Distribution of 19 major virulence genes in *Legionella pneumophila* serogroup 1 isolates from patients and water in Queensland, Australia

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The distribution of 19 major virulence genes and the presence of plasmids were surveyed in 141 *Legionella pneumophila* serogroup (SG) 1 isolates from patients and water in Queensland, Australia. The results showed that 16 of the virulence genes examined were present in all isolates, suggesting that they are life-essential genes for isolates in the environment and host cells. The 65 kb pathogenicity island identified originally in strain Philadelphia-1^T was detected more frequently in isolates from water (44.2%) than in those from patients (2.7%), indicating that the 65 kb DNA fragment may aid the survival of *L. pneumophila* in the sampled environment. However, the low frequency of the 65 kb fragment in isolates from patients suggests that the pathogenicity island may not be necessary for *L. pneumophila* to cause disease. Plasmids were not detected in the *L. pneumophila* SG1 isolates from patients or water studied. There was an association of both *lvh* and *rtxA* with the virulent and predominant genotype detected by amplified fragment length polymorphism, termed AF1, whereas the avirulent common isolate from water termed AF16 did not have *lvh* or *rtxA* genes, with the exception of one isolate with *rtxA*. It was found that a PCR detection test strategy with *lvh* and *rtxA* as pathogenesis markers would be useful for determining the infection potential of an isolate.

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INTRODUCTION

Legionellae, the aetiologic agents of legionellosis, are ubiquitous worldwide in rivers and lakes (Fliermans *et al.*, 1981), as well as in man-made water systems such as cooling towers and spas (Fields *et al.*, 2002). The vast majority of cases of legionellosis are caused by *Legionella pneumophila*, mostly serogroup (SG) 1 strains (Yu *et al.*, 2002). To understand the pathogenicity of *L. pneumophila*, a number of virulence genes of *L. pneumophila* have been well-characterized and extensively reviewed (Cianciotto, 2001; Dowling *et al.*, 1992). The virulence factors characterized include genes required for the whole infection process, such as bacterial cell attachment to host cells, survival and intracellular replication and cell-to-cell spread. The products of genes involved in the initial attachment to host cells and early stages of intracellular infection include type

IV pili, the 60 kDa heat-shock protein Hsp60, the pore-formation protein RtxA, the macrophage infectivity potentiator Mip and the macrophage-specific infectivity protein MilA (Cianciotto & Fields, 1992; Cirillo *et al.*, 2001; Garduño *et al.*, 1998; Harb & Abu Kwaik, 2000). The genes required for bacterial survival and intracellular replication are a group of genes called *icm* (intracellular multiplication) (Segal *et al.*, 1998) or *dot* (defect in organelle trafficking) (Vogel *et al.*, 1998). They form a type IV secretion system to deliver effectors to host cells to control organelle trafficking (Segal & Shuman, 1998). A number of effectors have been characterized, including RalF (Nagai *et al.*, 2002), LidA (Conover *et al.*, 2003) and LepA/B (Chen *et al.*, 2004) and other recently identified effectors (Shohdy *et al.*, 2005).

In addition, the type II protein secretion system is required for both intracellular infection and promotion of virulence in animal models, where the secretion system is prepilin peptidase PilD dependent (Rossier & Cianciotto, 2001; Rossier *et al.*, 2004). *L. pneumophila* can live in various environmental waters and has multiple iron-uptake systems.

Abbreviations: AFLP, amplified fragment length polymorphism; SG, serogroup.

Sequences of primers used are available as supplementary material in JMM Online.

Iron acquisition genes *feoB*, *iraA* and *ccmC* can be determinants of intracellular growth and virulence (Robey & Cianciotto, 2002; Viswanathan *et al.*, 2000, 2002). Moreover, some regulators such as RpoS, a stationary-phase sigma factor, and LetA, a GacA homologue response regulator, are required for efficient infection and intracellular multiplication of *L. pneumophila* (Bachman & Swanson, 2004; Gal-Mor & Segal, 2003). Other virulence factors include a 65 kb DNA fragment encoding some known virulence factors as a pathogenicity island (Brassinga *et al.*, 2003) and various plasmids (Brown *et al.*, 1982).

