Acd, a peptidoglycan hydrolase of Clostridium difficile with N-acetylglucosaminidase activity

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A gene encoding a putative peptidoglycan hydrolase was identified by sequence similarity searching in the Clostridium difficile 630 genome sequence, and the corresponding protein, named Acd (autolysin of C. difficile) was expressed in Escherichia coli. The deduced amino acid sequence of Acd shows a modular structure with two main domains: an N-terminal domain exhibiting repeated sequences and a C-terminal catalytic domain. The C-terminal domain exhibits sequence similarity with the glucosaminidase domains of Staphylococcus aureus Atl and Bacillus subtilis LytD autolysins. Purified recombinant Acd produced in E. coli was confirmed to be a cell-wall hydrolase with lytic activity on the peptidoglycan of several Gram-positive bacteria, including C. difficile. The hydrolytic specificity of Acd was studied by RP-HPLC analysis and MALDI-TOF MS using B. subtilis cell-wall extracts. Muropeptides generated by Acd hydrolysis demonstrated that Acd hydrolyses peptidoglycan bonds between N-acetylglucosamine and N-acetylmuramic acid, confirming that Acd is an N-acetylglucosaminidase. The transcription of the acd gene increased during vegetative cellular growth of C. difficile 630. The sequence of the acd gene appears highly conserved in C. difficile strains. Regarding deduced amino acid sequences, the C-terminal domain with enzymic function appears to be the most conserved of the two main domains. Acd is the first known autolysin involved in peptidoglycan hydrolysis of C. difficile.

INTRODUCTION

Bacterial peptidoglycan hydrolases (PGHs), also called autolysins, are endogenous enzymes that can break covalent bonds in the peptidoglycan of their own cell walls (Ghuysen et al., 1966). PGHs have been implicated in different cellular functions that require cell-wall remodelling, such as cell-wall expansion, peptidoglycan turnover, daughter cell separation and sporulation (Shockman & Holtje, 1994; Smith et al., 2000; Ward & Williamson, 1984), and also in antibiotic-induced autolysis (Moreillon et al., 1990). Various PGHs are described on the basis of their specific site of cleavage in the peptidoglycan: N-acetylmuramidases, N-acetylglucosaminidases, N-acetylmuramoyl-L-alanine amidases and endopeptidases. It has also been suggested that autolysins may contribute to bacterial pathogenesis by generating inflammatory cell-wall degradation products (Lenz et al., 2003; Myhre et al., 2004), by releasing virulence factors and by mediating bacterial adherence through the non-catalytic domain of some staphylococcal PGHs (Allignet et al., 2002; Heilmann et al., 1997; Hell et al., 1998). This implication of PGHs in the physiology, and probably in the pathogenicity of bacteria, further reinforces the importance of understanding bacterial autolysis.

Autolytic systems of several Gram-positive low G+C bacteria have been studied (Oshida et al., 1995; Smith et al., 2000; Tomasz, 2000). Bacillus subtilis is considered to be a model system for investigating the roles of autolysins in the cell-wall metabolism of endospore-forming Gram-positive bacteria (Smith et al., 2000). Members of the genus Clostridium, which belong to anaerobic microflora of humans and are a potential cause of human infections, belong to this Gram-positive bacteria low G+C phylum.
few PGHs are described in members of this genus, such as an amidase (SleC) and a muramidase (SleM) in *Clostridium perfringens* (Chen et al., 1997; Miyata et al., 1995). However, to our knowledge, the autolytic system of *C. difficile*, which is recognized as a major nosocomial enteric pathogen causing pseudomembranous colitis and many cases of antibiotic-associated diarrhoea (George, 1984), has not been investigated.

The aim of this study was the search for a putative PGH gene in the available *C. difficile* 630 genome, and the molecular characterization and expression of Acd, an autolysin of *C. difficile* with N-acetylglucosaminidase activity.

**METHODS**

**Bacterial strains and culture conditions.** *C. difficile* 630 was used (i) in all experiments for cloning and characterization of Acd and (ii) as a substrate in renaturing SDS-PAGE. An additional set of 11 *C. difficile* strains selected from a previous study (Lemée et al., 2004a) was used to study genetic polymorphism of the *acd* gene. All the *C. difficile* strains were grown in brain heart infusion (BHI) broth (Difco) at 37°C under anaerobic conditions.

