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PLASMIDS AND PUTATIVE VIRULENCE CHARACTERS IN PROTEUS MIRABILIS

Mohammadi,A.A., Smith , D.G. and

Rowbury, R.J.

ABSTRACT. **The correlation of plasmid carriage with a range of putative virulence properties in 145 isolates of Proteus mirabilis was investigated.As expected, the plasmid carrying (P^+) P. mirabilis isolates were more likely to be antibiotic-resistant than the p^- strains and also tended to carry more resistances. Plasmid carriage also enhanced production of enzymes, such as haemolysin, lecithinase and protease but appeared to greatly reduce ammonia production by urease. Most strikingly, the presence of plasmids markedly reduced swarming although a few P^+ P. mirabilis strains gave large swarming zones.**

Key words: PLASMIDS/PROTEUS MIRABILIS/BACTERIA/VIRULENCE-RESSITANCE

INTRODUCTION

Proteus mirabilis is a member of the Enterobacteriaceae, normally having the form of a short highly motile rod. It is found in sewage and in the gastrointestinal tract of man and animals and possibly in soil

(Williams and Schwarzhoff, 1978). This species is a common cause of urinary tract infections and can also be isolated from wounds, burns and discharging abscesses (Harmon et al., 1989); from the latter situations, Proteus mirabilis can go on to cause septicaemia and meningitis and is now a very common cause of systemic infections.

A feature of the physiology of this organisms,which can make it a problem during bacterial isolation, is its tendency to swarm, as rafts of long multinucleate filaments, on solid media (Smith, 1972). It has been demonstrated by Hesslewood and Smith (1974) that the plasmid R46 can enhance the swarming of P. mirabilis F67, whilst plasmid R6K can reduce swarming. Here an attempthas been made to correlate the extent of swarming with plasmid carriage.

A biochemical characteristic associated with P. mirabilis is the ability to form urease, which, in the female urinary tract, permits organisms to resist the normal acidity (pH4.5-6.0) and is associated with virulence (Senior et al., 1980; McLean et al., 1988). Other properties which may be associated with virulence are ability to attach to the epithelium of the urinary tract, increased hydrophobicity, iron chelation, production of other enzymes such as protease, haemolysin and lecithi - nase, serum resistance and the ability to swarm (Senior, 1979; Silverblatt, 1974; Pazin and Bruade, 1974; Shand et al., 1985; Mobley and Chippendale, 1990 . In the clinical situation, antibiotic resistance would also favour the survival of infecting organisms.In the present study, some of the above putative virulence characters have been examined in a collection of Proteus mirabilis

strains and an attempt to correlate plasmid carriage with some of them has been made.

MATERIALS AND METHODS

Bacterial strains

All strains used were of Proteus mirabilis;137 were isolates from urinary tract infections and supplied by the Microbiology Department, University College Hospital, London, whilst 8 were faecal isolates from the Animal Disease Diagnostic Laboratory, Razi Institute, Tehran,Iran, and the National Type Culture Collection.

Growth and culture media

The growth media used were Nutrient Broth (Oxoid No. 2), Nutrient Agar (Oxoid), Sensitest Agar (Oxoid), CLED Medium (Cysteine, lactose, electrolyte-deficient medium; Oxoid) and Proteus isolation medium (Hawkey et al.,1986). Blood Agar Base (Oxoid) and Urea Agar Base (Oxoid) were used for haemolysin and urease tests (see below).

Growth for these proteus strains was at 37°C except that swarming was studied with organisms growing at 30°C.

For antibiotic resistance tests, 0.1ml aliquots of overnight cultures were spread on sensitest agar plates and then multodiscs (Oxoid) or single antibiotic discs werre placed on the agar. Sensitivity or resistance was assessed after incubation for 18h at 37°C.

