Role of the Streptococcus agalactiae ClpP serine protease in heat-induced stress defence and growth arrest

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The main causes of microbial death after heat exposure are not well understood. Here, it is shown that the heat-shock protein ClpP plays a major role in heat-induced growth arrest in Streptococcus agalactiae. A mutant lacking the ClpP protease was more sensitive to the inhibitory effects of heat, salt and oxidative stress than the isogenic wild-type strain. During growth arrest, this mutant displayed important modifications of its total protein content, including a decreased level of essential metabolic enzymes such as the alcohol dehydrogenase. Analysis of protein carbonylation demonstrated that the ClpP protease plays a role in preventing accelerated protein oxidation. Higher levels of oxidized DnaK, a key modulator of the heat-shock regulon, were observed in the ClpP mutant and these were increased following heat shock. Accumulation of oxidized/inactivated DnaK might explain why the ClpP mutant was unable to properly synthesize DNA and proteins, and why it exhibited an aberrant cell morphology. Even though ClpP plays a minor role in the virulence of S. agalactiae in a murine infection model, the data presented here point to the importance of ClpP in oxidative stress defence in preventing heat-induced cell alterations.

INTRODUCTION

The heat-shock response involves the induction of a set of heat-shock proteins (HSPs) and chaperone regulators of proteolysis that allow the cell to adapt to cellular damage caused by heat, chemical or oxidative stress. The spectrum of HSPs and chaperones synthesized in different organisms after a stress challenge displays notable similarities (Morimoto, 1998; Morimoto et al., 1994). The Clp/HSP100 family of molecular chaperones constitutes a large family of highly conserved and universal proteins that are found in eukaryotic and prokaryotic cells (Squires & Squires, 1992). The Clp ATPases act as molecular chaperones and are involved in the folding, assembly and proteolysis of proteins (Schirmer et al., 1996). Four families of ATP-dependent proteases have been characterized in bacteria, namely Clp (ClpAP, CP, XP), HslUV (ClpQY), AAA (PstH) and the Lon family (Lon). The proteolytic activity of these enzymes resides in the ClpP and ClpQ subunits, while substrate recognition and unfolding is attributed to the ATPase subunits. The ClpX, ClpC and ClpA ATPases are involved in the regulation of ATP-dependent proteolysis by targeting specific proteins for degradation in association with the ClpP serine protease (Gottesman et al., 1998; Maurizi et al., 1990; Woo et al., 1989; Zhou et al., 2001). The ClpXP protease complex of Caulobacter crescentus is essential for growth, survival and normal cell division, and the loss of ClpP or ClpX results in cell-cycle arrest (Jenal & Fuchs, 1998). Previously, we have identified the Clp/HSP100 stress proteins as playing an important role in the adaptation to environmental conditions as well as in the virulence of the intracellular pathogen Listeria monocytogenes (Gaillot et al., 2000; Nair et al., 2000a; Rouquette et al., 1998). In this bacterium, ClpC and ClpE act synergistically in controlling cell division and ClpP acts as a serine protease in the degradation of misfolded proteins.

Group B Streptococcus (GBS), also known as Streptococcus agalactiae, is part of the normal human microflora colonizing the respiratory, gastrointestinal and urogenital tracts. This extracellular bacterium is one of the leading causes of invasive infections, bacterial sepsis and meningitis in neonates (Schuchat, 1998). The physiopathology of GBS infections implies that this bacterium can adapt rapidly to various growth conditions, including pH, osmolarity and temperature variations (Tamura et al., 1994). In this study, we investigated the contribution of oxidative stress due to

Abbreviations: GBS, Group B Streptococcus (Streptococcus agalactiae); PI, propidium iodide.

The GenBank accession number for the Streptococcus agalactiae clpP gene sequence reported in this paper is AJ413168.
heat exposure in GBS. We identified the clpP gene of *S. agalactiae* and showed that its product, the serine protease ClpP, is involved in the regulation of growth at high temperatures and survival under stress conditions. During heat shock, an *S. agalactiae* ΔclpP mutant was growth-arrested and displayed important modifications of its total protein content, including a decreased level of essential metabolic enzymes such as the alcohol dehydrogenase. Under these conditions, ClpP also contributed to cell division and septum formation. Finally, we demonstrated that in the absence of the ClpP protease, the level of carbonylated DnaK was increased. Our results suggest that, during heat shock, GBS ClpP might play an important role in the synthesis of functional proteins in the cell.

**METHODS**

**Bacterial strains, plasmids, media and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *S. agalactiae* NEM316 was responsible for a fatal septicaemia and belongs to the capsular serotype III (Gaillot et al., 1997). *Escherichia coli* DH5α was used for cloning experiments. *S. agalactiae* and *E. coli* were cultured in brain–heart infusion (BHI) broth or on BHI agar (Difco Laboratories) at 37°C. Unless specified otherwise, antibiotics were used at the following concentrations: for *E. coli*, 100 μg ampicillin ml⁻¹, 150 μg erythromycin ml⁻¹, 50 μg kanamycin ml⁻¹ and 60 μg spectinomycin ml⁻¹; for *S. agalactiae*, 10 μg erythromycin ml⁻¹, 1000 μg kanamycin ml⁻¹ and 250 μg spectinomycin ml⁻¹. *S. agalactiae* liquid cultures were grown in standing, filled flasks. Stress conditions at 37°C in the presence or absence of 1 M NaCl or 0.5 mM H₂O₂ were as described previously (Nair et al., 1999).

