Expression of phospholipase D, the major virulence factor of *Corynebacterium pseudotuberculosis*, is regulated by multiple environmental factors and plays a role in macrophage death

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Corynebacterium pseudotuberculosis is the aetiological agent of caseous lymphadenitis, a disease affecting sheep and goats. Phospholipase D (Pld), a major virulence determinant of *C. pseudotuberculosis*, is believed to play a critical role in dissemination of bacteria from the site of infection to the lymph nodes. Although the *pld* gene has been studied for some time, it is only recently that it has been identified as being down-regulated following heat shock from 37 to 43 °C. To gain insights into the mechanisms of Pld action, this study investigated how it was regulated under varying environmental conditions. Studies measuring *pld* mRNA levels or utilizing a reporter construct containing the *pld* promoter upstream of a *gfp* gene were performed. These showed that *pld* was upregulated in a cell-density-dependent manner, was regulated by heat shock at all cell-culture densities, and was highly expressed in a tissue-culture macrophage-infection model. Finally, the expression of Pld by intracellular *C. pseudotuberculosis* was shown to play a small but significant role in the reduction of macrophage viability following infection. This study demonstrates that the regulation of *C. pseudotuberculosis pld* is complex. This regulatory complexity may play an important role in allowing the pathogen to successfully adapt to the changing host environment during infection, migration, establishment and disease progression.

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INTRODUCTION

Corynebacterium pseudotuberculosis is a Gram-positive, intracellular, facultative anaerobe that causes disease in sheep, goats, horses and occasionally other species, including cattle and humans (Batey, 1986). In sheep and goats, *C. pseudotuberculosis* infection is manifested as caseous lymphadenitis (CLA). CLA is one of the most prevalent bacterial diseases of sheep, and results in lost revenue due to reduced wool production, carcase condemnation and carcase trimming (Paton *et al.*, 1988). Infection typically occurs via skin or mucous-membrane wounds, followed by dissemination of the bacteria to the superficial lymph nodes, in which caseous abscesses develop and necrosis occurs. In some cases, other sites, in particular within the visceral organs, may also be infected (Batey, 1986).

To date, the most important virulence determinant identified in *C. pseudotuberculosis* is phospholipase D (Pld). Pld, with a molecular mass of 31.4 kDa, is a secreted exotoxin that possesses sphingomyelinase activity (Hodgson *et al.*, 1990). Pld has been shown to increase vascular permeability *in vivo*, have dermonecrotic properties, exhibit synergistic haemolysis of sheep blood cells in the presence of products from *Rhodococcus equi*, and reduce the viability of ovine neutrophils *ex vivo* (Batey, 1986; Yozwiak & Songer, 1993). Studies with *C. pseudotuberculosis* strains with inactivated Pld have convincingly demonstrated the necessity of Pld for establishment of CLA (Hodgson *et al.*, 1999, 1992, 1994; McNamara *et al.*, 1994; Simmons *et al.*, 1998). Mutant strains are unable to cause abscessation of the lymph nodes. Additional evidence for the importance of Pld *in vivo* comes from the observation that vaccination with formulations in which Pld is the major component provides protection against subsequent disease challenge (Eggleton *et al.*, 1991). The chromosome

Abbreviation: CLA, caseous lymphadenitis.

contains a single copy of the gene and is transcribed as a monocistronic RNA of 1.1 kb (Hodgson *et al.*, 1990). It has recently been established that expression of the *pld* gene is strongly down-regulated following heat shock (McKean *et al.*, 2007). No other aspects of *pld* regulation have been reported to date.

In addition to *pld*, only one other gene has been shown to be essential for establishment of CLA by *C. pseudotuberculosis*. This gene, *fagB*, encodes an iron permease component (Billington *et al.*, 2002). Other suggested virulence determinants include a serine protease (Wilson *et al.*, 1995) and cell wall components (Muckle & Gyles, 1983). Although the pathogenesis of CLA is relatively well understood, little is known about how these virulence determinants are regulated, or indeed about the role of other virulence determinants in the disease process.

During the course of infection, *C. pseudotuberculosis* is exposed to a range of very different environments from the point of entry, via trafficking through the lymphatic system, to establishment of caseous lesions within organs. The niches in which the bacteria must survive range from the aerobic environment of the point of initial infection to intracellular replication within macrophages. Presumably, changes in gene expression play a role in the adaptations and modifications necessary for successful infection. This study demonstrates that regulation of expression of the main virulence factor Pld is complex, and responds to various environmental cues. Apart from the previously identified heat-shock regulation, the gene is also regulated by culture density and is highly expressed in infected macrophages, in which it has a small but significant effect on macrophage viability.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* TOP 10F' (Stratagene) was used as the host for plasmid constructions. *E. coli* strains were grown at 37 °C in Terrific Broth or maintained on Luria-Bertani agar (Sambrook *et al.*, 1989). DNA was transformed into *C. pseudotuberculosis* by electroporation (Songer *et al.*, 1991). The wild-type *C. pseudotuberculosis* strain C231 (Burrell, 1978), the *pld* mutant strain Toxminus (Hodgson *et al.*, 1992), and the *pld*-complemented

Toxminus strain (Toxminus + pTB111) (Tachedjian *et al.*, 1995) were grown at 37 °C in brain heart infusion (BHI) broth or maintained on BHI agar. For flow-cytometry and tissue-culture experiments, the media were supplemented with 1% Tween-80 to reduce cell aggregation. Antibiotics, ampicillin (100 µg ml⁻¹) and kanamycin (50 µg ml⁻¹), were added to the media as required. For heat-shock experiments, exponentially growing cultures were split into two, one half being placed at 37 °C and the other transferred to 43 °C, and incubated for the desired amount of time. To prepare *C. pseudotuberculosis* cultures for flow-cytometry analysis, overnight cultures were cooled on ice, mildly sonicated (XL-Series Sonicator; Heat Systems, using a standard microprobe set at 4) to break up clumps of cells, diluted to OD₆₀₀ 0.1, and grown at 37 °C. Prior to analysis, cultures were again briefly sonicated and diluted up to 500-fold in PBS. All bacterial optical density measurements were made in 1 cm path length cuvettes using a Beckman DU640 spectrophotometer. Samples were diluted in fresh growth media, so that the readings were between 0.1 and 1.0.