The genomes sequenced show that *L. pneumophila* has high genome plasticity. The difference between strain-specific genes in isolates within the same serogroup varied from 10 to 14% (Cazalet *et al.*, 2004). In a previous study, a predominant genotype termed AF1, detected by amplified fragment length polymorphism (AFLP), was identified in isolates from both patients (40.5%) and water (49.0%) (Huang *et al.*, 2004). All AF1-type isolates have been found to have the virulence genes *lvh*, a new type IV secretion gene locus where the products of *lvh* genes interact with components of the *icm/dot* system (Segal *et al.*, 1999), and *rtxA*, encoding the *dot/icm*-regulated, pore-forming toxin for bacterial entry into cells (Zink *et al.*, 2002). The second most-frequent type (36.5%), termed AF16, identified in isolates from water, has not been detected in isolates from patients. *lvh* and *rtxA* genes are not present in AF16 isolates, with the exception of one isolate with *rtxA*. The interaction of the products of both *lvh* and *rtxA* with the major *dot/icm* virulence factors and the strong association of both *lvh* and *rtxA* with the virulent AF1 strain suggest that *lvh* and *rtxA* could be used as indicators (markers) of infection potential for *L. pneumophila* SG1 isolates (Huang *et al.*, 2004).

Although the relationship between *icm/dot* type IV protein secretion genes and other virulence factors associated with the pathogenicity of *L. pneumophila* has been widely studied (Bandyopadhyay *et al.*, 2004; Segal *et al.*, 1999), the distribution of virulence genes in clinical or environmental isolate collections has not been examined. Therefore, the aims of this study were to determine whether there was a different distribution pattern between the clinical and environmental isolates of the well-studied virulence genes that play important roles in various steps of the whole infection process, and to examine whether these genes, together with *lvh* and *rtxA*, could be used as indicators of infection potential for *L. pneumophila* SG1 isolates.

METHODS

Collection of *L. pneumophila* SG1 isolates. The *L. pneumophila* SG1 isolates used were the same as those typed using AFLP in our previous study (Huang *et al.*, 2004). Legionellosis is a notifiable disease in Australia. A total of 141 *L. pneumophila* SG1 isolates were used in this study. They included 37 isolates from patients with clinical symptoms of Legionnaires' disease who had been referred to our laboratory during 1990–2003, and 96 isolates from water that were detected between July and December 2003 and eight isolates that

were referred to our laboratory between 1994 and 2003. Water samples were taken from free-flowing water in cooling water towers, according to the current sampling method for Australian/New Zealand Standards (Standards Australia, 2000). All isolates were epidemiologically unrelated, except for one water isolate from 1994 that was epidemiologically related to a case. These isolates from patients and water were from Brisbane and neighbouring regions in Queensland.

PCR primer design and reaction conditions. The virulence genes were chosen on the basis of two factors: (1) genes involved in different infection steps and (2) genes cited in published *Legionella* genome papers (Cazalet *et al.*, 2004; Chien *et al.*, 2004). In total, 19 gene targets were examined, including *lvh*, *rtxA*, 65 kb fragment, loci 1 and 2 of the type IV secretion system, loci 1 and 2 of the type II secretion system and *pilB*, *pilD*, *ralF*, *lidA*, *lepA*, *rpoS*, *letA*, *feoB*, *iraA*, *ccmC*, *hsp60* and *milA* (see Table 1). The *lvh* and *rtxA* regions were detected using the same primers as in previous studies (Samrakandi *et al.*, 2002). The 65 kb pathogenicity island encodes a total of 64 genes; two genes, *magA* and *traD*, were chosen for detection of the 65 kb fragment. The primer pairs MagF/MagR (referred to as F MagA5134/R MagA5603 in the original paper) and F9/R9 were used to amplify *magA* and *traD*, respectively, as described by Brassinga *et al.* (2003). The other primer pairs used were designed using the three *L. pneumophila* SG1 genome sequences published (Cazalet *et al.*, 2004; Chien *et al.*, 2004). The type II protein secretion system has two loci; primer pairs designed for *lspD* and *lspE* were used for the first locus (*lspDE*) and primer pairs for *lspG* and *lspJ* for the second locus (*lspFGHIJK*). The two loci of the *icm/dot* type IV secretion system in the genome were tested. The genes in locus 1 include *icmTSRQOMLKEGCDJBF*; primers were designed to detect *icmB*, *icmK* and *icmT*. The genes in locus 2 include *dotABCD-icmVWX*; primers were designed to detect *dotA*, *dotC* and *icmX*. Primers for detection of type IV pili of *L. pneumophila* were designed for *pilD* and *pilB*. (Sequences of the primers used are available in Supplementary Table S1 in JMM Online.) PCR reaction conditions were the same as those described by Samrakandi *et al.* (2002). The amplified PCR products were sequenced to verify their authenticity.

Plasmid isolation. Plasmids have been identified in *L. pneumophila* isolates (Bezanson *et al.*, 1992). They encode specific virulence factors that may contribute to the pathogenicity of this organism (Brown *et al.*, 1982). The extraction method used was described by Brown *et al.* (1982) and the plasmid-positive strain *Legionella bozemanii* 336/H7 was included as a control (Institute of Medical and Veterinary Science, Australia).