*Escherichia coli* strain M15 harbouring pREP4, which constitutively expresses the Lac repressor protein encoded by the *lacI* gene (QiaExpress System; Qiagen), was used as a recipient. Luria–Bertani broth (Difco) was used for cultivation of *E. coli* strain M15 harbouring pREP4, which constitutively expresses the Lac repressor protein encoded by the *lacI* gene (QiaExpress System; Qiagen), was used as a recipient. Luria–Bertani broth (Difco) was used for cultivation of *E. coli* M15(pREP4) as an N-terminal hexa-His-tagged protein with the expression vector pQE-32. After amplification with primers *acd* F and *acd* R (Table 1) were used for the amplification of a DNA fragment encoding Acd devoid of its signal peptide sequence and for the construction of hexa-His fusion protein. A 1790 bp DNA fragment was amplified with these primers from *C. difficile* 630 total DNA. PCR was performed on a GeneAmp System 2700 thermal cycler (Applied Biosystems) in a final volume of 50 µl containing 0.5 µM of each primer, 200 µM each deoxynucleotide triphosphate and 1 U *Pfu* DNA polymerase polymerase reaction buffer [20 mM Tris/ HCl, pH 8.8, 10 mM KCl, 2 mM MgSO<sub>4</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>]. The PCR mixtures were denatured (2 min at 95°C), then the amplification procedure followed, consisting of 30 s at 95°C, annealing for 2 min at 60°C and ending with an extension step at 68°C for 4 min. A total of 40 cycles were performed. DNA sequences were determined with an Applied Biosystems 310 automated DNA sequencer using an ABI-PRISM Big Dye Terminator Sequencing kit (Perkin Elmer). Primers (MWG-Biotech) used are listed in Table 1.

**Cloning, expression and purification of Acd-His-tagged protein in *E. coli.*** Acd-His-tagged protein was expressed and purified using a QiaExpress type IV kit (Qiagen), according to the manufacturer’s instructions. The protein was overexpressed in *E. coli* M15(pREP4) as an N-terminal hexa-His-tagged protein with the expression vector pQE-32. After amplification with primers *acd* F and R, PCR fragments were digested by BanHI and PstI introduced with the primers and cloned in-frame downstream of the hexa-His box sequence in the pQE-32 vector, precut with the same enzymes.

### Table 1. Primers used in this study

<table>
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<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5’→3’)</th>
<th>Position</th>
<th>Amplicon size (bp)</th>
<th>Restriction site</th>
<th>Use</th>
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<td>2038</td>
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<td>acd F</td>
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<td>1790</td>
<td>BanHI Cloning, expression</td>
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</table>

*Optimal recognition sequences for restriction endonucleases are in italic, restriction sites are underlined.
†Position 37.
‡Position 1824.
§Sequences from Lemée et al. (2004b).
E. coli M15/pREP4) competent cells were transformed with the resulting plasmid. E. coli recombinant strains were grown at 37°C in LB medium containing ampicillin and kanamycin. Protein expression was achieved by induction with 1 mM IPTG and a subsequent incubation of the culture for 5 h at a temperature of 28°C to avoid the formation of Acd-His inclusion bodies. The protein was purified by affinity chromatography on Ni²⁺-nitrilotriacetic acid (Ni-NTA) columns (Qiagen) under denaturing conditions after solubilization in 8 M urea, according to the manufacturer’s instructions. The purity of the His-tagged protein was confirmed by SDS-PAGE. The purified His-tagged protein was dialysed against sodium phosphate buffer (pH 8-0) before further use.