Isolation of RNA. Bacterial RNA was extracted using RNeasy (Tel-Test) as recommended by the supplier, with minor modifications. Bacterial pellets containing ~10⁹ cells were resuspended in 1 ml RNeasy, and transferred to a 2 ml screw-capped tube containing 1 ml of 0.1 mm diameter glass beads. The sample was homogenized in a Mini-BeadBeater-8 (Biospec Products) at maximum speed for 3 min. RNA isolation from the homogenate was then performed exactly as per the RNeasy protocol. RNA was resuspended in diethyl pyrocarbonate-treated water. RNA concentration and purity were determined by measuring A₂₆₀ and A₂₈₀. RNA was treated with RNase-free DNase I (DNA-free; Ambion) to remove trace DNA contamination.

Northern blot analysis. RNA was electrophoresed under denaturing conditions through a 1.2% agarose gel containing formaldehyde, then transferred to a Hybond-N+ membrane (Amersham), as described by Sambrook *et al.* (1989). Two micrograms *pld* PCR product (generated using primers 55 and 77; Table 1) was labelled with DIG Chem-Link from the DIG Chem-Link Labelling and Detection set (Roche), and then hybridized to the membrane for 16 h at 50 °C in the presence of DIG Easy Hyb (Roche). The membrane was washed at low stringency in 2 × saline sodium citrate (SSC)/0.1% (w/v) SDS at room temperature (2 × 5 min), and then at high stringency in 0.1 × SSC/0.1% (w/v) SDS at 68 °C (2 × 15 min). The bound probe was detected using an anti-digoxigenin (DIG)-alkaline phosphatase conjugate and the alkaline phosphatase substrate CSPD (Roche).

Real-time quantitative PCR. Reverse transcription was performed using a TaqMan Reverse Transcription Reagents kit (Applied Biosystems). One microgram of DNase I-treated RNA was reverse-transcribed

Table 1. Primers used in this work

Primer	Sequence (5'→3')*	Application
18	CCATGCTCCGCATCCCTTGCA	Upper primer for obtaining a <i>pld</i> promoter fragment for cloning into pSM20
55	CGAAGATCAGCGGTGA	Lower primer to obtain a <i>pld</i> fragment for Northern analysis
75	GCGGATCCACTTCTCCCTCATTTTAC	Lower primer for obtaining a <i>pld</i> promoter fragment for cloning into pSM20
77	GATGCATGCGCTATGGATACCCTAAAG	Upper primer to obtain a <i>pld</i> fragment for Northern analysis
101	GATTGCCACCGCGTTT	Upper primer to detect <i>pld</i> by real-time PCR
102	TCGCACCGATCGCAACT	Lower primer to detect <i>pld</i> by real-time PCR
103	CCTGTATAAGAAGCACCGGCTAA	Upper primer to detect 16S rRNA by real-time PCR
104	ACGCTCGCACCTACGTATT	Lower primer to detect 16S rRNA by real-time PCR

*When present, added restriction site is underlined.

in a 50 µl reaction containing 1 × RT buffer (Applied Biosystems), 5.5 mM MgCl₂, 445 µM dNTPs, 2.5 µM Random hexamers, 20 U RNase inhibitor and 75 U Multiscribe reverse transcriptase. The reaction was incubated for 10 min at 25 °C, 45 min at 48 °C and 5 min at 95 °C. For each RNA sample, an identical control reaction was performed without Multiscribe reverse transcriptase. cDNA was diluted 1 in 10 with water prior to use in PCR reactions. Primers for real-time PCR were designed using Primer Express version 1.5 software (Applied Biosystems) to meet optimal design parameters (Table 1). Real-time PCR reactions of 50 µl were performed in a 96-well plate format in an ABI PRISM 7700 Sequence Detector under universal cycling conditions (2 min at 50 °C, followed by 10 min at 95 °C to activate the DNA polymerase, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C). PCR reactions contained 1 × SYBR Green Master Mix (Applied Biosystems), 50 nM forward and reverse primers, and 10 µl diluted cDNA, and were set up in triplicate. Two negative controls were routinely performed. DNA contamination was determined by performing the PCR on negative control reverse-transcriptase reactions (in which no enzyme had been added to the reverse transcription reaction). Environmental contamination was monitored in no-template controls, in which water was added to the reaction instead of DNA. Data were analysed using Sequence Detector version 1.7 software (Applied Biosystems). The relative amount of *pld* mRNA in each sample was calculated using the comparative C_T method (Applied Biosystems User Bulletin #2), with 16S rRNA values being used to correct for differences in the amount of RNA in each RT reaction.