RESULTS AND DISCUSSION

Virulence gene PCR products were separated in agarose gels (Fig. 1). The results in Table 1 show that the following genes were detected in all *L. pneumophila* SG1 Queensland isolates examined: *icm/dot* type IV and type II protein secretion systems, type IV pili genes (*pilD* and *pilB*), virulence effector genes (*ralF*, *lidA* and *lepA*), virulence regulator genes (*rpoS* and *letA*), iron acquisition genes (*feoB*, *iraA* and *ccmC*) and surface protein genes (*hsp60* and *milA*). The data suggest that these genes, although they have been identified as essential virulence genes, are also crucial for survival, because mutations of these genes result in significant reduction of growth or no growth *in vitro* or in host cells (Conover *et al.*, 2003; Gal-Mor & Segal, 2003; Rossier *et al.*, 2004; Viswanathan *et al.*, 2000). They do not appear to be useful as indicators of the infection potential

Table 1. Detection of virulence genes in *L. pneumophila* SG1 isolates from patients and water

Data for *lvh* and *rtxA* are taken from Huang *et al.* (2004), with the permission of the American Society for Microbiology. Primer sequences are available in Supplementary Table S1 in JMM Online.

Isolate	No. of isolates	<i>lvh</i>	<i>rtxA</i>	65 kb	Plasmid	Type IV Locus 1	Type IV Locus 2	Type II Locus 1	Type II Locus 2	The rest*
Patient										
AF1	15	14/15	15/15	1/15	0/15	15/15	15/15	15/15	15/15	15/15
AF2–15	14	14/14	14/14	0/14	0/14	14/14	14/14	14/14	14/14	14/14
	7	0/7	7/7	0/7	0/7	7/7	7/7	7/7	7/7	7/7
	1	1/1	0/1	0/1	0/1	1/1	1/1	1/1	1/1	1/1
Summary	37	29/37	36/37	1/37	0/37	37/37	37/37	37/37	37/37	37/37
Water										
AF1	51	51/51	51/51	41/51	0/51	51/51	51/51	51/51	51/51	51/51
AF16	38	0/38	1/38	0/38	0/38	38/38	38/38	38/38	38/38	38/38
AF17–22, 3 and 14	9	9/9	9/9	3/9	0/9	9/9	9/9	9/9	9/9	9/9
	6	0/6	6/6	2/6	0/6	6/6	6/6	6/6	6/6	6/6
Summary	104	60/104	67/104	46/104	0/104	104/104	104/104	104/104	104/104	104/104

*Includes *pilD*, *pilB*, *ralF*, *lidA*, *lepA*, *rpoS*, *letA*, *feoB*, *iraA*, *ccmC*, *hsp60* and *milA* genes.

Fig. 1. PCR products of virulence genes in strains ATCC 33152^T (Philadelphia-1^T) and 03M1684 (an isolate from water). Lane M, GeneRuler DNA ladder mix (Fermentas). (a) PCR of strain ATCC 33152^T. Lanes 1–12 show amplified bands using primers for the following genes: 1, *icmX*; 2, 65 kb DNA fragment of MagF/MagR; 3, 65 kb DNA fragment of F9/R9; 4, *dotA* and *icmT*; 5, *lidA* and *hsp60*; 6, *lepA1* and *letA*; 7, *rpoS*, *ralF* and *lepA2*; 8, *icmK*, *icmB* and *dotC*; 9, *lspD*, *pilB* and *lspJ*; 10, *lspE*, *pilD* and *lspG*; 11, *feoB*, *iraA* and *ccmC*; and 12, *milA*. Amplified PCR bands of the virulence genes are stated from high molecular mass to low molecular mass in a multiplex-PCR lane. In addition, two primer sets (*lepA1* and *lepA2*) were used to detect the *lepA* gene because of its large size. (b) PCR of strain 03M1684 using the same primer pairs as in (a). No products were detected in lanes 2 and 3 (65 kb DNA fragment of MagF/MagR and F9/R9), suggesting that the 65 kb DNA fragment was not present in this isolate.

of an isolate, as they were present in all isolates of *L. pneumophila* studied.

However, the survey of the 65 kb DNA fragment in these isolates demonstrated a difference in detection rates (Table 1). Only one AF1-type isolate from all the isolates from patients tested was found to have the 65 kb DNA fragment (2.7%, 1/37). In contrast, the 65 kb DNA fragment was frequently present in the isolates from water, with a detection rate of 44.2% (46/104). In particular, the 65 kb DNA fragment was more frequently present in the predominant AF1-type from water, with a detection rate of 80.4% (41/51). Interestingly, the 65 kb DNA fragment was not present in isolates of the second most-frequent AF16-type from water (0/38). Although Southern blotting would be the method of choice to verify the 65 kb PCR-negative isolates, these detection results were obtained by using two pairs of primers to detect *magA* and *traD* genes, respectively, within the 65 kb DNA fragment to minimize possible mutation in one of the primer annealing regions. Therefore, the probability of false-negative detection of the 65 kb DNA fragment was significantly reduced. The results suggest that the 65 kb DNA fragment-bearing predominant AF1 strain is better adapted for survival in the sampled environment. The low frequency of the 65 kb fragment in the isolates from patients suggests that the pathogenicity island may not be necessary for *L. pneumophila* to cause disease (see Table 1). This contradicts the presumption that the pathogenicity island in the 65 kb DNA fragment would enhance the ability to cause disease in humans, as suggested by Brassinga *et al.* (2003).