**Genetic techniques and DNA manipulations.** Recombinant plasmid DNAs were introduced into *E. coli* by transformation (Sambrook et al., 1989). Electrocompetent cells of *S. agalactiae* were prepared as described by Cruz-Rodz & Gilmore (1990). IncP mobilizable shuttle vectors (pTCV-int, pAT113/Sp and their derivatives) were transferred by conjugation from the mobilizing donor strain *E. coli* HB101(pRK24) to *S. agalactiae* recipients (Poyart & Trieu-Cué, 1997). Plasmid DNA from *E. coli* (Sambrook et al., 1989) and total DNA from *S. agalactiae* (Poyart-Salmeron et al., 1992) were extracted as described.

**Cloning and sequencing of ClpP.** Based on the alignment of the ClpP amino acid sequences of *Streptococcus pyogenes*, *Streptococcus salivarius*, *Streptococcus mutans* and *Streptococcus pneumoniae*, we designed two degenerate oligonucleotide primers, designated P1 and P2. These primers were used to amplify a 573 bp DNA fragment internal to the streptococcal clpP gene. PCR was carried out in a final volume of 50 μl containing 50 ng *S. agalactiae* DNA, 0.1 mM each primer, 200 μM each dNTP and 2 U AmpliTag Gold DNA polymerase (Applied Biosystems) in 1× amplification buffer. DNA was sequenced with an ABI model 310 automated DNA sequencer using the ABI PRISM dye terminator cycle sequencing kit (Applied Biosystems). Sequence analysis of the cloned PCR fragment revealed that it was highly related to known *clpP* genes present in the databases (data not shown). Southern blot analysis of *S. agalactiae* NEM316 chromosomal DNA using the cloned PCR fragment as a

**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant properties</th>
<th>Reference/origin</th>
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<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>recA1 gyrA (Nal) Δ(lacZYA–argF) [Δ80Alac(lacZ)M15]</td>
<td>Gibco-BRL</td>
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<td><em>E. coli</em> HB101</td>
<td>F− hsd-20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 (Str) xyl-5 mtl-1 supE44</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
</tr>
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<td><em>S. agalactiae</em> NEM316</td>
<td>Serotype III isolated from neonate blood culture</td>
<td>Gaillot et al. (1997)</td>
</tr>
<tr>
<td><em>S. agalactiae</em> NEM1968</td>
<td>NEM316 clpPΔaphA+3, Km (ΔclpP)</td>
<td>This work</td>
</tr>
<tr>
<td><em>S. agalactiae</em> NEM1969</td>
<td>NEM1969::pAT113/SpΔclpP, Km, Sp; ClpP⁻/ClpP⁺ (ΔclpPc)</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
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<td>pUC18</td>
<td>Ap; ColE1 replicon, MCS LacZ⁺</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pAT113/Sp</td>
<td>Sp, Mob⁺ (IncP); oriR pACYC184, attTn1545, LacZ⁺, MCS pUC19</td>
<td>Celli &amp; Trieu-Cué (1998)</td>
</tr>
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<td>pAT113/SpΔclpP</td>
<td>Sp, Mob⁺ (IncP); a pAT113 derivative with a 1·3 kb fragment carrying <em>clpP</em> from NEM316</td>
<td>This work</td>
</tr>
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<td>pTCV-int</td>
<td>Em, Km, Mob⁺ (IncP); a pTCV-erm derivative with a 1·8 kb EcoRI–PstI fragment carrying pMMB57pMMB57</td>
<td>Poyart et al. (2001a)</td>
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<td>pG⁺host5</td>
<td>Em; ColE1 replicon, thermosensitive derivative of pGK12, MCS pBluescript</td>
<td>Biswas et al. (1993)</td>
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*Bacteria were grown in BHI broth or on BHI agar.

†Ap, ampicillin resistance; Cm, chloramphenicol resistance; Em, erythromycin resistance; Km, kanamycin resistance; Nal, nalidixic acid resistance; Sp, spectinomycin resistance; Str, streptomycin resistance; Tc, tetracycline resistance; attTn, transposon-attachment site; MCS, multiple-cloning site.
probe revealed that the clpP gene was located on a 1500 bp SspI DNA fragment (data not shown). Therefore, the remaining portion of the clpP gene was obtained by inverse PCR of chromosomal DNA digested with SspI. Self-ligated DNA was used as a template in PCRs carried out with primers P3 and P4. The resulting 950 bp PCR fragment was cloned into pUC18 and sequenced using custom-synthesized oligonucleotides. Assembly of the sequences of the DNA fragments amplified with the primer pairs P1/P2 and P3/P4 yielded a 1458 bp SspI DNA fragment containing clpP. To verify that we had correctly assembled the PCR fragments, primers P5 and P6 were used to amplify a 1316 bp fragment from S. agalactiae NEM316 chromosomal DNA; three clones were sequenced in their entirety on both strands.

