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Urease production as a marker of virulence in *Pseudomonas aeruginosa*

R. S. BRADBURY*, D. W. REID† and A. C. CHAMPION‡
School of Medical & Applied Sciences, Central Queensland University, Rockhampton, Queensland, †QIMR Berghofer Medical Research Institute, Herston, Queensland; and ‡University of Wollongong, Wollongong, New South Wales, Australia

Pseudomonas aeruginosa remains an intractable problem as a cause of respiratory infection in cystic fibrosis (CF) patients. While respiratory infection with *P. aeruginosa* in the majority of non-CF patients represents an acute, potentially fatal illness with risk of systemic dissemination, in CF patients it represents a chronic colonisation syndrome in which much of the local tissue damage is due to host response to the bacteria, rather than metabolic products of the bacteria themselves.

In the CF lung, *P. aeruginosa* grows in a biofilm state under anaerobic conditions.[1] After extended infection in the lung, CF isolates of *P. aeruginosa* are often hypermutable,[2] and mutation of regulator genes may occur over an extended period of colonisation, leading to a relatively dormant, slow growing phenotype. Strains show a gradual but marked change over time towards mucoid, non-motile and antibiotic resistant phenotypes.[3]

In recent decades, multidrug-resistant clonal strains of this organism have been described infecting CF patients on several continents.[4–10] At least one clonal strain (Australian epidemic strain 3; AES3) has been shown to result in adverse clinical outcomes for patients.[10] The mechanisms by which such clonal strains establish infection in the CF lung, and sometimes supplant other non-clonal isolates of *P. aeruginosa*, remain to be fully elucidated. Reliable mechanisms for the detection of these clonal strains in routine diagnostics are yet to be determined.

Ceftazidime resistance was noted as a marker for the Liverpool epidemic strain (LES) in 1996,[6] but not all LES strains are resistant to this antibiotic,[11] and non-clonal CF isolates may often express such resistance.[12]

P. aeruginosa isolates from CF respiratory infections also gradually cease to express virulence factors over time, leading to a less virulent phenotype, as demonstrated by immunoblot analysis of type III secretion system effector enzyme expression[13] and whole cell virulence in a *Dictyostelium discoideum* eukaryotic virulence model.[14] The progressive nature of whole cell virulence loss has also been demonstrated in a *D. discoideum* eukaryotic virulence model.[15]

Almost all non-CF isolates of *P. aeruginosa* express the enzyme urease. This enzyme acts to catalyse the hydrolysis of urea, producing ammonia and carbamic acid, which is in turn hydrolysed to form bicarbonate. This enzyme is produced by numerous pathogenic bacteria and fungi, and has recently been the focus of interest as a major virulence factor in some respiratory pathogens.[16] In *Cryptococcus neoformans*, pH changes due to ammonia derived from urease activity have a role in immune evasion.[16] Ammonia is toxic to host cells, promoting dissemination from the lung to the bloodstream and to other organs.[16] Urease production in *Mycobacterium bovis* assists immune evasion by decreasing both localisation in the lysosome and cell surface expression of major histocompatibility complex class II.[16] In *P. aeruginosa*, urease plays a role in pH homeostasis within biofilm growth, and *ureB*-negative mutants show poor biofilm formation.[17] Expression of urease in *P. aeruginosa* is controlled by *rpoN*.[18]

Progressive loss of *rpoN* expression of dependent surface factors in *P. aeruginosa* has been shown to occur during the course of colonisation of the CF lung.[19] Down-regulation of *P. aeruginosa* Sigma-factor 54 (σ^{54}), coded by *rpoN*, has been described in the Danish CF clonal strain DK2. It has been postulated that a combination of mutations in *rpoN*, *mucA* and *lasR*, possibly combined with mutations conferring resistance to antibiotics, have resulted in the success of this clonal strain in colonising and disseminating within the Danish CF population.[3] Such mutants are slower growing and have defects in flagella and type IV pili, resulting in a non-motile phenotype.