Detection of cell-wall lytic enzymes by SDS-PAGE and renaturing SDS-PAGE. Cell-wall lytic enzymes were extracted by the following two procedures. LiCl extractions were carried out as described by Groicher et al. (2000). Briefly, a pellet of 200 ml anaerobic C. difficile 630 cell culture (overnight at 37°C) was resuspended in 5 ml 5 M LiCl solution and shaken at 200 r.p.m. for 20 min. After centrifugation (20 000 g, 20 min, 4°C), the supernatant was dialysed overnight at 4°C against 0-01 M potassium phosphate buffer (pH 7-0) and concentrated 20-fold in a Centricon-3 concentrator (Amicon). SDS extractions were carried out as described by Leclerc & Asselin (1989). Briefly, a pellet of 200 ml anaerobic C. difficile 630 cell culture (overnight at 37°C) was resuspended in 75 ml 4 % (w/v) SDS. The suspension was shaken at 150 r.p.m. for 90 min at room temperature and twice sonicated on ice for 1 min. The extract was heated at 90°C for 15 min and centrifuged at 12 000 g for 15 min at room temperature. The supernatants from LiCl and SDS extractions were stored at −80°C.

SDS-PAGE was performed as described by Laemmli (1970) with 15% polyacrylamide separating gels. Lytic activity was detected by using Acd-His recombinant protein (370 kDa) as described by Groicher et al. (1999). Muropeptides were analysed by MALDI-TOF MS using a Voyager-DE STR mass spectrometer (Perseptive Biosystems) as reported previously (Huard et al., 2003).

RT-PCR. Total RNA extraction was performed at various times of broth culture of C. difficile 630 by using RNAProtect Bacteria Reagent and an RNasey Mini Kit (Qiagen), according to the manufacturer’s instructions. First, for RNA stabilization, 1 ml BHI cell culture was added to 2 ml RNAProtect Bacteria Reagent and the mixture was incubated for 5 min at room temperature. The pelleted cells were resuspended in 100 μl TE (10 mM Tris/Cl, 1 mM EDTA, pH 8-0) containing lysozyme (50 mg ml⁻¹), incubated for 10 min at room temperature and then treated with 350 μl RLT buffer (Qiagen) for lysis. After addition of 250 μl ethanol to the lysate, RNase-free DNase I (Qiagen) treatment was performed for DNA removal. The RNA was purified in succeeding steps with spin columns and finally eluted with 50 μl RNase-free water. The RNA was quantified spectrophotometrically and stored at −70°C. RT-PCR was performed using the OneStep RT-PCR kit (Qiagen). Amplification of a constant amount of total RNA (20 ng) was performed with primers designated in Table 1 and in a final volume of 25 μl, according to the manufacturer’s instructions, including 0-5 μM each RT 16S primer or 1 μM each RT act (this study) and tpi (Lemé et al., 2004b) primer. A reverse transcription reaction for 30 min at 50°C was followed by an initial heating step of PCR amplification at 95°C for 15 min to activate the DNA polymerase as well as to inactivate the reverse transcriptase. Then a touch-down procedure followed, consisting of 30 s at 95°C, annealing for 30 s at temperatures decreasing from 55 to 45°C during the first 11 cycles (with 1°C incremental steps in cycles 1 to 11) and ending with an extension step at 72°C for 30 s. A total of 40 cycles were performed. The analysis of act expression at various stages of growth was performed in two experiments. Simultaneously, PCR was performed on RNA samples with the same oligonucleotides to exclude false-positive amplification from residual DNA.