Construction of an allelic GBS ClpP mutant. To construct the S. agalactiae ΔclpP strain, we inserted a promoterless and terminatorless kanamycin-resistance cassette, aphA-3, within DNA segments internal to clpP. This was done by ligating, after digestion with the appropriate enzymes, the ampiclons P7–P8, KanK–KanB and P9–P10. The corresponding EcoRI–PstI fragments were cloned into pGEMH5; the resulting recombinant vector, pSN600, was introduced into S. agalactiae NEM316 by electroporation. The double-crossover events leading to the expected gene replacements were screened and obtained as described previously (Biswas et al., 1993; Rouquette et al., 1998). In the resulting S. agalactiae mutant NEM1968 (ΔclpP), the promoterless kanamycin-resistance cassette used to inactivate clpP is likely to be transcribed from Pclp. Southern analysis of restriction-enzyme-digested chromosomal DNA revealed that the mutant strain was devoid of sequences related to P+ host5 and that insertion of the resistance cassette had occurred at the expected location (data not shown).

For complementation analysis, we used the following strategy. Primer pair P5/P6 was used to amplify clpP together with its upstream promoter region (409 bp), Pclp. The corresponding 1316 bp fragment was cloned into pAT113/Sp to give pAT113/Spclp. This vector was conjunctively transferred (Poyart et al., 2001b) from HB101/pPK24 to S. agalactiae ΔclpP/pTK-cTV-into to restore the ClpP activity in this mutant strain. The plasmid insertion site was characterized in three integrants harbouring a single copy of pAT113/Spclp inserted at different loci. This was done by inverted PCR as described previously (Poyart et al., 2001b). The complemented strain NEM1969 (ΔclpPc) was chosen for further studies because in this strain pAT113/Spclp was not inserted within a protein-coding sequence (data not shown).

RNA preparations and Northern blot analysis. Total RNAs were extracted as described previously (Gailloit et al., 1997) from exponential phase (OD600 0.6) cultures of S. agalactiae grown in BHI broth at 37 or 41 °C without agitation. For Northern blot analysis, 40 µg RNA were separated through a 1-3% formaldehyde/agarose gel (Sambrook et al., 1989) and transferred to a Hybond- N+ membrane (Amersham). The filters were baked for 2 h at 80 °C in an oven. Pre-hybridization and hybridization were performed under stringent conditions as described by Sambrook et al. (1989). The DNA probe used was a PCR fragment obtained from NEM316 genomic DNA by using primers P1 and P2. DNA fragments were labelled with [α-32P]dCTP by using the Nick Translation Kit (Amersham).

Flow cytometry analysis. GBS strains were cultivated in BHI broth, at either 37 or 41 °C, and collected during the exponential and stationary phases of growth. Samples were fixed by the addition of cold ethanol to a final concentration of 70% and stored for up to one week at 4 °C in ethanol. When appropriate, fixed cultures were centrifuged and resuspended in PBS (10 mM potassium phosphate; 150 mM sodium chloride; pH 7.0). Dilutions were mixed with 10 µM propidium iodide (PI) and cells were analysed on a Becton Dickinson Calibur System. Excitation was at 485 nm and fluorescence was measured at 495 nm. Flow cytometry data were collected and analysed using CELLQUEST software (Becton Dickinson).

Carbonylation assays. Exponential and stationary phase cultures (20 ml) were pelleted and crude protein extracts were prepared using the Fastprep Bio101 machine and kit (Ozyme). The carbonyl groups in the protein side chains were derivatized, using the OxyBlot kit (Oncor), to 2,4-dinitrophenylhydrazine (DNP-hydrazine) by reaction with 2,4-dinitrophenylhydrazine as described by Dukan & Nystrom (1998). The DNP-derivatized crude protein extracts were separated by SDS-PAGE and subsequently transferred to PVDF membranes by using a semi-dry blotting system. The filters were incubated with primary antibody, specific to the DNP moiety of the proteins, and subsequently incubated with a secondary (goat-anti-rabbit) horseradish peroxidase–antibody conjugate directed against the primary antibody. For detection, the filters were treated with the ECL + chemiluminescence blotting substrate (Amersham Pharmacia Biotech).