Construction of the *pld* promoter probe vector pSM27. The promoter probe vector pSM20 has been described elsewhere (McKean *et al.*, 2005). The *pld* promoter was PCR-amplified from genomic C231 DNA using primers 18 and 75 (Table 1). A *Bam*HI restriction site was engineered into primer 75 to allow directional cloning into pSM20. The PCR product was digested with *Bam*HI and ligated into pSM20, cut with *Eco*RV and *Bam*HI, to generate pSM27.

Flow cytometry and fluorescence-activated cell sorter (FACS) analysis. Flow cytometry and FACS analysis were performed using a Becton-Dickinson FACSCalibur instrument with the 488 nm argon laser for excitation and a 530/30 nm emission filter. Data were collected and analysed using Cellquest software. NaCl (0.9 %, w/v) was used as sheath fluid.

Infection of J774 macrophages with *C. pseudotuberculosis*. The mouse macrophage-like cell line J774A.1 (ATTC no. TIB-67) was grown at 37 °C, 5 % carbon dioxide in Dulbecco's Modified Eagle's Medium (DMEM; TRACE Scientific) supplemented with 2 mM glutamine, 10 % (v/v) fetal calf serum and 50 µM β-mercaptoethanol. Macrophages were routinely grown in tissue-culture flasks to 80–100 % confluence.

An infection assay based on the gentamicin killing assay (Elsinghorst, 1994) was established. For assessment of *pld* promoter activity by intracellular *C. pseudotuberculosis*, macrophages were plated in six-well plates at 7 × 10⁵ cells per well at 18 h prior to addition of bacteria. For the neutral red assay of cell viability, cells were plated in 24-well plates at 1.5 × 10⁵ cells per well. Exponential-phase bacteria were mildly sonicated to disrupt clumps, diluted in pre-warmed DMEM, and added to the macrophage monolayer. Following incubation for 1 h, the monolayer was washed three times with 37 °C PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄), and then incubated with DMEM containing 100 µg gentamicin ml⁻¹. The infection was analysed at further time points.

For flow-cytometry analysis of individual intracellular bacteria, infected macrophages were washed in PBS. PBS (0.5 ml) was added to each well, and the cells were dislodged with a cell scraper. The

contents of the well were transferred to a 1.5 ml tube and sonicated to release the bacteria from the macrophages and disrupt bacterial clumps. Sonicated samples were diluted in PBS, then analysed by flow cytometry.

A neutral red assay was used to measure cell viability. Medium was removed from the cells 9.5 h post-infection and replaced with PBS containing 0.1 % (w/v) neutral red and 100 µg gentamicin ml⁻¹. After incubation for 1 h, the cells were washed three times in PBS. Acidified 2-propanol (300 µl) was then added to each well to lyse the macrophages and solubilize the incorporated neutral red. Absorbance was measured at 540 nm.

RESULTS

Regulation of *pld* expression by heat shock

Using a DNA macroarray approach, we have previously identified *pld* as being down-regulated following a 30 min heat shock from 37 to 43 °C (McKean *et al.*, 2007). Northern analysis was performed to determine the time course of *pld* mRNA down-regulation following heat shock. An exponentially growing culture of strain C231 was heat-shocked by incubation at 43 °C. RNA samples were taken at 0, 5, 10, 20 and 60 min after initiation of the heat shock. *pld* mRNA was detected in all 37 °C samples; however, following heat shock at 43 °C, down-regulation of *pld* expression was apparent by 10 min, as indicated by decreased band intensity (Fig. 1). The level of *pld* mRNA continued to decrease past this time point, to a minimal level detected at 20 and 60 min.

Measurement of *pld* expression using a *gfp* reporter construct

To allow us to readily assess *pld* expression under a variety of environmental shocks, a reporter plasmid was constructed by inserting the *pld* promoter into the promoter probe vector pSM20, to generate pSM27 (Fig. 2). pSM20 is an *E. coli*/*C. pseudotuberculosis* shuttle vector which contains a UV-optimized *gfp* gene as the reporter (McKean *et al.*, 2005). Using this construct, it was possible to measure *pld* promoter activity in broth culture by flow cytometry (Fig. 3). To demonstrate that pSM27 was a suitable reporter for monitoring *pld* expression, attempts

Fig. 1. Time course of *pld* regulation following heat shock. RNA was extracted from *C. pseudotuberculosis* control (37 °C) and heat-shocked samples (37 to 43 °C) at 5, 10, 20 and 60 min post initiation of the shock. Total RNA (8 µg) was analysed by Northern analysis using a DIG-labelled *pld* PCR product.

Fig. 2. Physical map of the *pld* promoter reporter plasmid pSM27. Relevant genes and encoded products are: *pld pro*, *pld* promoter region; *gfp*, reporter protein Gfp; *repA*, replication protein RepA; *kan*, kanamycin-resistance gene. *rrnB T1T2* and *trpA tt* encode transcription terminators.

were made to monitor the effect of thermoregulation on the expression of *pld*. A similar experiment to that described for the Northern analysis was performed, but at a lower cell density. A lower cell density was chosen because analysis of *C. pseudotuberculosis* by flow cytometry appears to be most efficient for low-density cultures, as a result of less bacterial clumping. An overnight culture of strain C231(pSM27) was diluted to OD₆₀₀ 0.1 and then grown at 37 °C for 1 h. Bacteria were then either incubated further at 37 °C or transferred to 43 °C. *pld* promoter activity was assessed by measuring Gfp fluorescence by flow cytometry at 0, 0.5, 1, 2 and 3.5 h post initiation of the heat shock (Fig. 4). Using this system, a small decrease in *pld* promoter activity at 43 °C was observed, at the later time

Fig. 3. Flow-cytometry analysis of Gfp expression from *C. pseudotuberculosis* harbouring the *pld* promoter construct pSM27 or the promoterless construct pSM20. For each strain 30 000 events were counted. Fluorescence intensity, on the *x* axis, is a measure of the mean fluorescence for each bacterial clone. Data were gated on forward and side scatter, such that plotted data primarily represent single bacteria.