RESULTS

Identification of a putative PGH gene in the C. difficile 630 genome sequence

An ORF encoding a putative PGH was identified in the C. difficile 630 genome sequence through sequence similarity analysis with other PGHs of Gram-positive bacteria. This 1824 bp ORF is preceded by a putative ribosome-binding site and a putative promoter as identified by −35 and −10 boxes is present upstream of the GTG start codon. The corresponding gene was named acd. We further amplified and sequenced the acd gene from C. difficile 630 and confirmed that its nucleotide sequence was identical to in silico ORF sequence data. The acd gene would encode a protein of 607 aa with a deduced molecular mass of 65 825 Da and a pI of 9-52. The Acd protein has a structural organization with two main domains, an N-terminal domain exhibiting repeated sequences (from 55 up to 67 aa) and a putative C-terminal catalytic domain (between aa 415 and 607) (Fig. 1). The first 22 N-terminal residues of Acd were determined as a fraction was further digested with mutanolysin (2500 U ml⁻¹) (Sigma). The soluble muropeptides obtained after digestion were reduced with sodium borohydride at a final concentration of 8 ng ml⁻¹ (Attri et al., 1999). The reduced muropeptides were then separated by RP-HPLC with an LC Module I system (Waters) and a Hypersyl PEP100 C18 column (250 × 4-6 mm, particle size 5 μm) (Thermo Finngan) as described by Atrih et al. (1999). Muropeptides were analysed by MALDI-TOF MS using a Voyager-DE STR mass spectrometer (Perseptive Biosystems) as reported previously (Huard et al., 2003).
putative signal sequence by SignalP (http://www.cbs.dtu.dk/services/SignalP/), with a possible cleavage site determined between aa 22 and 23. They could also constitute an N-terminal signal anchor sequence, since a transmembrane helix is predicted in positions 6–22 by HMMTOP (http://www.enzim.hu/hmmtop/). The amino acid sequence of the deduced protein from *C. difficile* 630 was aligned with amino acid sequences of *Staphylococcus aureus* Atl (Oshida et al., 1995) and *B. subtilis* LytD (Rashid et al., 1995). This alignment revealed significant similarity in the C-terminal amino acid regions (53–6 and 57–2 % respectively) (Fig. 2), suggesting that the corresponding acd gene encodes a putative PGH with N-acetylglucosaminidase activity, as described in *S. aureus* and *B. subtilis*.

Cloning of the acd gene and expression of the corresponding protein Acd in *E. coli*

A 1790 bp fragment from *acd* was cloned into the BamHI and PstI restriction sites of the expression vector pQE-32 in *E. coli*M15(pREP4). Plasmid extracts of recombinant clones were submitted to nucleotide sequencing to confirm that they had inserted the actual sequence of the whole 1790 bp fragment. The expression of the fusion recombinant protein with a hexa-His tag at the N terminus was induced by IPTG and the protein was then purified by affinity chromatography under denaturing conditions. A single 64 kDa protein band was visualized in SDS-PAGE by Coomassie-stained gels from the induced culture (Fig. 3a, lane 1), but was not detected in the uninduced culture (results not shown).

Acd, a protein with bacteriolytic activity

The Acd-His purified protein gave a clear hydrolysis band (estimated protein size 64 kDa) in SDS-PAGE experiments using renaturation buffer at pH 8.0 with *M. lysodeikticus* autoclaved cells (Fig. 3b, lane 1). We also investigated its bacteriolytic activity in SDS-PAGE using *B. subtilis* and *C. difficile* LytD and found that the recombinant protein generated the same hydrolytic band with both substrates (data not shown). The cell-surface-associated proteins released from LiCl and SDS extracts of *C. difficile* 630 (Fig. 3a, lanes 2 and 3) were also assayed for bacteriolytic activity on renaturing SDS-PAGE with *M. lysodeikticus* autoclaved cells (Fig. 3b, lanes 2 and 3). A hydrolysis band was detected in both LiCl and SDS extracts, whose molecular size was very close to the recombinant protein, suggesting that it most probably corresponds to Acd. An additional hydrolytic band (estimated size 48 kDa) was obtained in the LiCl extract and could represent a degradation product or another *C. difficile* PGH.