Plasmid transfer and curing

Cultures of P^+ and P^- strains were grown at 37°C to exponential phase and then 1ml aliquots of each incuba - ted with 10ml broth for 4 h or 16 h at 37°C . Diluted samples were then plated on selective media containing

appropriate antibiotics. Putative plasmid-containing (p^+) transconjugants were checked for the presence of plasmids by agarose gel electrophoresis (see below). Curing by sodium dodecylsulphate heat treatment (Salisbury et al. 1972) was not effective but some p^+ strains were cured by growth with acridine orange; curing was achieved by incubating culture dilutions in broth plus 50 g/ml acridine orange plus 1mM Tris HCL, the pH being adjusted to 8.5 (Goodson and Rowbury, 1978). After growth overnight at 37°C, cultures were plated on nutrient agar and colonies formed at 37°C replica-plated to NA plates, supplemented with appropriate antibiotics, to score for loss of resistance. Where loss of antibiotic resistance appeared to indicate plasmid curing, this was checked by agarose gel electrophoresis.

Swarming

Nutrient agar plates dried at 60°C for 10 min were inoculated centrally or at a margin with loopfuls of overnight culture. Plates were incubated at 30°C and , after 24 h, swarming distances measured from the inocu - lation point to the leading swarm edge.

Plasmid detection

Plasmid DNA isolated according to Kado and Liu(1981) was subjected to horizontal electrophoresis on 0.7% agarose gels together with plasmid DNA from a marker strain containing plasmids of known molecular weights (4.2,23.9, 42.0 and 98.0 md) and aliquots of phage DNA cut with EcoRI or Hind III restriction endonucleases. Ethidium bromide stained gels were photographed and molecular weights of Proteus plasmids determined from their mobilities .

Qualitative urease tests

Urea Agar Base (Oxoid, 24 gm) was added to 950 ml distilled water, and the suspension sterilized. Then 50 ml of 40% (w/v) filter-sterilized urea solution was added and the agar mixed and distributed in 10 ml lots in screw-cap bottles to form slopes. Loopfuls of organisms were then streaked on the slopes and a pink colour in the agar after 24 h incubation at 37°C was taken to indicate a ureas-positive phenotype.

Assessment of ammonia production by urease

The aim was to assess, semi-quantitatively, ammonia production by urease on solid and in liquid media.

For assessment of urease on solid medium, medium was prepared as above and distributed in 20 ml lots in perti dishes. Dried plates were inoculated centrally and incubated at 37°C; the pink zones which formed were measured at 2 h intervals during incubation.

To assess ammonia production by urease in liquid medium, nutrient broth plus 2% w/v urea was inoculated with overnight culture (1ml plus 20ml broth) and the pH measured at 2 h intervals during incubation wiht shaking at 37°C.

Assessment of secreted enzymes

To assess the level of protease in culture supernatants, 1 ml of overnight culture grown at 37°C in broth was centrifuged (12000 r/m) in an Eppendorff centrifuge for 10 min and 100 l aliquots of supernatant (with 0.3% w/v sodium azide added) were pipetted into wells in plates of nutrient agar containing 10% v/v skimmed milk. On incubation at 37°C the sizes of the zones of clearing at 24 and 48 h were taken as an indication of protease

activity. The same technique was used to assess lecithinase in culture supernatants using nutrient agar containing 5% v/v egg yolk emulsion (Oxoid).Haemolysin was assessed by streaking(to give single colonies) loopfuls of overnight 37°C broth cultures on nutrient agar or blood agar Base (Oxoid), each containing 0.7% (v/v) defibrinated horse or hseep blood. Incubation was at 37°C for 48 h with zones of clearing being measured at 24 and 48 h.

RESULTS

In the present study, 137 strains of Proteus mirabilis isolated from patients with urinary tract infections and 8 faecal isolates have been examined.

The results in Table 1 indicate the resistance pattern to 9 antibacterial agents or combinations of antibacterial agents; in addition, all 145 P. mirabilis strains were resistant to sulfafurazole and polymixin B at the concentrations tested. On the basis of the 24 h qualitative urease agar slope test (see Materials and Methods) all 145 P.mirabilis strains were urease positive.Most of them were able to swarm and swarming zone sizes of some of them are indicated on Table 5. Of the clinical isolates, 40 had one or more plasmids whilst of the faecal isolates P. mirabilis (RM1-RM6, RM8 and P33), 4 carried a plasmid (Table 1).