Protein analysis. Protein extracts were prepared from cultures of the wild-type, ΔclpP and ΔclpPc strains grown at 37 or 41 °C using the Fastprep kit (Ozyme) and the BIO 101 machine. The concentration of protein in each soluble extract was measured using the Bradford assay and equal amounts of protein were loaded into each lane. SDS-PAGE and Western blot analyses were done as described previously (Nair et al., 2000a). Polyclonal antibodies raised against pneumococcal Dnak (Kim et al., 1998) were diluted 1 : 1000 in PBS. Comparative two-dimensional gel analysis of proteins was performed in a Protean II xi 2D-cell apparatus (Bio-Rad). IEF was prepared with ampholines covering pH 3–10. The Panvera Protease Activity Detection kit was used to calculate overall protease activity. This kit uses an FTC-labelled casein as a substrate, which decreases in size as a result of degradation causing a change in spectrophotometric absorbance and fluorescence. Experiments were repeated at least three times on different protein extracts.

Protein sequencing was done by Edman degradation at the Protein Sequencing Laboratory, Pasteur Institute.

Electron microscopy. Exponential or stationary phase growing bacteria (37 and 41 °C) were processed for thin-sectioning and examined under the electron microscope as described by Frehel & Leduc (1987).

Mouse virulence assays. Six- to eight-week-old pathogen-free ICR female Swiss mice (Janvier, Le Geneset St Isle) were used in this study. Groups of 10 mice were inoculated intravenously with appropriate antibiotic(s) as described previously (Poyart et al., 2001b). Mice were killed by cervical dislocation in accordance with the policies of the Animal Welfare Committee of the Faculte Necker (Paris), and the experiment was performed twice.

Oligonucleotides. The sequences (5’ to 3’) of the relevant oligonucleotides used in this study were: P1, ATGATCTCTTGATGTTGATTTGAACAAAC; P2, CCATATTACAATGATGATTGACT; P3, CATGAAGCCGTCACCAAGCAG; P4, CACCTTGATATTGGCTCCATCG; P5, GTTACCCTGAAGTATGGATACAAG; P6, CCTGATCATAATGATATTGACT; P7, ATGATTCTGTGTTATATTGAACAAACAAAAG; P8, AGCGGTACCCCGTACGTCAGTCGCTG.
GTGGATCCATGAACCTTTATATGCCGACG; P10, ATGCTGCA-GTGGATGAGCCATAATCAAGTG. The sequences of the restriction sites added for molecular cloning are shown in bold.

RESULTS

Sequence analysis of clpP from S. agalactiae NEM316

Degenerate oligonucleotide primers (P1/P2; see Methods) and inverse PCR were used to clone a 1458 bp fragment from S. agalactiae NEM316 (GenBank accession no. AJ413168). Structural analysis of this fragment revealed that it contained three ORFs with the same polarity of transcription (Fig. 1a). orf1 was truncated during molecular cloning and the corresponding 96 aa polypeptide exhibited significant homology (75% identity) with the uracil phosphoribosyltransferase of Bacillus subtilis. The second ORF encoded a 196 aa protein that shared homology with the ClpP proteins of S. mutans (88-8% identity), S. pyogenes (89-3%), S. pneumoniae (91-8%), S. salivarius (91-8%), B. subtilis (55-8%), L. monocytogenes (61%) and E. coli (53-65%). Thus, we concluded that this second ORF encoded the S. agalactiae ClpP. In addition, all these proteins contained the amino acid triad S–H–D constituting the catalytic site of the ClpP serine proteases (Wang et al., 1997). The 306 bp long orf3 was located 40 bp downstream from clpP. This ORF had no obvious translational signals and the corresponding putative protein displayed no significant homology with proteins of known function present in the databases. We therefore assumed that orf3 is not a protein-coding sequence. A palindromic sequence forming a possible stem–loop transcriptional terminator (DG at 37°C = -212.6 kcal mol⁻¹) was detected 17 bp downstream from orf3, which suggested that clpP is transcribed as a monocistronic mRNA.

Transcriptional and translational signals characteristic of Gram-positive bacteria were detected upstream of the clpP start codon (Fig. 1b). In particular, a nearly canonical σA-type -35 and -10 promoter recognition sequence (TTGACC-X₁₇-TATAAT) was detected 32 bp upstream from clpP, which is likely to constitute the promoter of this gene (PclpP). The transcription of clpP from NEM316 was studied in bacteria cultivated at 37 and 41°C in BHI broth. A single transcript of approximately 1 kb in size was detected in bacteria cultivated at 37°C (lane 1) and 41°C (lane 2). RNAs were extracted from exponential phase (OD₆₀₀ 0.5) cultures. Forty micrograms of RNA were applied per lane. The DNA probe used was a 600 bp fragment corresponding to the NEM316 clpP gene obtained by PCR using primers P1 and P2.
detected with the P1–P2 DNA probe and the intensity of the signal was higher with the RNA extracted from the culture grown at 41 °C than from that grown at 37 °C (Fig. 1c). In contrast, following de-hybridization and re-hybridization with a probe corresponding to NEM316 16S rDNA, the resulting signals were similar in each lane (data not shown). We therefore concluded that the transcription of NEM316 clpP is heat-induced. The clpC, clpP and clpE genes of B. subtilis (Derre et al., 1999), L. monocytogenes (Nair et al., 2000b) and S. pneumoniae (Chastanet et al., 2001) are negatively controlled by CtsR, the class III stress repressor. CtsR binds to a recognition sequence (RGTCAAANANRGTCAAA) which often overlaps the −35 and −10 sequences. Interestingly, we identified a putative CtsR box, a heptameric direct repeat (GGTCAAcAAAGGTCAAA), that overlaps the −35 sequence of PclpP and whose orientation is opposite to that indicated by the arrows in Fig. 1(b), as is also the case in B. subtilis. This suggests that the transcription of the S. agalactiae clpP gene is also regulated by a CtsR-like protein, a hypothesis that remains to be proven. The 1 kb hybridization signal in Fig. 1(c) is consistent with a clpP transcript initiated at the promoter indicated in Fig. 1(b) and ending at the hairpin loop depicted in Fig. 1(a).

