Urease activity may contribute to the ability of *Actinobacillus pleuropneumoniae* to establish infection

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Abstract

The contribution of urease activity to the pathogenesis of *Actinobacillus pleuropneumoniae* was investigated using 2 different urease-negative transposon mutants of the virulent serotype 1 strain, CM5 Nal^r. One mutant, *cbiK*::Tn*10*, is deficient in the uptake of nickel, a cofactor required for urease activity. The other mutant, *ureG*::Tn*10*, is unable to produce active urease due to mutation of the urease accessory gene, *ureG*. In aerosol challenge experiments, pigs developed acute pleuropneumonia following exposure to high doses (10^6 cfu/mL) of the parental strain, CM5 Nal^r, and to the *cbiK*::Tn*10* mutant. When low dose (10^3 cfu/mL) challenges were used, neither urease-negative mutant was able to establish infection, whereas the parental strain was able to colonize and cause lesions consistent with acute pleuropneumonia in 8 of the 20 pigs challenged. These findings suggest that urease activity may be needed for *A. pleuropneumoniae* to establish infection in the respiratory tract of pigs.

Résumé

*Le rôle de l'uréase d'*Actinobacillus pleuropneumoniae *dans la pathogénie de la condition associée à ce micro-organisme fut étudié à l'aide de deux mutants uréase-négative de la souche virulente de type 1 CM5 Nal^r obtenus à l'aide d'un transposon. Un des mutants,* cbiK : :*Tn*10, *était déficient pour l'acquisition du nickel, un cofacteur requis pour l'activité de l'uréase. L'autre mutant,* ureG : :*Tn*10, *était incapable de produire une uréase active étant donné une mutation au niveau du gène accessoire* ureG. *Lors d'infections expérimentales par aérosol, les porcs ont développé une pleuropneumonie aiguë suite à l'exposition à des doses élevées (10^6 ufc/mL) de la souche parentale CM5 Nal^r et du mutant* cbiK : :*Tn*10. *Lorsque des doses plus faibles (10^3 ufc/mL) étaient utilisées, aucun des mutants ne fut capable de causer une infection, alors que la souche parentale a colonisé le tissu et a causé des lésions compatibles avec une pleuropneumonie aiguë chez 8 des 20 animaux infectés. Ces résultats suggèrent que l'activité de l'uréase serait nécessaire pour qu'*A. pleuropneumoniae *puisse causer une infection au niveau du système respiratoire des porcs.*

(Traduit par docteur Serge Messier)

Introduction

Actinobacillus pleuropneumoniae is a strict respiratory tract pathogen of swine. It is the causative agent of porcine pleuropneumonia, a disease that can range in severity from peracute to chronic, depending, in part, on the serotype, dose of infection, and the immune status of the host (1,2). Acute pleuropneumonia is characterized by high fever, coughing, dyspnea, anorexia, ataxia, and severe respiratory distress with cyanosis (1). While chronically infected pigs may show no signs of disease, they can harbor the organism in nasal passages, tonsilar crypts, and/or lung lesions (1). The organism does not survive for long in the environment and is transmitted from pig to pig via aerosols or direct contact (2).

The pathogenesis of pleuropneumonia is multifactorial, with RTX toxins, capsule polysaccharide (CPS), lipopolysaccharide (LPS), and various outer membrane proteins (OMPs) implicated in the disease process (3). To date, vaccines based on these virulence factors have not been very effective, suggesting that additional components may be important (4,5). Since virtually all strains of *A. pleuropneumoniae* produce urease (6,7), and it is implicated in the pathogenesis of other organisms, we hypothesized that this enzyme might contribute to the virulence of *A. pleuropneumoniae*. To date, there has only been a single study by Tascon Cabrero et al (8), in which it was found that urease mutants were not attenuated. These authors, however, used a high dose intratracheal inoculum to test the virulence of a single, uncharacterized, urease-negative transposon mutant. In order to further evaluate the possible role of urease in the pathogenesis of pleuropneumonia, 2 urease-negative transposon mutants were characterized and their ability to cause disease following a low dose aerosol challenge was investigated.