Fig. 4. Measurement of *pld* promoter activity in *C. pseudotuberculosis*, using the reporter construct pSM27. Duplicate cultures of strain C231(pSM27) were grown at 37 °C from OD₆₀₀ 0.1 for 1 h. One culture was incubated further at 37 °C (■), while the second was transferred to 43 °C (▲) (*t*=0). At various time points, Gfp fluorescence of the cultures was measured by flow cytometry. For each sample, 30 000 events were counted, and the mean fluorescence of the population determined to give relative Gfp fluorescence.

points of 2 and 3.5 h. Interestingly, in samples continuously incubated at 37 °C, a greater reduction in Gfp fluorescence was observed. At 43 °C, *C. pseudotuberculosis* had a reduced growth rate (data not shown). This, in conjunction with a Gfp half-life of >24 h (Andersen *et al.*, 1998; Corish & Tyler-Smith, 1999), means that it is likely that Gfp persists within the bacteria, thus masking the reduced transcription that occurs at the *pld* promoter. The results indicate that *pld* expression may be culture-density related; we therefore further investigated this aspect of *pld* expression. Overnight cultures of C231(pSM27) and the control strain C231(pSM22) were diluted to OD₆₀₀ 0.1 and grown at 37 °C for 8 h. pSM22 contains the *srp* promoter (Ezaz-Nikpay *et al.*, 1994), which drives Gfp constitutive expression. Fluorescence of the cultures was measured by flow cytometry every hour, and OD₆₀₀ was also determined. The two strains exhibited the same growth curve (Fig. 5a), which indicated that they had the same density, and that growth was not affected by the amount of Gfp in the cell. C231(pSM27) showed decreased fluorescence to OD₆₀₀ 0.8, which was indicative of low expression from the *pld* promoter (Fig. 5b). This was followed by a rapid increase in fluorescence, which indicated increased promoter activity. On the other hand, Gfp expression from C231(pSM22), which contains *gfp* expressed under control of the constitutive promoter *srp*, was essentially constant until the culture OD₆₀₀ reached 2. At this culture density, an increase in Gfp fluorescence was observed. This may correspond to a slowing in cell growth, leading to Gfp accumulation. For the first 6 h of the culture period, the growth rate was constant, so the changes seen in gene expression in the early phase could not be attributed to a response to growth rate. The basic chemical characteristics of the culture, such as pH and osmolarity, did not change

Fig. 5. Culture-density-dependent *pld* promoter activity. Cultures of strain C231(pSM22) (■) and C231(pSM27) (▲) were diluted to OD₆₀₀ 0.1 and then incubated at 37 °C. At hourly intervals, the growth of the culture was determined by measuring OD₆₀₀ (a), and the level of Gfp fluorescence of each culture was determined by flow cytometry (b). For each sample, 30 000 events were counted, and the mean fluorescence of the population determined to give relative Gfp fluorescence. To measure *pld* mRNA levels in cultures of different densities, *C. pseudotuberculosis* was grown from OD₆₀₀ 0.1 for 30 min, 3 h and 6 h (corresponding to mean OD₆₀₀ 0.16, 0.47 and 1.5, respectively). At each time point, RNA was extracted from duplicate cultures. Total RNA (8 µg) was analysed by Northern analysis using a DIG-labelled *pld* PCR product (c).

significantly in the early culture period. These results indicate that culture-density-dependent expression of *pld* does indeed occur.

The density-dependent expression of *pld* was further confirmed at the mRNA level by Northern analysis. Cultures of strain C231 were subcultured to OD₆₀₀ 0.1, then grown at 37 °C. At 30 min, 3 h and 6 h, duplicate samples (corresponding to mean OD₆₀₀ 0.16, 0.47 and 1.5, respectively) were harvested, and RNA extracted. Northern analysis was performed and confirmed that *pld* expression increased with cell density (Fig. 5c). In terms of correlating the Northern and flow cytometry data, it is likely that the decrease in fluorescence initially observed by flow cytometry

corresponded to low expression levels observed at the first two time points of the Northern analysis. This is likely to represent a slower rate of Gfp production than cell growth, such that the Gfp already present in the cells was dissipated during cell division. At the later time points, *pld* promoter activity was increased, thus leading to the rate of Gfp synthesis being greater than that of cell division, hence an increase in fluorescence was detected.