Determination of Acd hydrolytic bond specificity

Sequence homology analysis suggested that *acd* could encode an N-acetylglucosaminidase. To determine Acd hydrolytic specificity, the recombinant enzyme was used to digest cell walls from *B. subtilis* 168 HR. Mutanolysin, an autolysin with muramidase activity, was also used as a digestion control. As expected, the soluble muropeptides released from mutanolysin digestion were identical to those described by Atrihi et al. (1999) (data not shown), but were
completely different from soluble muropeptides released from Acd digestion, as revealed by RP-HPLC analysis (Fig. 4a). We could thus conclude that Acd does not possess a muramidase activity. Half of the Acd-soluble muropeptide fraction was further analysed by RP-HPLC, which revealed three major peaks, named peaks 1, 2 and 3 (Fig. 4a). MALDI-TOF MS generated molecular ions with m/z values of 892-26, 1815-74 and 1814-74 for these three peaks, respectively (Table 2). According to previously described data (Atrih et al., 1999; Huard et al., 2003), these m/z values correspond to a disaccharide tripeptide muropeptide with one amidation for peak 1, and to a disaccharide tripeptide disaccharide tetrapeptide with one or two amidations for peaks 2 and 3, respectively (Table 2, Fig. 5). The other half of the Acd-soluble muropeptide fraction was incubated with mutanolysin. The RP-HPLC digested muropeptides and pattern revealed new peaks (4, 5, 6, 7 and 8) (Fig. 4b). MALDI-TOF analysis of these peaks generated molecular ions with m/z values of 689-23, 1409-58, 1612-82, 1408-64 and 1611-91, respectively (Table 2). These values correspond to a disaccharide tripeptide with one amidation and missing one N-acetylglucosamine for peak 4, to a disaccharide tripeptide disaccharide tetrapeptide with one amidation and missing two or one N-acetylglucosamines for peaks 5 and 6, respectively, and to a disaccharide tripeptide disaccharide tetrapeptide with two amidations and missing two or one N-acetylglucosamines for peaks 7 and 8, respectively (Table 2, Fig. 5). These results reveal that the muropeptides generated by Acd hydrolysis could be further cleaved by a muramidase (mutanolysin), indicating that N-acetylglucosamine is present on the reducing end of the disaccharide of these muropeptides. Finally, these results demonstrate that Acd has an N-acetylglucosaminidase activity, as initially suggested by the sequence homology data.

**Variation of acd gene expression during cellular growth**

Transcriptional analysis of acd during cellular growth was investigated by RT-PCR from C. difficile 630 cells at different stages of cellular growth (Fig. 6) with two transcriptional controls: the 16S rDNA gene and the tpi (triosephosphate isomerase) gene, which has been
demonstrated to be species-specific (Dhalluin et al., 2003). Since one single copy of the \textit{tpi} gene exists in the \textit{C. difficile} 630 genome, its use avoids potential hampering of analysis due to multiple copies of 16S rRNA. We detected 230, 270 and 350 bp amplification products corresponding to the expression of \textit{tpi}, 16S rRNA and \textit{acd} genes, respectively (Fig. 6). The amplification signal of \textit{acd} increased during the exponential growth phase, was maximal at the end of the exponential growth phase and then decreased at the stationary phase, whereas 16S rRNA and \textit{tpi} amplification signals did not vary throughout bacterial growth. These results suggest that the expression of \textit{acd} is enhanced during vegetative cell growth of \textit{C. difficile} 630.

Polymorphism analysis of the \textit{acd} gene in strains of \textit{C. difficile}

We sequenced a 1641 bp fragment of the \textit{acd} gene in 12 \textit{C. difficile} strains (including \textit{C. difficile} 630). The \textit{acd} gene appears to be highly conserved, since the 12 sequences displayed at least 97.9\% homology, with 99 (6.0\%) polymorphic sites. The deduced amino acid sequences revealed 5.5\% of non-synonymous amino acids substitutions. Most of these non-synonymous substitutions occur in the N-terminal domain, whereas the C-terminal domain appears to be more conserved with only four non-synonymous substitutions.

**Table 2.** Calculated and observed \(m/z\) values for sodiated molecular ions of muropeptides obtained after hydrolysis of \textit{B. subtilis} peptidoglycan by Acd or by Acd followed by mutanolysin and purification by RP-HPLC

<table>
<thead>
<tr>
<th>Peak</th>
<th>Ion</th>
<th>(m/z)</th>
<th>(\Delta m) (Da)*</th>
<th>Muropeptide identification†</th>
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<tr>
<td>1</td>
<td>(M + Na)⁺</td>
<td>892-26</td>
<td>892-38</td>
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</tr>
<tr>
<td>2</td>
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<td>1813-74</td>
<td>1815-77</td>
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<tr>
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<td>892-38</td>
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<tr>
<td>5</td>
<td>(M + Na)⁺</td>
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<td>1815-77</td>
<td>-406-19</td>
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<tr>
<td>6</td>
<td>(M + Na)⁺</td>
<td>1612-82</td>
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<td>7</td>
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<tr>
<td>8</td>
<td>(M + Na)⁺</td>
<td>1611-91</td>
<td>1814-79</td>
<td>-202-88</td>
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</table>

*\(\Delta m\), Difference between calculated and observed \(m/z\) values.
†ds, Disaccharide (MurNAc-GlcNAc); tri, tripeptide; tetra, tetrapeptide; NH₂ indicates the presence of an amidation on the peptidic chain, most probably on mDAP according to Atrih et al. (1999).

