensions of representative cultures of PF-positive and PF-negative strains were used as mixtures or alone and added to ECA as in the experiment described in Table I. The mixtures were incubated and used for modification of red blood cells. Repeated experiments revealed that PF-positive cultures were equally active in the presence or absence of PF-negative cultures. Thus, an inhibitor was not detected in the latter strains and PF production is not shared by all species of this genus.

Several mechanisms may be considered to explain the mode of action of PF on ECA. (i) PF may affect the erythrocytes; (ii) it may alter the haptenic determinant; or (iii) it may affect parts of the molecule other than the haptenic determinant. Accordingly, erythrocytes were treated with PF and subsequently with ECA; hemagglutination was not affected, indicating that PF does not alter the red blood cells. To elucidate the possible effect on the haptenic determinant or parts of the molecule that alter the reaction of ECA with the corresponding antibody, the antibody neutralizing capacity of PF-treated and untreated ECA was determined. The results of a representative experiment are presented in Table III. It is evident that the antibody neutralizing capacity of treated ECA was significantly reduced, although it was not completely abolished.

Since all strains of *P. aeruginosa* tested produced PF it was of interest to determine whether mucoid strains of the species, often present in the respiratory tract of patients with cystic fibrosis (15), differ in PF production from nonmucoid isolates recovered from the same patients. Six strains of mucoid *P. aeruginosa* and four pairs obtained from single sputum specimens of four patients each were studied quantitatively. These titrations were done in accord with those recorded in Table III. Repeated experiments revealed that mucoid strains produced more PF than nonmucoid strains, the difference being at least 10-fold.

Discussion. Previous observations (12, 14) established the fact that *P. aeruginosa* produces a lipase which strikingly alters ECA, produced by almost all strains of the family Enterobacteriaceae. Based on the examination of 81 strains representing 20 different species it is shown here that not all species of the genus *Pseudomonas* produce this enzyme. The observation that from 5 to 9 different strains of several species yielded identical results suggests that, with the probable exception of mutants, the presence or absence of PF is characteristic of the species rather than of individual strains. From these observations (Table II) it is evident that production of PF is not restricted to pathogenic species, since all 11 strains of the opportunistic pathogens *P. cepacia, P. fluorescens, P. putida,* and *P. stutzeri* failed to produce this enzyme. It remains to be determined whether PF is produced *in vivo,* such as in the respiratory tract of patients with cystic fibrosis, in infected burns, or in infections of the urinary tract, and, if so, whether it affects ECA of strains of Enterobacteriaceae that may be present at the same site. The effect of PF antibodies under these conditions also deserves exploration.

TABLE III. EFFECT OF PF ON ANTIBODY-NEUTRALIZING CAPACITY OF ETHANOL-SOLUBLE ECA FROM *S. typhimurium* AS DETERMINED BY HEMAGGLUTINATION INHIBITION

ECA (μg)	*E. coli* 014 antiserum			
	1:160	1:640	1:160	1:640
	PF-treated ECA		Untreated ECA	
10	2[a]	0	0	0
5	3	0	0	0
2.5	3	1	2	0
1.25	3	2	2	0
0.62	3	3	3	1
0	3	3	3	3

Note. Abbreviations used: PF, *Pseudomonas* factor; ECA, enterobacterial common antigen.

[a] Data expressed as absent hemagglutination (0) or various degrees (1 to 3) of hemagglutination.

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