Down-regulation of *pld* expression by heat shock occurs at all cell densities

The studies performed to date have shown that *pld* is regulated by at least two mechanisms (thermoregulation and cell-density dependence). The heat-regulation experiments were performed at medium to high cell densities, thus raising the question as to whether thermoregulation also occurs at lower cell densities. An overnight culture of strain C231 was diluted to OD₆₀₀ 0.1, then incubated for 1, 3.5 and 6.5 h at 37 °C. For the last 30 min period of each time point, replica samples were transferred to 43 °C. The relative levels of *pld* mRNA and 16S rRNA were determined by reverse transcription, followed by real-time PCR. As previously demonstrated by Northern analysis, expression of *pld* mRNA increased with increasing cell density (5.6-fold over the time course of the experiment; Fig. 6). Thermoregulation of *pld* expression occurred following heat shock at all cell densities, with fold reductions of 4.2, 10.2 and 17.8 at 1, 3.5 and 6.5 h, respectively. However, the level of *pld* mRNA detected was essentially equal in all heat-shocked samples, which suggests that heat shock reduced

Fig. 6. Effect of temperature on expression of *C. pseudotuberculosis pld* at different cell densities. An overnight culture of strain C231 was diluted to OD₆₀₀ 0.1, then incubated at 37 °C. At 30 min, 3 h and 6 h, aliquots of the culture were transferred to 43 °C for 30 min. RNA was extracted from the heat-shocked samples (hatched bars) and those incubated at 37 °C (black bars) for the same period of time (i.e. 1, 3.5 and 6.5 h). The levels of *pld* mRNA and 16S rRNA were determined by reverse transcription followed by real-time PCR. *pld* expression was normalized to 16S rRNA levels, to take into account differences in starting amounts of RNA. Results represent the average and range of duplicate samples.

pld expression to a basal level. This study demonstrated that down-regulation by heat shock overrides upregulation of expression of *pld* at higher cell densities.

Macrophage infection

Given that *C. pseudotuberculosis* is primarily an intracellular pathogen residing within host macrophages, we were interested to determine whether *pld* was expressed within macrophages, and whether it demonstrated a similar density-related expression within this environment to that observed *in vitro*. An assay based on the gentamicin killing assay was utilized (Elsinghorst, 1994). J774 macrophages were infected at m.o.i. 4 with either C231(pSM22) or C231(pSM27) for 1 h, and washed, after which fresh medium containing 100 µg gentamicin ml⁻¹ was added to kill extracellular bacteria. At further time points, macrophage monolayers were washed, lysed and mildly sonicated to disrupt bacterial clumps. The sonicates were diluted in PBS, and Gfp fluorescence of individual bacteria was measured by flow cytometry (Fig. 7). Gfp fluorescence from the control C231(pSM22) strain was constant over the time course of the experiment. Fluorescence from C231(pSM27) was high throughout the time course of the experiment; however, a decreased level of fluorescence at the 1.5 h time point compared to that of the added bacteria (*t*=0 h) was observed. Fluorescence of C231(pSM27) at the remaining time points gradually increased to the level initially observed in the added bacteria, indicating that *pld* is highly expressed in an essentially unregulated manner by intracellular *C. pseudotuberculosis*.

Fig. 7. *pld* promoter-directed *gfp* expression from *C. pseudotuberculosis* inside infected J774 macrophages. Macrophages were infected with exponentially growing strain C231(pSM22) (■) or C231(pSM27) (▲) at m.o.i. 4. Following an incubation period of 1 h, the macrophages were washed and gentamicin-containing medium was added. At various time points, the fluorescence of individual bacteria was determined by flow cytometry. For each sample, 50 000 events were measured, and the mean fluorescence of the bacterial population determined to give relative Gfp fluorescence (log scale).

Intracellular *pld* expression reduces macrophage viability

The previous experiment showed that *pld* was expressed at a high level by *C. pseudotuberculosis* inside macrophages. Given the properties of this enzyme, it is likely that intracellular Pld affects macrophage function and viability, either by destruction of macrophage membranes or by activation of mammalian cell-signalling pathways. Treatment of ovine neutrophils with Pld has been shown to reduce their viability. In particular, intracellular Pld (delivered as an oil emulsion) has been shown to be more toxic to neutrophils than extracellular Pld (delivered in aqueous solution) (Yozwiak & Songer, 1993). This study was performed using relatively large amounts of purified protein, so the biological significance of the results is not clear. We were interested to determine whether intracellular *C. pseudotuberculosis* produces sufficient Pld to alter macrophage viability.

To study the effects of *C. pseudotuberculosis* Pld on macrophage cell function, a Pld-negative strain called Toxminus was utilized (Hodgson *et al.*, 1992). Toxminus is a mutant of the wild-type strain C231, in which the *pld* gene has been inactivated by insertion of an antibiotic-resistance gene. A third strain, Toxminus+pTB111, has a *pld* gene supplied *in trans* on a plasmid, and demonstrates a wild-type phenotype (Tachedjian *et al.*, 1995).

J774 macrophages were either left untreated or infected at m.o.i. 4 or 8 with C231, Toxminus or Toxminus+pTB111 for 1 h. Cells were then washed and incubated in gentamicin-containing media for an additional 9.5 h. Neutral red was included for the final hour of this incubation. At 10.5 h post-infection, macrophage viability was significantly reduced, following infection with all three strains of *C. pseudotuberculosis* at both m.o.i. values (Fig. 8). As expected, the percentage of surviving cells was lower following infection at the higher m.o.i. At both m.o.i. values, C231 had a greater effect on macrophage viability than did Toxminus. The wild-type phenotype was completely re-established following infection with Toxminus+pTB111 at m.o.i. 4. At m.o.i. 8, infection with Toxminus+pTB111 caused significantly more cell death than Toxminus; however, the percentage of surviving macrophages was still significantly higher than that following infection with C231. These data indicate that the high Pld expression observed in intracellular *C. pseudotuberculosis* has a small but significant effect on macrophage survival.

DISCUSSION

The primary aim of the work described in this paper was to gain an understanding of some of the environmental factors affecting *pld* expression. The observation that *pld* is expressed by *C. pseudotuberculosis* inside macrophages led to a study with the secondary aim of elucidating the functional significance of this finding.