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Table I. Oligonucleotides used for polymerase chain reaction

Oligonucleotide	Position[a]	Strand	Length	Sequence 5′–3′
Kan	NA	NA	21	CCTCTTCCGACCATCAAGCAT
59F1	318–340	+	22	GGCTAATAACCGGCGGAGAAAC
59R1	5632–5612	−	20	CCGTAAGTCGCTACATACTG
59R2c	5223–5243	+	20	TTCTGCTGCTCTCTCTCAAG
Urease/Xba	11 200–11 100	−	32	CCGGCCTCTAGAAGCGAAGCACGAATTAAGTT
Tn*10*	NA	NA	21	GATCATATGACAAGATGTGTA

Kan — kanamycin; NA — not applicable

[a] Position 5′ to 3′ within the compiled urease and upstream sequences (see Figure 1). Note that the Kan and Tn*10* primers were designed from sites within the Kan cassette of, and near the end of, the mini-Tn*10* transposon sequence, respectively

Materials and methods

Bacterial strains and culture conditions

A spontaneously nalidixic acid-resistant derivative of the virulent *A. pleuropneumoniae* serotype 1 strain CM5, CM5 Nal^r, was routinely grown at 37°C with 5% CO_2 on brain-heart infusion (BHI) (Difco Laboratories, Detroit, Michigan, USA) medium supplemented with 0.01% nicotinamide adenine dinucleotide (NAD) and 20 μg/mL nalidixic acid (BHIV-Nal). *Escherichia coli* S17-1(λ-pir), used as the conjugal donor strain for the plasmid pLOF/Km, was grown at 37°C on Luria-Bertani (LB) medium supplemented with 50 μg/mL kanamycin (Kan) and 100 μg/mL ampicillin (Amp). Transconjugants were grown on BHIV supplemented with 20 μg/mL nalidixic acid and 50 μg/mL kanamycin (BHIV-NalKan).

Transposon mutagenesis

The suicide plasmid pLOF/Km, which carries a mini-Tn*10* transposon with an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible transposase located outside the mobile element (9), was conjugally transferred from *E. coli* S17-1 (λ-pir) into CM5 Nal^r, as described previously (10). Transconjugants were selected on BHIV-NalKan and loss of the suicide plasmid was confirmed by patching colonies onto BHIV-NalKanAmp. Individual isolates were cultured in wells of a microtitre plate containing 100 μL/well of BHIV-NalKan broth and were stored at −70°C with 8% dimethylsulfoxide. Detection of urease-negative mutants was done by subculturing isolates in 96-well plates containing 120 μL/well of BHIV-NalKan broth. After overnight incubation at 37°C, 30 μL/well of 5X urea base medium (Difco) was added. Urease-positive isolates turned the medium pink, whereas urease-negative isolates turned the medium yellow. Urease-negative isolates were confirmed by plating onto BHIV-NalKan agar and urea agar.

Mapping of transposon insertions

Transposon insertions were characterized by PCR and by Southern blotting. For Southern blot analysis, total cellular DNA was digested with *Xho*I and transferred as described previously (11). The blots were probed with the kanamycin resistance cassette from pLOF/Km labeled with digoxigenin (DIG) (Boehringer Mannheim Canada, Laval, Quebec). Hybridizations were carried out at 65°C overnight. After 2 × 5-minute washes at room temperature with 2 × SSC, followed by 2 × 15-minute washes at 65°C with 0.1 × SSC, the blots were developed using anti-DIG-alkaline phosphatase and CSPD® (Boehringer Mannheim) according to manufacturer's instructions. The precise locations of the Tn*10* insertions in the urease mutants used in this study were mapped by sequencing specific DNA fragments generated by polymerase chain reaction (PCR), by using a combination of primers designed to span the urease gene cluster and the 5 kbp upstream region (Figure 1), together with primers designed from sequences within the transposon (Table I).