Knockout mutation in *rpoN* will have a significant impact on quorum sensing[20] and virulence in *P. aeruginosa*, as well as halting urease and glutamate synthase expression.[18] Additionally, pilin[21] and flagella[18] genes will not be produced, leading to a non-motile phenotype. Expression of *lasR* and *lasI* is controlled by *rpoN*, and *rpoN*-negative mutants show increased production of these genes at low cell densities, but these effects are reversed at high cell densities. Expression of *lasB* (coding for elastase) *hcnA* (cyanide synthase) and *rhlA* (precursor of rhamnolipid) is derepressed in such mutants.[20] Loss of *rpoN* function has also been shown to confer the capacity for growth under anaerobic conditions, as found in the CF lung.[1]

Correspondence to: Dr Richard Bradbury
Email : r.bradbury@cqu.edu.au

In the present study, strains of *P. aeruginosa* were selected to provide a broad range of clinical or environmental sources.[14] Genotypes and virulence in a *D. discoideum* model had been determined in a previous study.[10,14] Isolates were subcultured from minimal maintenance media on to Mueller Hinton agar for 48 h at 37°C in air. A single, well-isolated colony was then inoculated on to the surface of a Christensen's urea agar slope (Oxoid, Thebarton, South Australia). Agar slopes were then incubated at 21°C under aerobic conditions with increased moisture and protection from light for seven days, in concordance with the conditions previously employed in the *D. discoideum* virulence assay.[14]

Following incubation, slopes were examined visually for a colour change. Strong pink colouration in the agar was considered to be a positive result, while no change (or a yellow colour) was considered a negative result. All tests were performed in duplicate. *Proteus vulgaris* ATCC 8427 was used as a positive control, while *Escherichia coli* ATCC 10418 acted as the negative control. *Klebsiella pneumoniae* and *K. aerogenes* clinical strains, used as controls in the *D. discoideum* virulence assay, were also tested.

The significance of the relationships between urease production, *D. discoideum* virulence assay results, ceftazidime sensitivity, CF or non-CF source, and clonality of isolates were determined by Student's *t*-test. The significance of urease as related to source and genotype of isolates was determined using Fisher's exact test. All statistical analyses were performed using Microsoft Office Excel 2003. This work was approved by the Tasmanian Human Research Ethics Committee (Approval number: H0009813).

Eighteen of the 32 *Pseudomonas aeruginosa* isolates (56%) involved in this study produced detectable levels of the urease enzyme on urea agar slopes. The only urease-negative non-CF strain was isolate 147. While a number of CF strains were able to elaborate significant amounts of the urease enzyme, 13 out of 19 CF isolates (68%) were urease-negative, with all (n=8) AES3 isolates being urease-negative. Discrepant urease testing results were obtained in duplicate tests of isolates U33b and R44a; consequently a triplicate was employed and the average result of the three was used for statistical analysis.

Cystic fibrosis isolates of *P. aeruginosa* were more likely ($P<0.001$) to be urease- negative than were non-CF isolates, and AES3 clonal strain isolates were more likely ($P<0.05$) to be urease-negative than were panmictic CF isolates, which in turn were more likely ($P<0.05$) to be urease-negative than were non-CF isolates. This incapacity to elaborate urease was significantly associated ($P<0.01$) with a less virulent phenotype in the previously performed *D. discoideum* virulence models. Both urease-positive control strains of *Klebsiella* used for the virulence assay allowed luxuriant growth and full development of *D. discoideum*.

Only one urease-positive *P. aeruginosa* isolate, R43, showed decreased virulence, but this isolate still did not support the development of *D. discoideum* beyond the single-celled amoeba life stage. Only one non-CF isolate, 147, was urease-negative. This isolate was recovered from a swimming pool and showed decreased virulence in the *D. discoideum* assay.