**Fig. 5.** Structure of the muropeptides from \textit{B. subtilis} peptidoglycan obtained after Acd digestion (peaks 1, 2 and 3) or Acd and mutanolysin digestion (peaks 4–8). Peak numbers refer to the peaks on the chromatograms presented in Fig. 4.
putative signal peptide, or a retention signal with a domain of the deduced Acd sequence might constitute a housekeeping function. The present results indicate that the acd gene is involved in cellular physiological functions. The aim of this study was to characterize the first known autolysin involved in peptidoglycan hydrolysis of C. difficile. We cloned this gene and expressed the recombinant protein. The lytic activity of the purified Acd-His recombinant protein was confirmed with M. lysodeikticus, B. subtilis and C. difficile cells as substrates (Leclerc & Asselin, 1989). Moreover, cell-surface-associated proteins released from LiCl and SDS extracts revealed a hydrolytic band corresponding to the recombinant protein.

The C-terminal domain (residues 415–607) of Acd exhibits significant homology with the catalytic domain of LytD, a glucosaminidase from B. subtilis (39% identity, 57-2% similarity) and the C-terminal glucosaminidase domain of S. aureus Atl (40% identity, 53-6% similarity). Recent results with B. subtilis LytG (Horsburgh et al., 2003) or L. lactis AcmB (Huard et al., 2003) indicate that PGHs presenting sequence homology may have different activities and that it is necessary to determine their hydrolytic bond specificity experimentally by analysis of the produced muropeptides. Since the purified recombinant Acd protein was found to hydrolyse B. subtilis vegetative cell walls in renaturing SDS-PAGE experiments, the hydrolytic bond specificity of Acd was further investigated in B. subtilis vegetative peptidoglycan, whose molecular structure has been previously studied in detail (Atrihi et al., 1999). RPHPLC and MALDI-TOF MS analysis of muropeptides generated by Acd hydrolysis clearly confirmed the N-acetylg glucosaminidase activity that was suggested through sequence homology analysis.

Regarding the deduced Acd amino acid sequences from 12 C. difficile strains, Acd is highly conserved, particularly in the C-terminal catalytic domain which includes the enzymic site of Acd. This supports the hypothesis that Acd has an important function in the physiology of C. difficile. The variation of acd expression during C. difficile vegetative growth was studied by RT-PCR, with 16S rRNA and tpi genes chosen as transcriptional controls owing to their housekeeping function. The present results indicate that acd is mostly transcribed during vegetative growth and suggest that the acd gene is involved in cellular physiological actions (Wren, 1991). These repeated sequences contain four putative GW modules, which constitute another motif for cell-surface anchoring. GW modules might interact with cell-wall polymers such as teichoic or lipoteichoic acids and are present in many surface proteins produced by Gram-positive bacteria, including L. monocytogenes InIB and Ami (Braun et al., 1997; Cabanes et al., 2002; Milohanic et al., 2001) and the four staphylococcal surface autolysins, S. aureus Atl (Oshida et al., 1995), S. caprae AtlC (Allignet et al., 2001), S. epidermidis AtlE (Heilmann et al., 1997) and S. saprophyticus Aas (Hell et al., 1998).