The first major correlation between plasmid carriage and isolate properties is that, as expected, plasmid-carrying(p^+) P.mirabilis strains were more likely to be antibiotic resistant. With reference to the 9 agents (or combinations of agents) of Table 1, 75% of the p^+ isolates

were resistant to one or more, with the mean number of resistances per p^+ P. mirabilis strain being 1.91±o.28 . For the p^- P. mirabilis strains, these figures were 35% resistant and 0.45±o.07 resistances per p^- strain. Most of the antibiotic-ressitant p^- P. mirabilis strains carried only a single resistance (77%) whereas only 27% of resistant p^+ ones had a single resistance. The faecal isolates (p^+ and p^-) showed some antibiotic resistance but none was ampicillin resistant in contrast to the clinical isolates (34% ampicillin-ressitant).

Swarming appeared to be affected by plasmid carriage a higher percentage of p^+ P. mirabilis strains (than p^- ones) failed to swarm (20.5% p^+ compared with 3.0% p^-) and the mean swarming zone size was greater for those that were p^- (4.0±0.17 cm) than for those that were p^+ (2.0±0.28 cm).

Accordingly, on the basis of the above tests on all 145 P. mirabilis strains, it appeared that plasmid carriage favoured antibiotic resistance and led to reduced swarming but (on the basis of the simple qualitative urease test used) did not appear to affect urease.

To ascertain for some isolates, which resistances were plasmid-encoded and how plasmids more precisely affected specific properties, firstly, several were cured of their plsmids and, secondly, some plasmids were transferred to p^- isolates; in each case, there sulting p^+/p^- pairs were compared.

Conjugal transfer of plasmids

The plasmid-carrying strains P. mirabilis PM4, PM5 and P49 (each of which has a single plasmid of molecular weights 42, 105 and 32 md respectively) were used as

donors with P. mirabilis strain G9 (a p^- isolate) as recipient. Examination of transconjugants (Table 2)indicated that pPM4 conferred resistance to cephaloridine and kanamycin, pPM5 resistance to tetracycline, cephaloridine and co-trimoxazole but not to nalidixic acid whilst pP49 carried resistance to tetracycline and cephaloridine. Because P. mirabilis strain G9 is ampicillin-resistant , it was not possible to assess whether any of these three plasmids carries ampicillin-resistance also.

Curing of plasmid-carrying strains

Table 2 also shows the antibiotic resistance pattern of 3 pairs produced by successful curing. Loss of pPM5 from P. mirabilis strain PM5 led to tetracycline and cephaloridine sensitivity as expected; the results also showed that ampicillin resistance is associated with this plasmid. Strikingly, whereas cotrimoxazole resistance was transferred by pPM5 (Table 2), curing did not eliminate resistance; presumably P. mirabilis strain PM5 carries both chromosomal an plasmid-encoded resistance.

Curing of P. mirabilis strain 49 confirmed the results obtained by conjugal transfer, adding ampicillin resistance to those resistances that are plasmid-encoded. P. mirabilis strain 991 was the third one cured ; it carries a single 78 md plasmid and 6 of its 7 resistances were lost on curing.

Urease in plasmid-free and plasmid-carrying P. mirabilis strains

Because of the proposed importance of urease in the virulece of Proteus (Senior, 1983), ammonia production by this enzyme was studied in more detail in 28 of the 145 isolates, 14 being p^+ and 14 p^-. Measurements of zone

sizes produced on urea agar after short periods of incubation indicated that p^+ P. mirabilis strains produce less ammonia from urea than p^- ones (Table 3). Measurements of pH in urea broth confirmed this; the pH values after 4 h incubation were significantly higher for the p^- than the p^+ P. mirabilis strains, indicating more ammonia production (Table 4).

Zone size measurements on the $p^+$and p^- pairs produced by curing and conjugal transfer confirmed that plasmid carriage reduces ammonia production by urease(Table4).

Plasmids and swarming

The measurements of swarming zone sizes on p^+/p^- pairs confirmed the results on the 145 original P. mirabilis strains; the presence of each of the plasmids pPM4, pPm5, pP49 and pP991 led to a reduction in swarming compared to the corresponding p^- P. mirabilis strain (Table 5).