Phenotypic characterization of mutants

The *cbiK*::Tn*10* and *ureG*::Tn*10* mutants were compared to the parental strain for various phenotypic characteristics. Growth rates were determined by monitoring the OD_{600} of matched cultures in BHIV. Hemolytic activity was assessed using a tube assay (12). Lipopolysaccharides and OMPs were extracted and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis by using a 4% stacking gel and a 12% separating gel as described previously (13). Duplicate gels were stained either with Coomassie blue or silver nitrate using a silver stain kit (Bio-Rad Laboratories Ltd., Mississauga, Ontario). The amount of CPS on the surface of each strain was assessed by transmission electron microscopy using poly-cationized ferritin (14). Cytosolic urease activity was measured using a urea nitrogen assay kit (Sigma Chemical Company, St. Louis, Missouri, USA), as described previously (7).

Challenge procedures

The relative virulence of the *cbiK* ::Tn*10* and *ureG* ::Tn*10* mutants was compared to that of the parental strain, CM5 Nal^r, in 3 separate experiments. For all experiments, the pigs used were obtained from a commercial, specific-pathogen-free (SPF) swine herd known to be free of *A. pleuropneumoniae* and *A. suis*. Prior to challenge, serum samples were collected from all pigs and tested for the presence of Apx antibodies by enzyme-linked immunosorbent assay (15). None of the pigs had antibodies to the Apx toxins prior to infection, indicating that they had not been exposed previously to *A. pleuropneumoniae* or to other organisms, such as *A. suis*, which could have conferred protective immunity (16). In the first experiment, 2 groups of 2 pigs each were exposed to a high dose (approximately 10^6 cfu/mL) aerosol challenge with the parental strain, CM 5 Nal^r,

Table II. Clinical scores and lung scores for pigs exposed to an aerosol of urease-positive or urease-negative *A. pleuropneumoniae*

Experiment #1	CM5 Nal^r (n = 2)	*cbiK*::Tn*10* (n = 2)
Dose (cfu/mL)	1×10^6	1×10^6
Clinical score	8	8
Lung score	6.0 ± 2.0	7
Experiment #2	CM5 Nal^r (n = 10)	*cbiK*::Tn*10* (n = 10)
Dose (cfu/mL)	7×10^3	5×10^3
Clinical score	5.4 ± 1.8	0
Lung score	2.4 ± 2.5	0.70 ± 0.56
Experiment #3	CM5 Nal^r (n = 10)	*ureG*::Tn*10* (n = 10)
Dose (cfu/mL)	1×10^3	3×10^3
Clinical score	4.1 ± 1.9	1.0 ± 0.90
Lung score	2.9 ± 3.1	0.30 ± 0.50

and with the *cbiK*::Tn*10* mutant, as previously described (17). In the second experiment, 2 groups of 10 pigs each were exposed to a low dose (approximately 10^3 cfu/mL) aerosol challenge with these 2 strains. In the third experiment, 2 groups of 10 pigs each were exposed to a low dose challenge of the *ureG*::Tn*10* mutant and the parental strain. In all of the experiments, the pigs were monitored for clinical signs of disease every 2 to 6 h. Clinical signs were scored out of a possible total of 8, with 1 point given for each of the following: anorexia, lethargy, cough, nasal/oral discharge, vomiting/diarrhea, dyspnea, refusal to rise, and cyanosis (17). Any pigs showing severe signs of respiratory distress were euthanized and necropsied immediately. In the low dose challenge experiments, tonsil swabs, collected from pigs every 3 to 4 d post-challenge, were plated on selective blood agar containing 100 mg/mL bacitracin, 50 μg/mL nalidixic acid, 1 μg/mL crystal violet, and 0.05% NAD (18). The presence of *A. pleuropneumoniae* serotype 1 was confirmed using a latex agglutination test (19). Urease activity was assessed by plating bacteria on urea agar.

Pigs that survived the low dose challenges were euthanized and necropsied on day 21 (experiment 2) or day 15 (experiment 3). At necropsy, lung lesions were scored out of a possible total of 9, with 1 point given for each of the following: pleural effusion, fibrinous adhesions, tracheal froth, and pericarditis. Points were also given for the percentage of lung with consolidation, hemorrhage, and adhesions, determined visually, with a score of 1 for < 5%, 2 for 5 to 25%, 3 for 25 to 50%, 4 for 50 to 75%, and 5 for > 75% (17). Bacteriological samples, collected from nasal cavities, tonsils, and lungs of all pigs, were plated on selective agar. All procedures were done in accordance with the guidelines of the Guide to Care and Use of Experimental Animals of the Canadian Council on Animal Care.