Fig. 8. Effect on macrophage viability of intracellular *pld* expression by *C. pseudotuberculosis*. J774 macrophages were infected with strains C231, Toxminus or Toxminus+pTB111 for 10.5 h at m.o.i. 4 (a) or m.o.i. 8 (b). Results are presented as the mean $\pm$ SEM of six replicates. A one-way ANOVA followed by Tukey's test was performed. At both m.o.i. values, a significant difference between the number of untreated cells and treatments was observed ($P < 0.001$). At m.o.i. 4, significantly more macrophages remained following infection with Toxminus in comparison with C231 ($P < 0.01$), while there was no difference in the percentage of surviving macrophages following infection with C231 or Toxminus+pTB111. Toxminus was different from Toxminus+pTB111 at $P < 0.05$. At m.o.i. 8, all treatments were different from one another at $P < 0.001$, except for C231 and Toxminus+pTB111, which were different at $P < 0.05$.

Analysis of the heat-shock response of *pld* showed that its expression was rapidly reduced at 43 °C, such that very little *pld* mRNA could be detected by 20 min post initiation of the heat shock. This is indicative of a reduction in the rate of transcription from the *pld* promoter and rapid degradation of the *pld* mRNA already present in the cell. Thus, it appears that, as for other bacterial mRNA species, the half-life of *pld* mRNA is relatively short. The maximal repression of *pld* expression was maintained from 20 min for the remainder of the experiment (up to 60 min).

Many virulence determinants are regulated by more than one environmental factor. For example, expression of

invasin, the primary invasive factor of *Yersinia pseudotuberculosis*, is controlled by a number of environmental signals including temperature, growth phase, nutrient availability, pH and osmolarity (Nagel *et al.*, 2001). In order to allow us to more easily monitor the effects of environmental stimuli on *pld* expression, a reporter construct was generated by inserting the *pld* promoter into pSM20. pSM20 is a *C. pseudotuberculosis* promoter probe vector that contains a *gfp* gene as a reporter, and has been used to identify regulated genes of *C. pseudotuberculosis* by differential fluorescence induction (McKean *et al.*, 2005). Attempts to monitor the effect of increased temperature on transcription from the *pld* promoter using strain C231(pSM27) were unsuccessful, in that the large decrease in *pld* mRNA observed by Northern analysis following heat shock could not be detected as a substantial decrease in Gfp fluorescence. Although a small decrease was observed by flow cytometry, this did not correlate with either the timing or the magnitude of the decrease in *pld* mRNA. With the use of any reporter, a factor that must be taken into account is the half-life of the reporter protein. Although there are advantages in the long half-life of Gfp, such as the potential to see the transient upregulation of gene expression, there are also situations such as this one, in which Gfp protein stability can obscure the regulation that is occurring.

While it was not possible to monitor the heat-shock effect on *pld* expression using the reporter construct pSM27, it was possible to demonstrate a density-dependent regulation of *pld* expression. This was visualized using flow cytometry, as a decrease in detectable Gfp until OD₆₀₀ ~0.8 was reached, followed by a rapid increase. This phenomenon was confirmed by Northern analysis, which showed that *pld* expression gradually increased with increasing cell density.

Expression of *pld* at all investigated cell densities could be down-regulated by heat shock. This indicates that the mechanism of *pld* thermoregulation is able to override the density-dependent increase in *pld* expression. At all densities tested, heat shock at 43 °C was able to reduce *pld* expression to a similar basal level. Interestingly, the experiment showed that even in a low-density culture, in which *pld* expression is not high, the level can be further reduced.

The mechanisms by which expression from the *pld* promoter is controlled remain to be elucidated. The observation that *pld* is differentially regulated by a variety of environmental conditions suggests that the processes involved are likely to be multifactorial, and may involve mechanisms such as the binding of repressors or activators, or changes in DNA structure brought about by shifts in temperature. In a number of pathogenic bacteria, the control of virulence gene expression has been shown to occur by quorum sensing. Given that *pld* expression is greatest in high-density cultures, we were interested to determine whether its expression was regulated by quorum

sensing. However, by using conditioned media from high-density cultures, we were unable to demonstrate a role for quorum sensing, via diffusible signal molecules, in the regulation of *pld* expression (data not shown). Other mechanisms by which *pld* expression may be induced in high-density cultures include response to nutrient depletion or changes in bacterial growth rate. Many genes are growth-phase regulated, for example, those for ribosomal proteins and some fatty acid biosynthetic enzymes. *pld* expression may be regulated by similar environmental cues.

Using the reporter strain C231(pSM27), it was possible to show that *pld* is highly expressed by *C. pseudotuberculosis* resident in macrophages. Although a small decrease in Gfp fluorescence was observed between bacteria used to infect the macrophages and those resident within the macrophages at 2 h post-infection, *pld* expression was essentially unregulated during the experiment. It is likely that the decrease observed in macrophage-derived bacteria at the 2 h time point was the result of an initial decrease in expression in DMEM before the bacteria were phagocytosed by the macrophages. That no density-dependent expression was observed suggests that the density-dependent regulation mechanism is not operational within the macrophages, or that other factors dominate. It is likely that different regulatory systems are activated by the intracellular environment, which leads to activation of the *pld* promoter. Alternatively, perhaps the microenvironment of the phagosome appears as a high-density environment to the bacteria, thus leading to *pld* expression.