Plasmids and enzyme secretion

Tests have been made of enzyme secretion in 16 of the plasmid-containing and 15 of the plasmid-free P.mirabilis strains. These studies indicated that the presence of a plasmid (or plasmids) enhanced secretion of protease, haemolysin and lecithinase. For protease,zone sizes varied from 2.0-8.0 mm for p^- P.mirabilis strains and from 6.0-20.0 mm for p^+ ones but zone size means were 4.5±0.43 mm for those that were p^+ (difference signifi - cant at 99% confidence level). For haemolysin,zone sizes varied from 3.0-11.0 mm for those that were p^- strains and from 8.0-21.0 mm for those that were p^+ but the mean zone sizes were 7.7±0.59 mm (p^-) and 12.9±0.87 mm (p^+); this difference is significant at the 99% confidence

level. For lecithinase, zone sizes varied from 0-5.0mm for p^- and from 2.0-7.0 mm for p^+ P. mirabilis strains but mean zone sizes were 3.0±0.38 mm (p^-) and 4.9±0.41mm (p^+); difference significant at the 95% confidence level.

Studies of zone sizes produced by protease and haemyolysin for a group of P.mirabilis strains isogenic except for the presence of the plasmids pPM5,pP49 and pP991 suggested that the presence of these plasmids substantially enhances enzyme secretion. The effects were less clear cut for lecithinase (Table 6).

DISCUSSION

One physiological characteristic strikingly correlated with plasmid carriage, in the group of 145 P.mirabilis strains studied here, is reduced swarming ability. Many of those that were p^+ failed to swarm,the difference between the mean swarming zone sizes for the p^+ P. mirabilis strains (2.0±0.28 cm) and the p^-ones (4.0±0.17 cm) was significant to the 99% confidence level and for each of the four plasmids studied in more detail by curing and/or conjugal transfer, plasmid loss increased swarming whereas plasmid gain reduced it (Table 5). Although the correlation between plasmid carriage and reduced swarming is clear, the finding that several p^+ P.mirabilis strains (e.g.G11, P200 & 580) gave large swarming zones suggests that a few plasmids, at least, can, like the R46 plasmid studied by Hesslewood and Smith (1974), actually confer increased swarming ability.

Plasmids frequently a later the envelope properties of their host bacterium and it may be that, as suggested

by Hessleweel and Smith (1974), it is such changes which affect swarming ability. Swarming involves derepressed flagellar synthesis and the insertion of greatly increased numbers of flagella per unit surface area (Smith , 1972) a process which itself leads to marked changes in envelope composition (Armitage et al., 1975; 1979). Possibly, some plasmid-encoded envelope changes (to envlope proteins or lipopolysaccharide) may prevent or reduce the marked increase in flagellar insertion which is an essential feature of swarming.

There was also a marked correlation between plasmid carriage and ammonia production from urea . All 145 P. mirabilis strains in the full test group were urease-positive in the 24 h qualitative test but this clearly masked quite major differences in view of the subsequent findings, firstly, that p^+ P. mirabilis strains form significantly less ammonia from urea than p ones(Table 3) and, secondly, that loss of a plasmid by curing increased ammonia production by urease whilst gain by conjugal transfer reduced it (Table 4).

The finding tht plasmids produced enhanced secretion of protease, haemolysin and (less markedly) lecithinase may be of medical significance if these enzymes affect virulence. Of particular interest, is the mechanisms by which plasmids might enhance secretion. Hesslewood and Smith (1974) proposed that this might result from a weakened cell envelope in the p^+ strain. It is our intention to correlate changes in envelope composition with plasmid carriage in P. mirabilis .

Table No.1 Distribution of antibiotic resistance in p and p^+ strains of *P. mirabilis* .