Results

Genetic and phenotypic characterization of urease mutants

From a total of 6000 transconjugants, 19 urease mutants were obtained. Many of these were in the regions immediately upstream of the urease structural and accessory genes. Mutant 8G12 was found to contain a single transposon insertion within the *cbiK* gene, part of a putative nickel transport operon upstream of the urease cluster (Figure 1, Table I). The details and further characterization of this operon will be described elsewhere. Characterization of the *cbiK*::Tn*10* mutant suggests that only urease activity is affected by mutation of the nickel uptake system. This is consistent with nickel having only a limited role in cellular functions (20). It is not likely that *A. pleuropneumoniae* produces any other nickel-dependent enzymes. Nickel-containing enzymes other than urease and hydrogenase are found only in certain types of bacteria (21,22). Fermentation of carbohydrates under anaerobic conditions does not result in production of dihydrogen by *A. pleuropneumoniae* (23), and there is no other evidence of hydrogenase activity in *A. pleuropneumoniae*.

Mutant 16D4 has a transposon insertion within one of the urease accessory genes, *ureG*, (7) as well as a second insertion which was not mapped (Figure 1, Table I). No phenotypic changes could be associated with this second insertion. The hemolytic activities of the 2 urease-negative mutants were not significantly different from that of the parental strain: *cbiK*::Tn*10* (73.6 ± 6.49 hemolytic units (HU)), *ureG*::Tn*10* (74.9 ± 3.16 HU), and CM5 Nal^r (82.4 ± 9.16 HU). In addition, there were no detectable differences in the amount of CPS produced, or in the LPS or OMP profiles of the 3 strains (data not shown). The growth rates of both mutants were identical to that of the parental strain. The only phenotypic difference was the absence of detectable urease activity in cell-free lysates of the mutants, whereas the urease activity of the parental strain was 21 ± 2 U/mg protein. In an effort to complement the *ureG*::Tn*10* mutant, a 6.5 kbp fragment of DNA containing the urease gene cluster from strain CM5 Nal^r was generated by PCR using the primers urease/Sac (GCGGCGAGCTC-AGTTGGTTATTATTGGCGGTT) and urease/Xba (CCGGCCTCTAGA-AGCGAAGCACGAATTAAGTT). These primers introduced *Sac*I and *Xba*I sites at either end of the urease cluster, which were subsequently used to subclone the fragment into the shuttle vector pGZRS19 (24). The resultant plasmid, pURE19, was electroporated into *E. coli* DH5-α, and urease-positive transformants were selected on LB-Amp medium. However, despite numerous attempts, pURE19 could not be introduced into the *ureG*::Tn*10* mutant, whereas pGZRS19 containing no insert was capable of transforming the *ureG*::Tn*10* mutant.