ClpP is required for growth under different stress conditions

The ClpP proteins of various prokaryotes, including B. subtilis (Gerth et al., 1998) and the facultative intracellular pathogen L. monocytogenes (Gaillot et al., 2000), have been shown to play an essential role in stress tolerance. To determine the role of ClpP in GBS stress tolerance, we constructed an S. agalactiae clpP strain lacking a functional ClpP (ΔclpP) and an S. agalactiae ΔclpP complemented strain (ΔclpPc) (see Methods). Stress tolerances of the ΔclpP and ΔclpPc strains were compared to those of the wild-type. At 37 °C, the growth rate of ΔclpP was reduced when compared to that of the wild-type and ΔclpPc strains (Fig. 2a); in the presence of NaCl or H2O2, the growth rate of the ΔclpP strain was severely impaired (Fig. 2c, d). During heat shock at 41 °C, the mutant grew very slowly and after reaching an OD600 value of approximately 0-4 stopped growing, even after overnight incubation (Fig. 2b). Bacterial growth was fully restored in the complemented strain. Taken together, these results indicate that ClpP is required for growth of S. agalactiae under heat-shock, salt- and oxidative-stress conditions. However, in the presence of ethanol, which is supposed to induce a heat-shock response (Bochner et al., 1984), we observed that growth of the mutant was not as affected by this stress as compared to growth in the presence of NaCl or H2O2 (data not shown).

Role of ClpP in cell division and morphology

Since the ΔclpP strain struggled to grow under the different stress conditions, we checked whether it displayed stress-related morphological changes. The wild-type, ΔclpP and ΔclpPc strains were examined by electron microscopy during the exponential and stationary phases of growth when cultivated at 37 or 41 °C in BHI broth. Thin-section electron microscopy of the wild-type and ΔclpPc strains showed new septal planes formed parallel to the cell division plane and occurring at the mid-point of the elongating cells (Fig. 3a shows part of this analysis). This typical GBS morphology was observed with both strains whatever the growth temperature tested and during the different phases of growth. When cultivated at 37 °C, the ΔclpP mutant exhibited no relevant morphological changes (data not shown). In contrast, during exponential, and even more pronounced during stationary phase at 41 °C, the ΔclpP strain displayed an aberrant morphology due to the presence of large multiseptated cells containing non-parallel septa (Fig. 3b). In addition, while the wild-type and ΔclpPc strains were surrounded by a thin and heavily stained cell wall, the ΔclpP strain exhibited a thick and lightly stained cell wall. Surprisingly, these pleomorphic cells were only observed during growth of the mutant at 41 °C but not during the

Fig. 2. Growth curves of S. agalactiae NEM316 (wild-type, ■), NEM1968 (ΔclpP, ○) and NEM1969 (ΔclpPc, ◇) at (a) 37°C or (b) 41°C, and at 37°C in the presence of (c) 1 M NaCl or (d) 0-5 mM H2O2. Overnight cultures of bacterial strains grown at 37°C were diluted in BHI broth to an OD600 value of 0-01 and their growth was monitored over a period of 6 h. The vertical arrow in (c) and (d) indicates the time at which the salt or oxidative stress was applied.
other stress conditions tested in this study (NaCl or H₂O₂). Thus, during heat shock, lack of ClpP in GBS results in poor survival and in dramatic morphological alterations.

Flow cytometry has been used previously to identify temperature-sensitive mutants that are blocked at specific points in the cell cycle with respect to the replicative status of the chromosome (Winzeler & Shapiro, 1995). We observed that the ΔclpP strain was temperature-sensitive, did not separate, had multiple septa and appeared to be blocked in its cell cycle and cell division. We thus tested whether depleting cells for ClpP would result in an alteration of DNA content and block DNA replication under stress conditions. Flow cytometry was used to screen the different strains grown at 41 °C (OD₆₀₀ ~ 0.4, as shown in Fig. 2). The ΔclpP mutant strain displayed important morphological alterations (irregular cell division, abnormal cell-wall structure and increased size in the shape of ‘larvae’).