Like most of the previously described bacterial PGHs (Heilmann et al., 1997; Hell et al., 1998; Milohanic et al., 2001; Oshida et al., 1995), Acd has a modular structure with two main domains. The first 22 aa of the N-terminal domain of the deduced Acd sequence might constitute a putative signal peptide, or a retention signal with a transmembrane domain as described for a few proteins like cell-wall hydrolases in B. subtilis (Tjalma et al., 2004). Most known PGHs exhibit repeated sequences that could be involved in cell-wall binding. Although LysM domains involved in cell-wall targeting and peptidoglycan binding (Bateman & Bycroft, 2000) have been described in the major autolysin of Lactococcus lactis (AcmA) (Buist et al., 1995) and in a muramidase (MurA) of Listeria monocytogenes (Carroll et al., 2003), such repeated sequences were not found in Acd. The PGH of C. difficile is also devoid of LPXTG or other LPXTG-like motifs enabling covalent binding to peptidoglycan (Comfort & Clubb, 2004; Fischetti et al., 1990). However, the N-terminal domain exhibits repeated sequences probably involved in cell-wall interactions (Wren, 1991). These repeated sequences contain four putative GW modules, which constitute another motif for cell-surface anchoring. GW modules might interact with cell-wall polymers such as teichoic or lipoteichoic acids and are present in many surface proteins produced by Gram-positive bacteria, including L. monocytogenes InIB and Ami (Braun et al., 1997; Cabanes et al., 2002; Milohanic et al., 2001) and the four staphylococcal surface autolysins, S. aureus Atl (Oshida et al., 1995), S. caprae AtlC (Allignet et al., 2001), S. epidermidis AtlE (Heilmann et al., 1997) and S. saprophyticus Aas (Hell et al., 1998).

**DISCUSSION**

The aim of this study was to characterize the first known autolysin involved in peptidoglycan hydrolysis of C. difficile. The acd gene was identified by a sequence similarity search on the C. difficile 630 genome and was found to encode a putative PGH. We cloned this gene and expressed the corresponding Acd protein in E. coli M15(pREP4). The lytic activity of the purified Acd-His recombinant protein was confirmed with M. lysodeikticus, B. subtilis and C. difficile cells as substrates (Leclerc & Asselin, 1989). Moreover, cell-surface-associated proteins released from LiCl and SDS extracts revealed a hydrolytic band corresponding to the recombinant protein.

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**Fig. 6.** acd gene expression during cellular growth. (a) C. difficile 630 growth curve. Growth (c.f.u. ml⁻¹) was followed by measuring OD₅₆₅ and 1–8 represent the different times for total RNA extraction and RT-PCR. (b) Electrophoretic migration of RT-PCR amplification products at different growth times.
functions like peptidoglycan turnover or cell separation. Further investigation of the putative role of Acd in vegetative growth would require targeted disruption of the acd gene. However, the inability to transform this species, possibly due to the activity of endogenous restriction systems, hampers this type of investigation. Several genetic tools have been tentatively used to introduce heterologous DNA in C. difficile: (i) electroporation gene transfer (Ackermann et al., 2001), the efficiency of which was contested (Purdy et al., 2002); (ii) use of conjugal transposons (Mullany et al., 1994) or plasmids (Liyanage et al., 2001), which showed low efficiency; (iii) removal or methylation of restriction sites in vectors derived from C. difficile plasmids, although this had not yet been used for the disruption of genes (Purdy et al., 2002). An alternative antisense RNA approach was also elaborated, but was found to be insufficient to decrease the expression of the corresponding protein (Roberts et al., 2003). Therefore, further improvements are required in genetic manipulation to study the function of targeted genes in C. difficile.

Although PGHs are implicated in several physiological functions, previous studies also suggested that autolysins may play an indirect role in the pathogenesis of some bacteria. It has been suggested that autolysins of Strep- tococcus pneumoniae facilitate the release of potent pro-inflammatory agents such as cell-wall components or pneumolysin, the main pneumococcal toxin (Canvin et al., 1995; Diaz et al., 1992). Another study proposed that proteins lacking an N-terminal signal sequence could be exported from the cytoplasm by cell lysis or via the flagellar export machinery, the holin systems or other unidentified export systems (Tjalsma et al., 2004). The pathogenesis of C. difficile is mainly due to toxin A (enterotoxin) and toxin B (cytotoxin) (Lyerly et al., 1995), which are synthesized without any N-terminal signal peptide. The potential role of Acd in the pathology of C. difficile should be further investigated.

In conclusion, we report here the first molecular characterization of an autolysin of C. difficile, named Acd. This PGH displays N-acetylglucosaminidase activity and could thus play an important role in the physiology of this organism.

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