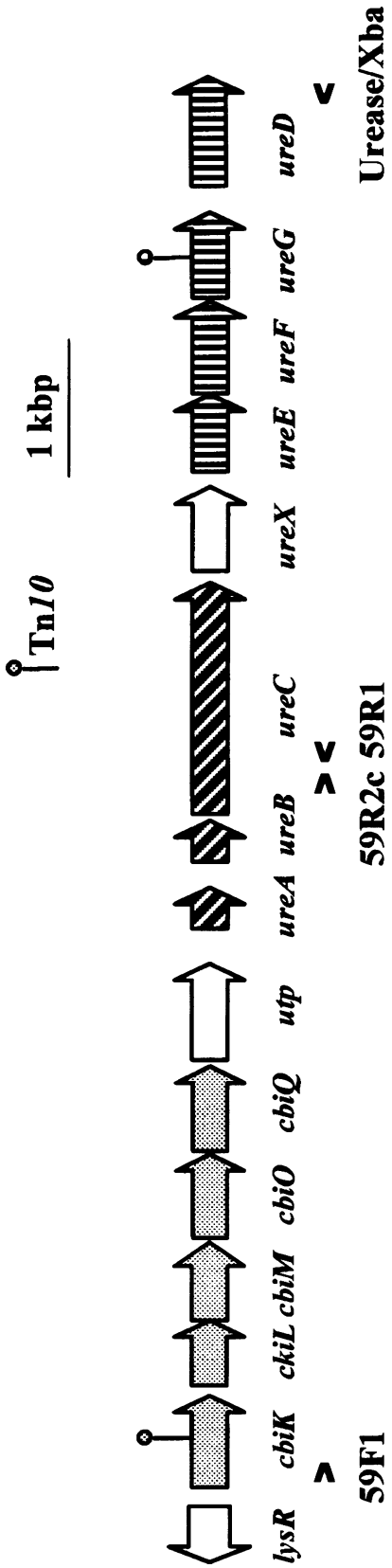

Figure 1. Locations of the relevant primers and Tn*10* insertions in *cbiK* and *ureG*. The upstream *cbi* genes are indicated in grey, urease structural genes with diagonal lines, and the urease accessory genes by vertical lines. The positions of the primers used to map the Tn*10* insertions are indicated by the ">" and "<" symbols; their precise position is listed in Table I.

Virulence in pigs

No difference in virulence was observed when pigs were exposed to high doses (10^6 cfu/mL) of either the urease-negative (*cbiK*::Tn*10*) or the parental strain (CM5 Nal^r) (Table II). A different result was obtained following challenge with lower numbers of bacteria. In low dose challenge experiments neither mutant was able to cause disease, whereas the parental strain caused clinical signs in 12 out of 20 animals (Table II). Six out of 20 pigs exposed to CM5 Nal^r showed signs of severe disease, and were euthanized between 2 and 5 d post-challenge. Six out of the remaining 14 pigs also had high clinical scores but were not euthanized until day 15 or 21. At necropsy, 8 of the 20 pigs had high lung scores (Table II) and lesions consistent with acute pleuropneumonia. In all cases, urease-positive *A. pleuropneumoniae* was recovered in pure culture from these lesions. In contrast, pigs exposed to the *cbiK*::Tn*10* mutant showed no clinical signs of disease. Likewise, there were no signs of disease in animals exposed to the *ureG*::Tn*10* mutant, except that 7 of the 10 animals developed a mild, short-lived diarrhea.

During the course of experiments 2 and 3, the parental strain was recovered from the tonsils of 15 out of 20 animals prior to euthanasia. In contrast, the *cbiK*::Tn*10* mutant was recovered from only 1 pig during the course of the experiment (3 d post-challenge) and the organism could not be cultured at post mortem. The low recovery rate of the *cbiK*::Tn*10* mutant from any of the sites tested at 21 d post infection led us to euthanize the pigs in experiment 3 at day 15, in case clearance of the infection in the second experiment was due to development of an active immune response (15). Even at 15 d post infection, the *ureG*::Tn*10* mutant could not be isolated from the nose, tonsils, or lungs of any of the pigs.

Discussion

In agreement with the report of Tascon Cabrero and colleagues (8), we found no differences in virulence between a urease-negative (*cbiK*::Tn*10*) mutant and the wild type parent when pigs were exposed to high doses (10^6 cfu/mL) of either strain (Table II). This result is perhaps not surprising, as the mutant still produced wild type levels of ApxI and ApxII. In addition to strong lytic activity, these RTX toxins can alter cell function of alveolar macrophages and neutrophils, reducing or eliminating the ability of these phagocytes to clear the infection (25,26)

A marked difference in virulence was seen, however, when pigs were exposed to a low dose (10^3) of the urease-negative mutants. In these experiments, the parental strain caused severe disease in approximately one third of the pigs and many of the remaining pigs had high clinical scores (Table II). Lesions consistent with acute pleuropneumonia were seen in 40% of these animals at post mortem and urease-positive *A. pleuropneumoniae* was recovered in pure culture in every case. None of the pigs exposed to the urease mutants showed any clinical signs of pleuropneumonia. The transient diarrhea seen in the *ureG*::Tn*10* challenged animals was most likely due to a mild incidental infection. Similarly, there were no signs of *A. pleuropneumoniae* infections at post mortem and none of the tissues tested were culture positive. The presence of very limited hemorrhage in some of the animals was likely an artifact of euthanasia.