**Fig. 3.** Electron microscopy of (a) *S. agalactiae* NEM316 (wild-type) and (b) *S. agalactiae* NEM1968 (ΔclpP) during growth at 41 °C (OD₆₀₀ ~ 0.4, as shown in Fig. 2). The ΔclpP mutant strain displayed important morphological alterations (irregular cell division, abnormal cell-wall structure and increased size in the shape of ‘larvae’).

DNA content (PI) (Fig. 4). At 37 °C, ΔclpP and ΔclpPc strains had similar DNA contents, albeit slightly higher than the wild-type. However, during heat shock, mutant cells displayed a dramatic increase in their DNA content, probably explaining the defects in morphology observed at this restrictive temperature (Fig. 4). This effect might be caused by any number of factors, including a failure to complete DNA replication and/or cell division. Thus, the results of the fluorescence-activated cell sorting (FACS) study support a role for ClpP in maintaining homeostasis within the cell with respect to proteins involved in the control of DNA synthesis and/or cell division. It is noteworthy that similar results were obtained for temperature-sensitive mutants of *Caulobacter* that were blocked in their cell cycle (Winzeler & Shapiro, 1995).

**ClpP is required for synthesis of functional proteins during heat shock**

Since new protein synthesis is required to initiate new rounds of DNA replication (Newton, 1972), we compared the protein profiles of the strains grown at 41 °C. Interestingly, SDS-PAGE analysis profiles of crude extracts revealed that several proteins were missing or present in a lower quantity in the ΔclpP mutant as compared to the wild-type or complemented strains, even though the same quantity of total protein extract was loaded on the gels for each strain as determined by the Bradford assay (Fig. 5a). In addition, smearing was observed for proteins of less than 43 kDa in size in the mutant strain. We interpreted these results as indicating that the protein extracts of the ΔclpP mutant probably contained more truncated proteins and/or low-molecular-mass polypeptides that could not be visualized as individual bands by SDS-PAGE. The presence of smeared low-molecular-mass peptides could explain why there were apparently less proteins in the ΔclpP extracts as compared with the wild-type strain, even though the same amount of proteins were loaded onto the gel, as quantified by the Bradford assay. When we extracted proteins from the same amount of cells, we still observed this difference in the protein patterns (data not shown). We measured total protease activity in crude extracts of the wild-type, ΔclpP and ΔclpPc strains grown at 41 °C. A significant decrease in overall protease activity was observed for the mutant (66 ± 8% versus 100 ± 12%). Thus, it is conceivable that the smeared proteins observed in the ΔclpP strain grown at 41 °C (Fig. 5) represent incomplete proteins made from truncated mRNAs that cannot be degraded rather than the degradation products of fully synthesized but unfolded proteins.

To obtain a further insight into the differences in protein profiles, we analysed the extracts of the wild-type and mutant strains by two-dimensional gel electrophoresis (Fig. 5b). This analysis confirmed that many proteins were either absent or present at lower levels in the ΔclpP mutant. Two proteins that were missing in the mutant were unambiguously identified as the 21.5 kDa GBS ClpP protein (MIPVVEQTSR) and the alcohol dehydrogenase protein (MKAVVQNQAS) of *S. agalactiae* (Glaser et al., 2002). The
Fig. 4. Flow cytometry analysis of *S. agalactiae* NEM316 (wild-type), NEM1969 (ΔclpPc) and NEM1968 (ΔclpP) grown at 37 °C (upper graphs) and 41 °C (lower graphs). Cell sample dilutions were mixed with PI and analysed by fluorescence-activated cell sorting (FACS). The relative cell count versus the DNA content (PI) is shown in the last graph. At 37 °C, wild-type and ΔclpPc cells (feint-black and dotted lines, respectively) have similar DNA contents compared to the ΔclpP cells (heavy-black line). In contrast, at 41 °C the ΔclpP mutant cells have a higher DNA content as compared to the wild-type and complemented strains. Experiments were repeated twice on different culture samples to confirm reproducibility; representative results are shown.

Fig. 5. Coomassie-blue-stained SDS-PAGE (a) and two-dimensional gel electrophoresis (b) of proteins extracted from *S. agalactiae* NEM316 (wild-type, lane 1), NEM1969 (ΔclpPc, lane 2) and NEM1968 (ΔclpP, lane 3). Proteins were extracted from bacteria cultivated in BHI broth at 41 °C and collected after 5 h growth as shown in Fig. 2. (a) The small arrowheads (inset) indicate the position of the approximately 40 kDa alcohol dehydrogenase that was barely detectable in the ΔclpP mutant. (b) Spots 1 and 2 correspond to ClpP and the alcohol dehydrogenase, respectively, which appear to be absent in the mutant. For each sample, equal amounts of protein were loaded onto the gels. Protein sequencing was repeated twice on two different protein extracts to confirm reproducibility.
latter protein was also sequenced following purification from the SDS-polyacrylamide gel (Fig. 5a).