Pld expressed by intracellular bacteria was shown to make a small but significant contribution to the *C. pseudotuberculosis*-induced reduction in macrophage viability. Several mechanisms may be proposed to explain this observation. Firstly, this effect may have been mediated by a reduction in the integrity of the macrophage plasma membrane, as a result of the sphingomyelinase activity of Pld. The plasma membrane of eukaryotic cells is asymmetric with regard to phospholipid content, such that the phospholipids phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol are primarily located in the inner layer of the membrane, while phosphatidylcholine and sphingomyelin are predominantly located in the outer layer (Devaux, 1991). Given that the Pld substrate is located in the outer layer of the plasma membrane, its effect may be mediated by an extracellular rather than intracellular mechanism. That is to say, that following the death of a macrophage, the cellular contents are released. Pld in the medium is then able to attack sphingomyelin located in the outer membrane of still viable macrophages. Alternatively, sphingomyelin is also a major phospholipid component of murine (J774 macrophages) phagosomal membranes (Desjardins *et al.*, 1994). Therefore, the effect of Pld on macrophage viability may also be mediated by reducing the integrity of intracellular compartments, potentially allowing the escape of bacteria from this restricted area. As a third mechanism, the actions of Pld within the macrophages may be primarily mediated through disruption of

mammalian signalling pathways. Mammalian cells possess two Pld proteins that are involved in cell signalling, in addition to membrane remodelling (Exton, 1997). That bacterial phospholipases may modulate cell-signalling pathways to the advantage of the pathogen has been demonstrated for the two phospholipase C genes of *Listeria monocytogenes* (Schwarzer *et al.*, 1998). In order to gain insights into whether the Pld of *C. pseudotuberculosis* modulates host signalling cascades, the Toxminus strain can be utilized. By comparing the effects of the wild-type C231 and Toxminus strains on host signalling cascades, perturbations that are directly attributable to Pld could be identified. This type of study may give insights into how *C. pseudotuberculosis* manipulates the host response.

The observation that Pld is a major virulence factor and protective antigen indicates that it must be expressed *in vivo*. It is thought that *in vivo* expression of *pld* leads to increased local vascular permeability, thus enhancing dissemination of bacteria from the site of infection to the lymph node (Batey, 1986). Although it is not known whether *pld* is expressed *in vivo* under all situations and in all locations, the studies presented in this paper demonstrate that it is expressed at high levels in macrophages. The observation that *pld* is expressed by intracellular bacteria suggests that Pld may enhance the formation of abscesses within the lymph node. Abscess formation involves cycles of phagocytosis, bacterial replication within the phagocyte, and phagocytolysis. The observation from this study that *pld* expressed by intracellular bacteria has a direct effect on macrophage viability further supports the hypothesis that Pld is important in abscess formation.

It is not known whether naturally infected animals demonstrate an increased temperature following infection; however, experimentally infected sheep experience a transient temperature increase in the first day post infection (Pepin *et al.*, 1991). It has also been postulated that in the early stages of infection, *C. pseudotuberculosis* replicates extracellularly (Batey, 1986). It could be envisioned that *pld* expression may not occur during the early stages of infection, as a result of low extracellular bacterial density and repression of *pld* expression by heat shock. This may be a way for the pathogen to replicate without causing excessive tissue damage that would result in the recruitment of immune cells before a sufficient number of bacteria are present to mount a successful infection.

For bacterial pathogens, infection consists of a series of steps. For *C. pseudotuberculosis*, the bacteria must first cross the skin, generally via wounds or other disruptions in skin integrity, then move from the site of infection to the lymph nodes. This is either preceded or followed by internalization of the bacteria within phagocytic cells. Within the lymph nodes, the bacteria must then undergo cycles of replication, phagocytolysis and reinfection of new phagocytes. The ultimate step is exit from the host, thereby allowing a new cycle of infection to begin. The external environment, blood, lymph nodes and intracellular environment of

macrophages each require adaptations from the bacteria to allow survival, and the scavenging of nutrients required for viability and replication. As is the case with many other major virulence factors expressed by bacterial pathogens, the control of *C. pseudotuberculosis* *pld* expression is regulated in response to a variety of environmental cues, thus allowing successful infection of the host.