Plasmids were not detected in the *L. pneumophila* SG1 isolates from water or patients analysed in this study (Table 1). Plasmid extraction was carried out using the method of

Brown *et al.* (1982) and was retested using another plasmid miniprep method (Sambrook *et al.*, 1989). A positive plasmid band was produced consistently on gels with the positive-control strain *L. bozemanii* 336/H7 using the two extraction methods. The isolates of *L. pneumophila* SG1 used in this study were primary isolates and were stored at -70°C . It is notable that plasmids are frequently present in isolates of *L. pneumophila* from water from other countries, with detection rates ranging from 20 to 60% (Bezanson *et al.*, 1992; Brown *et al.*, 1982; Maher *et al.*, 1983). This suggests that *L. pneumophila* isolates from Australia might be different from those from other countries in this regard.

In the AF1-type isolates, *lvh* and *rtxA*, respectively, were positive in 14 and 15 out of 15 isolates from patients and in 51 and 51 out of 51 isolates from water (Table 1). In the second most-common AF16-type isolates from water, a genotype that was not detected in patients, neither *lvh* nor *rtxA* was present in the 38 isolates, except for one isolate with *rtxA*. Therefore, *lvh* and *rtxA* may not be required for survival in water. Statistical analyses (Fisher's exact test) indicated that, in *L. pneumophila* SG1 isolates from water, there was a significant association of *lvh* and *rtxA* with AF1-type isolates ($n=51$, $P<0.0001$) and a significant association of the absence of *lvh* and *rtxA* with AF16-type isolates ($n=38$, $P<0.0001$). Because either *lvh* or *rtxA* or both were present in all *L. pneumophila* SG1 isolates from patients tested, no significant association of *lvh* or *rtxA* with AF1-type isolates was found ($n=15$, $P>0.07$), suggesting that *lvh* and *rtxA* could be used as indicators to assess the infection potential of an isolate. *lvh* genes encode a new type IV protein secretion system and do not resemble the homologous *icm/dot* type IV protein secretion genes that are required for intracellular growth of *L. pneumophila* in human macrophages and protozoan hosts (Segal *et al.*, 1999). However, they enhance intracellular growth and can replace the functions of some *icm/dot* genes in plasmid RSF1010 conjugation, suggesting that the components of the *lvh* type IV protein secretion system can interact with components of the *icm/dot* system (Segal *et al.*, 1999). In addition, *lvh* has a substantial effect on the efficiency of infection of host cells in a temperature-dependent manner. *lvh* is crucial for intracellular survival and replication when the bacteria are grown at 30°C and less so when they are grown at 37°C (Ridenour *et al.*, 2003). The *rtxA* gene product has eight RTX amino-acid repeats that have been identified in a number of RTX toxins of pathogens, such as the cytotoxin of *Bordetella pertussis*, the leukotoxin of *Actinobacillus actinomycetemcomitans* and the haemolysin of *Escherichia coli* (Cirillo *et al.*, 2000, 2001). Interestingly, the function of the pore-forming toxin gene *rtxA* is regulated by the *dot/icm* complex (Zink *et al.*, 2002). A *rtxA* mutant of *L. pneumophila* has been shown to have a reduced ability to attach to monocytes and epithelial cells, and reduced cytotoxicity and intracellular replication (Cirillo *et al.*, 2000, 2001). An isolate with the *rtxA* gene would have enhanced abilities for attachment, cytotoxicity and intracellular growth. Consequently, isolates bearing either *lvh* or

rtxA, or both, appear to be more virulent. Therefore, they could be considered as indicators of the infection potential of an isolate.

In summary, there was an association of both *lvh* and *rtxA* with the virulent and predominant AFLP genotype termed AF1. Functionally, there is an interaction of the products of *lvh* and *rtxA* with the products of the major virulence *icm/dot* system (Ridenour *et al.*, 2003; Zink *et al.*, 2002), conferring greater virulence on isolates bearing either *lvh* or *rtxA*, or both. We found that a test strategy for *lvh* and *rtxA* as pathogenesis markers using an easy PCR would be useful for determining the infection potential of an isolate.

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