	Tet	Cep	Nit	Kan	Nan	Cap	Str	Sxt	Amp
Strains resistant.	17	22	2	6	2	21	7	12	48
% of resistance.	11.7	14.5	0.7	4.1	0.7	14.5	4.8	8.3	33
p^+ Strains resistant.	9	18	1	6	1	11	6	10	26
% of p^+ resistance.	21	42	2.3	14	2.3	26	14	23	60
P^- Strains resistant.	8	4	1	0	1	10	1	2	22
% of p^- resistance.	8	4	1	0	1	10	1	2	22

Table 2. Antibiotic resistance of cured strains and transconjugants

Strain	Plasmid	Plasmid size (md)	Resistance or sensitivity to								
			Tet	Cep	Nit	Kan	Nal	Cap	Str	Sxt	Amp
PM5	pPM5	105	R	R	S	S	R	S	S	R	R
PM5c	none	N.A.	S	S	S	S	R	S	S	R	S
P49	pP49	32	R	R	S	S	S	S	S	S	R
P49c	none	N.A.	S	S	S	S	S	S	S	S	S
P991	pP991	78	R	R	S	R	S	R	R	R	R
P991c	none	N.A.	R	S	S	S	S	S	S	S	S
G9	none	N.A.	S	S	S	S	S	R	S	S	R
G9pPM4	pPM4	42	S	R	S	R	S	R	S	S	R
G9pPM5	pPM5	105	R	R	S	S	S	R	S	R	R
G9pP49	pP49	32	R	R	S	S	S	R	S	S	R

Table 3. Plasmid carriage and ammonia production by ureas.

Strain	Plasmid	Ammonia production as indicated by:	
		Zone size	Medium pH
PM1	-	1.1	9.46
PM2	-	1.2	9.38
PM4	1	0.4	8.47
PM5	1	0	7.22
P4	2	0.7	9.47
P33	-	0.9	9.46
P49	1	0	8.31
P991	1	0	9.24
RM1	1	1.1	9.38
RM2	1	0.8	8.95
RM3	1	0.6	9.42
RM4	-	1.5	9.58
RM6	-	1.2	9.55
RM8	-	0.9	9.62
G7	2	0.9	8.92
G8	2	0.4	9.62
G9	-	1.5	9.61
G10	-	1.3	9.65
G12	1	0.5	8.31
G16	-	0.9	9.27
G17	1	0.1	8.87
G30	1	0.8	8.51
G32	2	0.7	8.92
133	-	1.2	9.54
202	-	0.9	9.12
P405	-	1.2	9.60
P406	-	1.3	9.73

The mean medium pH was 8.83±0.17 for the 14 p^+ strains and 9.51±0.05 for the 14 p^- strains. The difference was significant (99% confidence). The corresponding mean zone sizes were 0.50±0.10 (for p^+ strains) and 1.17±0.06 (for p^- strains). This difference was also significant at the 99% confidence level.

Table 4. Ammonia production from urea in plasmid-free and plasmid-carrying pairs of strains

Strain	pH after growth + urea for 4 h.	Zone sizes due to ammonia at		
		2 h.	4 h.	6 h.
PM5	7.22	0	0	0
PM5c	9.51	0.9	1.5	2.8
P49	8.31	0	0	0
P49c	9.54	0.8	1.3	2.3
P991	9.24	0	0	0
P991c	9.58	0.6	1.0	2.5
G9	9.61	0.9	1.5	3.3
G9pPM5	7.92	0	0	0
G9pP49	9.44	0.2	0.9	2.8

Table 5. Effects of plasmids on swarming

Strain	Plasmid present	Swarming zone size(cm.)
PM4	pPM4	3.2
PM5	pPM5	0
P49	pP49	0
P991	pP991	0
PM5c	none	5.1
P49c	none	5.3
P991c	none	4.2
G9	none	7.8
G9 pPM4	pPM4	4.0
G9 pPM5	pPM5	3.2
G9 pP49	pP49	4.6