**Lack of ClpP protease induces DnaK protein carbonylation**

As shown previously, the growth of the ΔclpP mutant was blocked during heat shock. In *E. coli* and *Saccharomyces cerevisiae*, oxidative stress is involved in heat-induced cell death (Davidson *et al.*, 1996; Dukan & Nystrom, 1998, 1999). Therefore, we looked at the levels of oxidized proteins during heat shock and growth arrest of the ΔclpP mutant as compared to the wild-type and complemented strains. This was done by using an immunochemical assay to detect protein carbonylation groups (Tamarin *et al.*, 1998) in crude protein extracts obtained after overnight growth at 37 or 41°C. This analysis revealed a similar low level of protein carbonylation in the wild-type and complemented strains grown at both temperatures (Fig. 6a, lanes 1–1' and 2–2'). In contrast, higher levels of carbonylated proteins were detected in the clpP mutant and the patterns obtained clearly indicated that, in this strain, carbonylation is heat-induced (Fig. 6a, lanes 3–3'). Using SDS-PAGE, we further analysed the pattern of carbonylation of proteins extracted from the wild-type and mutant strains grown at 41°C. Three carbonylated protein bands were detected in all strains (Fig. 6b). This is in contrast to the many carbonylated proteins observed in *E. coli* by Dunkan & Nystrom (1998).

We took advantage of this situation to sequence carbonylated proteins directly off one-dimensional gels and in all cases, HPLC analysis of Edman degradation products clearly revealed single peaks corresponding to a single protein. Two of these bands were identified as DnaK (XKIIGIDLGTNNSAV) and glyceraldehyde-3-phosphate dehydrogenase (VVKVGINGFGRRGFLFRRIQNV) (Glaser *et al.*, 2002). It is worth noting that, at 41°C, the levels of oxidized DnaK were higher in the mutant than in the wild-type strain, indicating that in the absence of the ClpP protease there is an accumulation of oxidized DnaK (Fig. 6b). This result cannot be explained by an increased production of DnaK since a Western blot analysis demonstrated that the amount of this protein was very similar in the wild-type and mutant background (Fig. 6c).

**ClpP and GBS virulence**

We studied the role of ClpP in the virulence of *Streptococcus agalactiae* by intravenously infecting Swiss mice with the wild-type and ΔclpP mutant strains. By following the mortality after inoculation with increasing doses of bacteria, we found no significant difference between the LD50 of these strains (approx. 6×105 bacteria). We then followed, over a period of 4 days, the bacterial survival of these strains in the blood and organs (spleen, liver and brain) of mice infected with a sublethal dose of bacteria (106) (Fig. 7). Up to day 4 of infection, *S. agalactiae* wild-type and mutant strains persisted in the blood at levels of 3×104 c.f.u. ml⁻¹ and 1×103 c.f.u. ml⁻¹, respectively. Both strains were eliminated similarly from the brain to a level of about 1×102 c.f.u. ml⁻¹ by day 4 of infection. During the course of infection, the mutant strain was cleared slightly more rapidly than the wild-type strain from the spleen and liver. After 4 days infection, the levels of the wild-type and mutant strains were 5×103 and 1×103 c.f.u. ml⁻¹, respectively, in the spleen and 2×103 and 103 c.f.u. ml⁻¹, respectively, in the liver (Fig. 7). However, the differences between the bacterial counts of the wild-type versus mutant strain were always less than one order of magnitude. Thus, we consider these results to indicate that the absence of ClpP weakly attenuated the virulence of *S. agalactiae*.

**DISCUSSION**

In this work, we identified the clpP gene of *S. agalactiae* and showed that its product, the serine protease ClpP, is involved in the regulation of growth at high temperatures and survival under stress conditions, including heat shock, as already shown in the case of *B. subtilis* (Gerth *et al.*, 1998), *L. monocytogenes* (Gaillot *et al.*, 2000) and *S. pneumoniae* (Chastanet *et al.*, 2001). Consistently, in all these bacteria, the synthesis of ClpP is induced under stress conditions including heat shock (Chastanet *et al.*, 2001; Derre *et al.*, 1999; Gaillot *et al.*, 2001). The role of ClpP in regulated proteolysis and degradation has been clearly demonstrated in *E. coli* (Gottesman *et al.*, 1998; Maurizi *et al.*, 1990; Woo...
et al., 1989; Zhou et al., 2001). In this bacterium, the ClpP and Lon proteases account for up to 80% of protein degradation (Gottesman, 1996). In the present study, the fact that at 41 °C the overall protease activity of the $\Delta$clpP mutant was significantly lower than that of the wild-type GBS strain suggests that ClpP constitutes an essential heat-induced protease.