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REFERENCES

- Andersen, J. B., Sternberg, C., Poulsen, L. K., Bjorn, S. P., Givskov, M. & Molin, S. (1998). New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64**, 2240–2246.
- Batey, R. G. (1986). Pathogenesis of caseous lymphadenitis in sheep and goats. *Aust Vet J* **63**, 269–272.
- Billington, S. J., Esmay, P. A., Songer, J. G. & Jost, B. H. (2002). Identification and role in virulence of putative iron acquisition genes from *Corynebacterium pseudotuberculosis*. *FEMS Microbiol Lett* **208**, 41–45.
- Burrell, D. H. (1978). Experimental induction of caseous lymphadenitis in sheep by intralymphatic inoculation of *Corynebacterium ovis*. *Res Vet Sci* **24**, 269–276.
- Corish, P. & Tyler-Smith, C. (1999). Attenuation of green fluorescent protein half-life in mammalian cells. *Protein Eng* **12**, 1035–1040.
- Desjardins, M., Celis, J. E., van Meer, G., Dieplinger, H., Jahraus, A., Griffiths, G. & Huber, L. A. (1994). Molecular characterization of phagosomes. *J Biol Chem* **269**, 32194–32200.
- Devaux, P. F. (1991). Static and dynamic lipid asymmetry in cell membranes. *Biochemistry* **30**, 1163–1173.
- Eggleton, D. G., Middleton, H. D., Doidge, C. V. & Minty, D. W. (1991). Immunisation against ovine caseous lymphadenitis: comparison of *Corynebacterium pseudotuberculosis* vaccines with and without bacterial cells. *Aust Vet J* **68**, 317–319.
- Elsinghorst, E. A. (1994). Measurement of invasion by gentamicin resistance. *Methods Enzymol* **236**, 405–420.
- Exton, J. H. (1997). New developments in phospholipase D. *J Biol Chem* **272**, 15579–15582.
- Ezaz-Nikpay, K., Uchino, K., Lerner, R. E. & Verdine, G. L. (1994). Construction of an overproduction vector containing the novel *srp* (sterically repressed) promoter. *Protein Sci* **3**, 132–138.
- Hodgson, A. L., Bird, P. & Nisbet, I. T. (1990). Cloning, nucleotide sequence, and expression in *Escherichia coli* of the phospholipase D gene from *Corynebacterium pseudotuberculosis*. *J Bacteriol* **172**, 1256–1261.
- Hodgson, A. L., Krywult, J., Corner, L. A., Rothel, J. S. & Radford, A. J. (1992). Rational attenuation of *Corynebacterium pseudotuberculosis*: potential cheesy gland vaccine and live delivery vehicle. *Infect Immun* **60**, 2900–2905.
- Hodgson, A. L., Tachedjian, M., Corner, L. A. & Radford, A. J. (1994). Protection of sheep against caseous lymphadenitis by use of a single oral dose of live recombinant *Corynebacterium pseudotuberculosis*. *Infect Immun* **62**, 5275–5280.
- Hodgson, A. L., Carter, K., Tachedjian, M., Krywult, J., Corner, L. A., McColl, M. & Cameron, A. (1999). Efficacy of an ovine caseous lymphadenitis vaccine formulated using a genetically inactive form of the *Corynebacterium pseudotuberculosis* phospholipase D. *Vaccine* **17**, 802–808.
- McKean, S., Davies, J. & Moore, R. (2005). Identification of macrophage induced genes of *Corynebacterium pseudotuberculosis* by differential fluorescence induction. *Microbes Infect* **7**, 1352–1363.
- McKean, S. C., Davies, J. K. & Moore, R. J. (2007). Probing the heat shock response of *Corynebacterium pseudotuberculosis*: the major virulence factor, phospholipase D, is down-regulated at 43 °C. *Res Microbiol* **158**, 279–286.
- McNamara, P. J., Bradley, G. A. & Songer, J. G. (1994). Targeted mutagenesis of the phospholipase D gene results in decreased virulence of *Corynebacterium pseudotuberculosis*. *Mol Microbiol* **12**, 921–930.
- Muckle, C. A. & Gyles, C. L. (1983). Relation of lipid content and exotoxin production to virulence of *Corynebacterium pseudotuberculosis* in mice. *Am J Vet Res* **44**, 1149–1153.
- Nagel, G., Lahrz, A. & Dersch, P. (2001). Environmental control of invasion expression in *Yersinia pseudotuberculosis* is mediated by regulation of RovA, a transcriptional activator of the SlyA/Hor family. *Mol Microbiol* **41**, 1249–1269.
- Paton, M. W., Mercy, A. R., Wilkinson, F. C., Gardner, J. J., Sutherland, S. S. & Ellis, T. M. (1988). The effects of caseous lymphadenitis on wool production and bodyweight in young sheep. *Aust Vet J* **65**, 117–119.
- Pepin, M., Pardon, P., Lantier, F., Marly, J., Levieux, D. & Lamand, M. (1991). Experimental *Corynebacterium pseudotuberculosis* infection in lambs: kinetics of bacterial dissemination and inflammation. *Vet Microbiol* **26**, 381–392.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schwarzer, N., Nost, R., Seybold, J., Parida, S. K., Fuhrmann, O., Krull, M., Schmidt, R., Newton, R., Hippenstiel, S. & other authors (1998). Two distinct phospholipases C of *Listeria monocytogenes* induce ceramide generation, nuclear factor-kappa B activation, and E-selectin expression in human endothelial cells. *J Immunol* **161**, 3010–3018.
- Simmons, C. P., Dunstan, S. J., Tachedjian, M., Krywult, J., Hodgson, A. L. & Strugnelli, R. A. (1998). Vaccine potential of attenuated mutants of *Corynebacterium pseudotuberculosis* in sheep. *Infect Immun* **66**, 474–479.
- Songer, J. G., Hilwig, R. W., Leeming, M. N., Iandolo, J. J. & Libby, S. J. (1991). Transformation of *Corynebacterium pseudotuberculosis* by electroporation. *Am J Vet Res* **52**, 1258–1261.
- Tachedjian, M., Krywult, J., Moore, R. J. & Hodgson, A. L. (1995). Caseous lymphadenitis vaccine development: site-specific inactivation of the *Corynebacterium pseudotuberculosis* phospholipase D gene. *Vaccine* **13**, 1785–1792.
- Wilson, M. J., Brandon, M. R. & Walker, J. (1995). Molecular and biochemical characterization of a protective 40-kilodalton antigen from *Corynebacterium pseudotuberculosis*. *Infect Immun* **63**, 206–211.
- Yozwiak, M. L. & Songer, J. G. (1993). Effect of *Corynebacterium pseudotuberculosis* phospholipase D on viability and chemotactic responses of ovine neutrophils. *Am J Vet Res* **54**, 392–397.

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