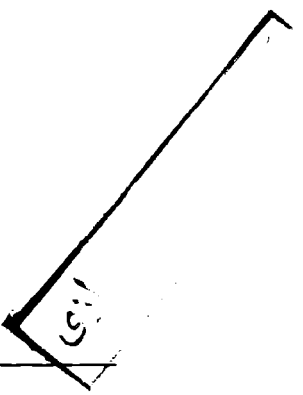

Table 6. Plasmid effects on enzyme secretion

Strain	Plasmid present	Enzyme secretion (zone size, m m) for:		
		Protease	Haemolysin	Lecithinase
PM5	pPM5	8	18	4
PM5c	none	2	10	3
P49	pP49	11	14	6
P49c	none	5	7	4
P991	pP991	13	12	7
p991c	none	6	8	4
G9	none	4	3	3
G9pPM5	pPM5	10	14	3
G9pP49	pP49	6	12	7

REFERENCES

1- Armitage, J.P. Rowbury, R.J., Smith, D.G. 1975 Indi - rect evidence for cell wall and membrane differences between filamentous swarming and short non-swarming cells of Proteus mirabilis . Journal of General Microbiology 89: 199-202.

2- Armitage, J.P., Smith, D.G.,Rowbury, R.J., 1979.Alterations in the cell envelope composition of Proteus mirabilis during the development of swarmer cells . Biochimica et Biophysica Acta . 584: 389-397.

3- Goodson, M., Rowbury, R.J., 1978. Reversal by cyclic adenosine monophosphate of the acridine-orange-indu - ced elimination of Flac from strains of Escherichia coli. Microbios Letters 4: 159-162.

4- Harmon, R.C., Rutherford, R.L., Wu, H.M., Collins, M. S. 1989. Monoclonal antibody-mediated protection and neutralization of motility in experimental Proteus mirabilis infection.Infection and Immunity 57: 1936-1941.

5- Hawkey, P.M., Mc Cormick,A., Simpson,R.A., 1986. Selective and differential medium for the primary isolation of members of the Proteeae. Journal of Clinical Microbiology, 23: 600-603.

6- Hesslewood, S.R., Smith, J.T., 1974. Envelope alterations produced by R factors in Proteus mirabilis. J. of General Microbiology . 85: 146-152.

7- Kado, D.I. Liu, S.T. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. of Bacteriology . 145: 1365-1373.

8- McLean, R.J.C., Nickel,J.C., Cheng,K.J., Costerton,J. W., 1988. The ecology and pathogenicity of urease-producing bacteria in the urinary tract. CRC Critical Reviews in Microbiology . 16: 37-79.

9- Mobley, H.L.T., Chippendale, G.R., 1990. Haemagglutinin, urease and haemolysin production by Proteus mirabilis from clinical sources. Journal of Infectious Diseases. 161: 525-530.

10-Pazin,G.J., Braude, A.I. 1974. Immobilizing antibodies in urine. II Prevention of ascending spread of P. mirabilis. Investigative Urology . 12: 129-133.

11-Salisbury, V., Hedges, R.W., Datta,N. 1972. Two modes of curing transmissible bacterial plasmids. Journal of General Microbiology . 70: 443-452.

12-Senior, B.W., 1979. The special affinity of particular types of Proteus mirabilis for the urinary tract. J.

of Medical Microbiology . 12: 1-8.

13-Senior, B.W., 1983. Proteus morganii is less frequently associated with urinary tract infections than P. mirabilis - an explanation. Journal of Medical Microbiology . 16: 317-322.

14-Senior.B.W., Bradford, N.C., Simpson, D.S. 1980. The ureases of Proteus strains in relation to virulence for the urinary tract. Journal of Medical Microbiology. 13: 507-512.

15-Shand , G.H., Anwar,H., Kadurugamuwa,J., Brown,M.R.W. Silverman, S.H.,Melling, J., 1985. In vivo evidence that bacteria in urinary tract infection grow under iron-restricted conditions. Infection and Immunity . 48: 35-39.

16-Silverblatt,F.J., 1974. Host-parasite interaction in the rat renal pelvis. A possible fole for pili in the pathogenesis of pyelonepharitis. Journal of Experi - mental Medicine. 140: 1696-1711.

17-Smith, D.G., 1972. The proteus swarming phenomenon . Science Progress. 60: 487-506.

18-Williams, F.D., Schwarzhoff, R.H., 1978. Nature of the swarming phenomenon in Proteus. Annual Reviews of Microbiology . 32: 101-122.