This study demonstrates that lack of urease expression in *P. aeruginosa* is significantly associated with decreased virulence in a *D. discoideum* model. Furthermore, urease-negative phenotype was almost exclusively seen in CF isolates, with only a low virulence environmental isolate also expressing this phenotype. The Tasmanian CF clonal strain of P. *aeruginosa*, AES3, was significantly more likely to be urease-negative than were other isolates.

Further work is required to explain the lack of

Table 1. Sources and urease test result of isolates in this study, compared to previously determined virulence in a *D. discoideum* eukaryotic cell virulence model.[14]

Bacterial isolate	Source	Urease result	Virulence in *D. discoideum* X22[14]
Proteus vulgaris	ATCC 8427	Pos	
Escherichia coli	ATCC 10418	Neg	
Klebsiella aerogenes	Clinical control	Pos	–
Klebsiella pneumoniae	Clinical control	Pos	–
AES 1	CF sputum	Pos	+
U17b*	CF sputum	Neg	–
U19b*	CF sputum	Neg	–
U32b*	CF sputum	Neg	–
U33b*	CF sputum	Neg	–
R44a*	CF sputum	Neg	–
R44b*	CF sputum	Neg	–
U50*	CF sputum	Neg	–
U115*	CF sputum	Neg	–
U32a†	CF sputum	Neg	–
U3a	CF sputum	Pos	+
U29b	CF sputum	Neg	–
R43	CF sputum	Pos	–
U16a	CF sputum	Neg	–
U61a	CF sputum	Pos	+
U156	CF sputum	Pos	+
U194a	CF sputum	Neg	–
U194b	CF sputum	Neg	–
82	CF sputum	Pos	+
20	Urine	Pos	+
30	Sputum	Pos	+
46	Soft tissue	Pos	+
67	Soft tissue	Pos	+
74	Suction unit	Pos	+
77	Urine	Pos	+
99	Urine	Pos	+
119	Sink	Pos	+
123	Sink	Pos	+
127	Sink	Pos	+
147	Pool	Neg	–
160	Stream	Pos	+
PA01	Soft tissue	Pos	+

AES: Australian epidemic strain
*AES3 isolate
†50% similarity to AES3 by RAPD PCR
+: Caused death of *D. discoideum*
–: Allowed growth of *D. discoideum*

development of *D. discoideum* strains AX2 and X22 observed on isolate R43, despite the large size of plaque produced. This does not appear to be a factor of the ammonia by-product of urease activity interfering with signalling in *D. discoideum* and halting culmination, as the strongly urease-positive control *Klebsiella* strains allowed virtually complete growth and development of both AX2 and X22. This suggests that factors other than urease activity alone are at play in the increased virulence of urease-positive *P. aeruginosa* strains.

The association of decreased virulence and urease negativity with CF isolates points towards down-regulation of expression of urease with other bacterial virulence factors. This may be associated with a mutation in *rpoN*, the global regulator of urease production, virulence and antimicrobial resistance expression, which has been implicated as the cause of clonal dissemination in the Danish CF clonal strain DK2. The product of *rpoN* is σ^{54}, the regulator of urease expression in *P. aeruginosa*.

rpoN mutants would show down-regulation of function in *lasR* and *lasI* at the high cell densities used in *D. discoideum* models, as expression of these genes is down-regulated in the absence of σ^{54} at such cell densities. *lasR*[22,23] *lasI* and *rhlR*[22] knockout mutants have already been shown to demonstrate such attenuated virulence in *D. discoideum* models.[22]

Given the previous association of *rpoN*-negative status and clonality, and the significant association in this study of AES3 with urease-negative phenotype, further investigation of a large cohort of CF clonal and panmictic *P. aeruginosa* isolates to determine if urease testing may be used as a simple and sensitive screening test for CF *P. aeruginosa* is warranted.

In summary, the present findings show that a urease-negative phenotype is a potential marker for attenuated virulence in *P. aeruginosa* isolates. Further investigation, including amplification and sequencing of *rpoN*, followed by correlation to quantitative measures of urease enzyme production, would determine if absence of urease enzyme expression is consequent on *rpoN* mutation in isolates of *P. aeruginosa*.

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