During heat shock, an S. agalactiae $\Delta$clpP mutant was growth-arrested and displayed important modifications to its protein content, which included a decreased level of essential metabolic enzymes such as the alcohol dehydrogenase. Pyruvate can be converted to ethanol via acetaldehyde, a reaction requiring alcohol dehydrogenase and generating NAD$^+$. If NAD$^+$ were not regenerated, glycolysis could not proceed beyond glyceraldehyde 3-phosphate, which means that no ATP would be generated (Lehninger, 1982; Lodish et al., 2000). Thus, the absence or availability of decreased levels of metabolic enzymes, such as the alcohol dehydrogenase, may be responsible for the growth arrest of the $\Delta$clpP mutant observed at 41 °C. As a major effect, heat shock may cause cellular oxidation and many of the heat-shock proteins may function to protect against oxidation (Bochner et al., 1984). Interestingly, we showed that there is an accumulation of oxidized GBS DnaK in the absence of ClpP. This might indicate that DnaK is a preferred target for oxidation and/or that oxidized DnaK is a preferred substrate for ClpP. Oxidized proteins lose their structural integrity and catalytic activity, and the levels of oxidized proteins increase exponentially with age (Stadtman, 1992). Mutations inactivating the genes encoding the chaperone proteins GroESL and DnaK are known to have pleiotropic effects on host metabolism, including defects in DNA and RNA synthesis, proteolysis and cell division. E. coli cells lacking DnaK die rapidly during stasis and fail to develop resistance to heat and oxidation (Georgopoulos et al., 1994). In particular, DnaK is important in the ‘resurrection’ of the activity of heat-inactivated RNA polymerase (Georgopoulos et al., 1994).

These findings are consistent with our proposal that, at 41 °C, the $\Delta$clpP GBS mutant synthesizes numerous aberrant and prematurely terminated peptides made from truncated mRNAs probably due to oxidized/inactive DnaK. However, the lack of ClpP, which, together with ClpX and ClpC, has to fulfill general quality control functions and possible regulatory functions, could also be the more-direct cause of these phenotypes. It has recently been shown that the S. pneumoniae $\Delta$clpP mutant is also sensitive to H$_2$O$_2$ (Robertson et al., 2002). In addition, micro-array analysis of this mutant showed regulatory phenotypes that included downregulation of the oxidative-stress response. Thus, the phenotypes we observed in the S. agalactiae $\Delta$clpP mutants were probably due to pleiotropic regulatory effects caused by the lack of ClpP.

The role of chaperone proteins in the cell division cycle is unknown, yet many of the division components are peripheral or integral inner-membrane proteins. Defects in cell division as a result of mutations in the clp genes have also been observed in B. subtilis and L. monocytogenes, suggesting that proteins involved in cell morphology or cell division are controlled either directly or indirectly by the ClpXP protease complexes (Gerth et al., 1998; Nair et al., 1999). In C. crescentus, ClpXP is required in vivo for the cell-cycle-dependent degradation of the regulatory protein CtrA (Jenal & Fuchs, 1998). Common strategies in cell-cycle control exist in prokaryotes and eukaryotes, suggesting that specific proteolysis/degradation events play a key role in the cell cycle. Similarly, we observed that, during heat shock, an S. agalactiae $\Delta$clpP mutant displayed important morphological alterations, indicating that, just like in eubacteria, the ClpP in GBS could play an essential role in the checkpoint mechanism of cell-cycle control. Under these growth conditions, the mutant also showed altered basic cell functions, as exemplified by the absence of the alcohol dehydrogenase and DnaK oxidation, which are likely to be responsible for the dramatic phenotypic modifications observed, including the growth arrest. Since the cytoplasm

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**Fig. 7.** Mouse virulence assays. Growth of S. agalactiae NEM316 (wild-type, ○) and NEM1968 (ΔclpP, ●) was followed in the blood, brain, liver and spleen of Swiss mice inoculated intravenously with 10$^6$ bacteria. Means of bacterial counts in the four organs per time point are shown, SD ≤ 0·25. Reproducible results were obtained in two independent experiments.

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becomes pro-oxidant during lethal heating, a role for GBS ClpP as an anti-oxidant in the protection of DnaK cannot be excluded.

Finally, our results showed that ClpP does not play a major role in the virulence of S. agalactiae, at least in our murine infection model. The slight difference observed between the kinetics of elimination of the wild-type and mutant strains is likely to reflect the growth defect of the ΔclpP mutant observed in vitro at 37 °C, i.e. the temperature of the bodies of the mice. Our results apparently conflict with those of Jones et al. (2000), who identified, by using signature-tagged mutagenesis, a GBS Clp regulatory subunit as an essential virulence factor. However, it should be noted that, whereas the proteolytic activity sensu stricto resides in the ClpP subunit, the regulatory subunits also act as molecular chaperones involved in the folding and assembly of proteins (Schirmer et al., 1996). The Clp chaperonin activity might thus be more important than its proteolytic activity for the proper development of the GBS infectious process. Thus, we have concluded that, as opposed to the Gram-positive intracellular pathogen L. monocytogenes (Gaillot et al., 2000), ClpP is not critical for the virulence of the extracellular pathogen S. agalactiae. This difference might reflect the difference in the lifestyles (extracellular versus intracellular) of these two pathogens.

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