In the 2 low dose challenge experiments, it was possible to isolate the parental strain from the tonsils throughout the course of the experiment. In contrast, with a single exception, neither of the urease mutants could be recovered from the tonsils following challenge. These data are consistent with the notion that urease mutants of *A. pleuropneumoniae* do not cause disease because they fail to colonize or are eliminated by innate host defenses. There are several possible reasons why urease mutants fail to colonize.

The outcome of infection (clearance versus colonization) appears to be determined in the first few hours following exposure to *A. pleuropneumoniae* (27). One of the first barriers that the organism must overcome is ciliary clearance. Studies by Narita et al (28) revealed that treatment with drugs that depress mucus production and ciliary activity increases the pigs' susceptibility to *A. pleuropneumoniae* infection. Likewise, impairment of mucociliary clearance by atmospheric ammonia (25 to 100 ppm) has been shown to enhance colonization by *Pasteurella multocida* in the respiratory tract of pigs (29–31). Although the contribution of ammonia liberated by ureolytic organisms to dysfunction of ciliated epithelium in the respiratory tract has not yet been investigated, Stalheim and Gallagher have demonstrated that ammonia liberated by the ureolytic activity of *Ureaplasma urealyticum* can cause ciliostasis and deciliation of oviductal epithelium (32).

The cytotoxic effects of ammonia generated by urea hydrolysis could also contribute to the ability of *A. pleuropneumoniae* to establish infection. *Actinobacillus pleuropneumoniae* appears to evade the bactericidal mechanisms of alveolar macrophages, and may even replicate in the cytoplasm, eventually causing the lysis of the phagocytes via liberation of toxins (25). Although, the RTX toxins are thought to play the major role in this process, Cullen and Rycroft (5) reported that a mutant of *A. pleuropneumoniae* serotype 2, deficient in production of ApxII and ApxIII, was still capable of damaging porcine alveolar macrophages in vitro. They suggested that the presence of an additional cell-associated 'toxin' may be responsible for this toxicity. It is possible that this cell-associated toxin is, in fact, ammonia, which may also act synergistically with Apx toxins to contribute to cytotoxicity, as well as to impair macrophage function (26,33,34). Such synergism between a cytolytic hemolysin and ammonia in epithelial cytotoxicity has been documented in several other species. For example, Mobley et al (35) showed that hemolysin-negative strains of *Proteus mirabilis* and *Proteus vulgaris* retain some ability to damage cultured epithelial cells, but only in the presence of urea.

The contribution of urease activity to nitrogen metabolism may also enhance the ability of *A. pleuropneumoniae* to infect the respiratory tract of pigs. In bacteria, urea hydrolysis by urease gives rise to ammonia, a preferred nitrogen source for many species (36,37). Urea is constantly available in serum and mucosal secretions and provides an excellent source of nitrogen to organisms capable of ureolysis (38,39). For example, Morou-Bermudes and Burne (40) showed that urease activity may confer a selective advantage to the oral pathogen, *Actinomyces naeslundii*, by providing an efficiently utilizable nitrogen source. Similarly, assimilation of ammonia into protein and other nitrogenous compounds by glutamine synthetase was found to be critical for viability of *Helicobacter pylori* (41), confirming the importance of urease activity in nitrogen metabolism in this bacterium.

Taken together, the results of these experiments strongly suggest that urease activity contributes to the ability of *A. pleuropneumoniae* to establish infection in the respiratory tract of pigs and to subsequently cause disease. Urease activity may contribute to pathogenesis by providing the organism with a preferred source of nitrogen and/or by directly or indirectly damaging host phagocytes and endothelial cells. Ideally, an isogenic urease-negative strain is needed to further characterize the role of urease in the pathogenesis of *A. pleuropneumoniae* infections. Based on the current study, this does not seem feasible by transposon mutagenesis. Similarly, repeated attempts to do allele replacement in *A. pleuropneumoniae* CM5 have not been successful.

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