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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INTRODUCTION

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.

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...
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 (McKeen 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 + pTR111) (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 RNAzolB (Tel-Test) as recommended by the supplier, with minor modifications. Bacterial pellets containing ~10⁹ cells were resuspended in 1 ml RNAzolB, 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-BeadBuster-8 (Biospec Products) at maximum speed for 3 min. RNA isolation from the homogenate was then performed exactly as per the RNAzolB 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

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*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 Cₚ 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 BamHI restriction site was engineered into primer 75 to allow directional cloning into pSM20. The PCR product was digested with BamHI and ligated into pSM20, cut with EcoRV and BamHI, 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 (Elingshorst, 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 × 10⁶ 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 disrupted 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 were made to use C. pseudotuberculosis phospholipase D namely to eliminate the C. pseudotuberculosis phospholipase D

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were made to monitor the effect of thermoregulation on the expression of \textit{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 \textit{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 OD$_{600}$ 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. \textit{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 \textit{pld} promoter activity at 43°C was observed, at the later time 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, \textit{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 \textit{pld} promoter. The results indicate that \textit{pld} expression may be culture-density related; we therefore further investigated this aspect of \textit{pld} expression. Overnight cultures of C231(pSM27) and the control strain C231(pSM22) were diluted to OD$_{600}$ 0.1 and grown at 37°C for 8 h. pSM22 contains the \textit{srp} promoter (Ezaz-Nikpay et al., 1994), which drives Gfp constitutive expression. Fluorescence of the cultures was measured 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 \textit{pld} promoter activity at 43°C was observed, at the later time 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, \textit{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 \textit{pld} promoter. The results indicate that \textit{pld} expression may be culture-density related; we therefore further investigated this aspect of \textit{pld} expression. Overnight cultures of C231(pSM27) and the control strain C231(pSM22) were diluted to OD$_{600}$ 0.1 and grown at 37°C for 8 h. pSM22 contains the \textit{srp} promoter (Ezaz-Nikpay et al., 1994), which drives Gfp constitutive expression. Fluorescence of the cultures was measured by flow cytometry every hour, and OD 600 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 OD$_{600}$ 0.8, which was indicative of low expression from the \textit{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 \textit{gfp} expressed under control of the constitutive promoter \textit{srp}, was essentially constant until the culture OD$_{600}$ 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.

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

**Fig. 3.** Flow-cytometry analysis of Gfp expression from \textit{C. pseudotuberculosis} harbouring the \textit{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 \textit{pld} promoter activity in \textit{C. pseudotuberculosis}, using the reporter construct pSM27. Duplicate cultures of strain C231(pSM27) were grown at 37°C from OD$_{600}$ 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.
significantly in the early culture period. These results indicate that culture-density-dependent expression of \( \text{pld} \) does indeed occur.

The density-dependent expression of \( \text{pld} \) was further confirmed at the mRNA level by Northern analysis. Cultures of strain C231 were subcultured to OD\(_{600} \) 0.1, then grown at 37 °C. At hourly intervals, the growth of the culture was determined by measuring OD\(_{600} \), and the level of Gfp fluorescence of each culture was determined by flow cytometry. For each sample, 30 000 events were counted, and the mean fluorescence of the population determined to give relative GFP fluorescence. To measure \( \text{pld} \) mRNA levels in cultures of different densities, \( C. \) pseudotuberculosis was grown from OD\(_{600} \) 0.1 for 30 min, 3 h and 6 h (corresponding to mean OD\(_{600} \) 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 \( \text{pld} \) PCR product.

**Down-regulation of \( \text{pld} \) expression by heat shock occurs at all cell densities**

The studies performed to date have shown that \( \text{pld} \) 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 OD\(_{600} \) 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 \( \text{pld} \) mRNA and 16S rRNA were determined by reverse transcription, followed by real-time PCR. As previously demonstrated by Northern analysis, expression of \( \text{pld} \) mRNA increased with increasing cell density (5.6-fold over the time course of the experiment; Fig. 6). Thermoregulation of \( \text{pld} \) 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 \( \text{pld} \) mRNA detected was essentially equal in all heat-shocked samples, which suggests that heat shock reduced the level of \( \text{pld} \) mRNA to a similar extent regardless of cell density.
**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).](image)

**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 \textit{pld} expression by \textit{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 ± 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 \textit{pld} showed that its expression was rapidly reduced at $43^\circ\text{C}$, such that very little \textit{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 \textit{pld} promoter and rapid degradation of the \textit{pld} mRNA already present in the cell. Thus, it appears that, as for other bacterial mRNA species, the half-life of \textit{pld} mRNA is relatively short. The maximal repression of \textit{pld} expression was maintained for 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 \textit{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 \textit{pld} expression, a reporter construct was generated by inserting the \textit{pld} promoter into pSM20. pSM20 is a \textit{C. pseudotuberculosis} promoter probe vector that contains a \textit{gfp} gene as a reporter, and has been used to identify regulated genes of \textit{C. pseudotuberculosis} by differential fluorescence induction (McKean et al., 2005). Attempts to monitor the effects of increased temperature on transcription from the \textit{pld} promoter using strain C231(pSM27) were unsuccessful, in that the large decrease in \textit{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 \textit{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 \textit{pld} expression using the reporter construct pSM27, it was possible to demonstrate a density-dependent regulation of \textit{pld} expression. This was visualized using flow cytometry, as a decrease in detectable Gfp until OD$_{600}$~0.8 was reached, followed by a rapid increase. This phenomenon was confirmed by Northern analysis, which showed that \textit{pld} expression gradually increased with increasing cell density.

Expression of \textit{pld} at all investigated cell densities could be down-regulated by heat shock. This indicates that the mechanism of \textit{pld} thermoregulation is able to override the density-dependent increase in \textit{pld} expression. At all densities tested, heat shock at $43^\circ\text{C}$ was able to reduce \textit{pld} expression to a similar basal level. Interestingly, the experiment showed that even in a low-density culture, in which Gfp protein stability can obscure the regulation that is occurring.

The mechanisms by which expression from the \textit{pld} promoter is controlled remain to be elucidated. The observation that \textit{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 \textit{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 phagocytosis. 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, phagocytosis 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**


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