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Molecular and Biochemical Characterization of a Protective 40-Kilodalton Antigen from *Corynebacterium pseudotuberculosis*

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A 40-kDa protein from *Corynebacterium pseudotuberculosis* has been previously identified as a protective antigen against ovine caseous lymphadenitis. From genomic DNA libraries of *C. pseudotuberculosis*, we have cloned and sequenced the 40-kDa protein gene, which was found to contain an open reading frame of 1,137 bp encoding a protein of 379 amino acids. No significant homology with previously published DNA or amino acid sequence data was found in databases, suggesting that this is a novel protein. Recombinant 40-kDa protein was overexpressed as a fusion protein to 15% of total cell proteins in *Escherichia coli*. Biochemical analysis of native and recombinant 40-kDa proteins has revealed associated proteolytic activity, which was shown to be of the serine protease type through the use of specific inhibitors. We suggest that this novel protective antigen be termed corynebacterial protease 40 (CP40).

Historically, human and animal health vaccines have been live attenuated or killed preparations of the infective organism which have proven to be very effective prophylactic agents (3). One of the biggest drawbacks with such vaccines is their variable efficacy due to ignorance of the protective antigens and undesirable side effects such as a local inflammatory response (31). These problems can be overcome with recombinant subunit vaccines which can be manufactured inexpensively with high batch-to-batch reproducibility. However, the ability to identify antigens that offer protection levels equal to that of whole cell culture vaccines is a major hurdle in the production of genetically engineered subunit vaccines (10).

Caseous lymphadenitis (CLA) is a chronic disease of sheep and goats that is caused by infection with *Corynebacterium pseudotuberculosis*. Vaccines of various compositions have been used against this disease. The well-characterized phospholipase D (PLD) toxin of *C. pseudotuberculosis* has been shown in crude and purified forms to provide significant levels of protection (5, 11, 12). It has been suggested that a cell-associated antigen is also required for optimal protection (5, 6); however, Eggleton et al. found that inclusion of heat-killed cells in a toxoid vaccine had no effect on protection levels in sheep (12). While some experiments provided evidence for an additional protective antigen(s) (5–8), the work of Eggleton et al. indicated that such antigens were not cell associated.

A 40-kDa protein from *C. pseudotuberculosis* has been identified in this laboratory as being an immunogenic protein in infected animals, and field trials with semipurified preparations of this secreted antigen have shown that it is highly protective (34). This finding demonstrates that a supernatant antigen other than PLD is protective against CLA, which is supported by the recent finding that sheep immunized with a genetically engineered strain of *C. pseudotuberculosis* deleted of the toxin gene were highly protected against the disease (16). As part of an effort to produce a recombinant subunit vaccine against CLA, we describe here the molecular cloning, sequencing, and expression in *Escherichia coli* of the novel 40-kDa protein of *C. pseudotuberculosis*. Furthermore, we present preliminary data as to the biochemical action of the 40-kDa protein.

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MATERIALS AND METHODS

Protein purification and amino acid sequencing. The 40-kDa protein was purified as described previously (34). Briefly, *C. pseudotuberculosis* WA1030 was grown in brain heart infusion (Difco) broth, the culture was centrifuged, and the supernatant was retained. The supernatant was brought to 50% ammonium sulfate, the precipitate was removed by centrifugation, and the soluble fraction was chromatographed on a hydrophobic chromatography column. The fraction containing the 40-kDa protein was electrophoresed on a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide minigel (Bio-Rad) (21) and visualized with 2 M KCl as a negative stain. The appropriate band on the gel was excised, and the 40-kDa protein was electroeluted from the gel slice in a 0.1 M ammonium carbonate buffer at 100 V for 1 h, using a commercial electroelution apparatus (Centricon). Purified protein was subjected to cyanogen bromide digestion in 70% formic acid in the dark for 16 h, and the resultant peptides were separated on an SDS-polyacrylamide step gradient gel (28), electroblotted onto a polyvinylidene difluoride sequencing-grade membrane (Millipore), and stained with Coomassie brilliant blue. The N-terminal amino acid sequences of both the peptides and the electroeluted protein were determined by using an Applied Biosystems model 470A protein sequencer.

Genomic DNA extraction. *C. pseudotuberculosis* genomic DNA was extracted by the method of Songer et al. (30). Briefly, *C. pseudotuberculosis* cells grown on solidified brain heart infusion plates were suspended in 2.5 mM Tris-HCl–2.5 mM EDTA with 5 mg of lysozyme (Boehringer Mannheim) per ml and incubated at 37°C for 2 h. The cells were pelleted and resuspended in 5 mM Tris-HCl–5 mM EDTA with 1% SDS and incubated at 50°C for 30 min. The lysed cells were then phenol and phenol-chloroform extracted; the DNA was precipitated with 2 volumes of 100% ethanol and then resuspended in 7 M guanidine-HCl. DNA was reprecipitated with ethanol and resuspended in 10 mM Tris-HCl–1 mM EDTA (pH 8.0).

Oligonucleotide synthesis. Forty-kilodalton protein-specific oligonucleotides deduced from N-terminal and internal amino acid sequence data and those used for sequencing were synthesized by using an Applied Biosystems model 391 DNA synthesizer. In an effort to decrease oligonucleotide redundancy, pools of oligonucleotides were synthesized and inosine was substituted in positions of high ambiguity (25). The oligonucleotides from the internal peptides were designed to be complementary to the strand of DNA for the N-terminally derived oligonucleotides.

PCR. PCRs were performed as described previously (17) in a programmable water bath machine (Bartelt, Melbourne, Victoria, Australia) with 30 to 60 cycles (92°C for 1 min, 50 to 60°C for 1 min, 72°C for 1 min). For each reaction, 100 ng of each oligonucleotide, in different combinations, was used with 1 ng of *C. pseudotuberculosis* genomic DNA as a template. PCR products were cloned into a dideoxythymidine vector, constructed by the method of Marchuk et al. (23), using pBluescript SK^- (Stratagene) for nucleotide sequencing.

Southern blot analysis. *C. pseudotuberculosis* genomic DNA was digested with restriction enzymes (Boehringer Mannheim), electrophoresed on a 0.7% agarose gel, and blotted onto nylon membranes (Amersham), using standard Southern blot techniques (27). Filters were hybridized in buffer (0.5 M $NaHPO_4$, 7% SDS, 1% bovine serum albumin, 1 mM EDTA, 50 μg of salmon sperm DNA per ml) (9) at 65°C with a ^{32}P-labelled probe overnight, then washed under high stringency (65°C, 0.1% SDS, 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), and autoradiographed.

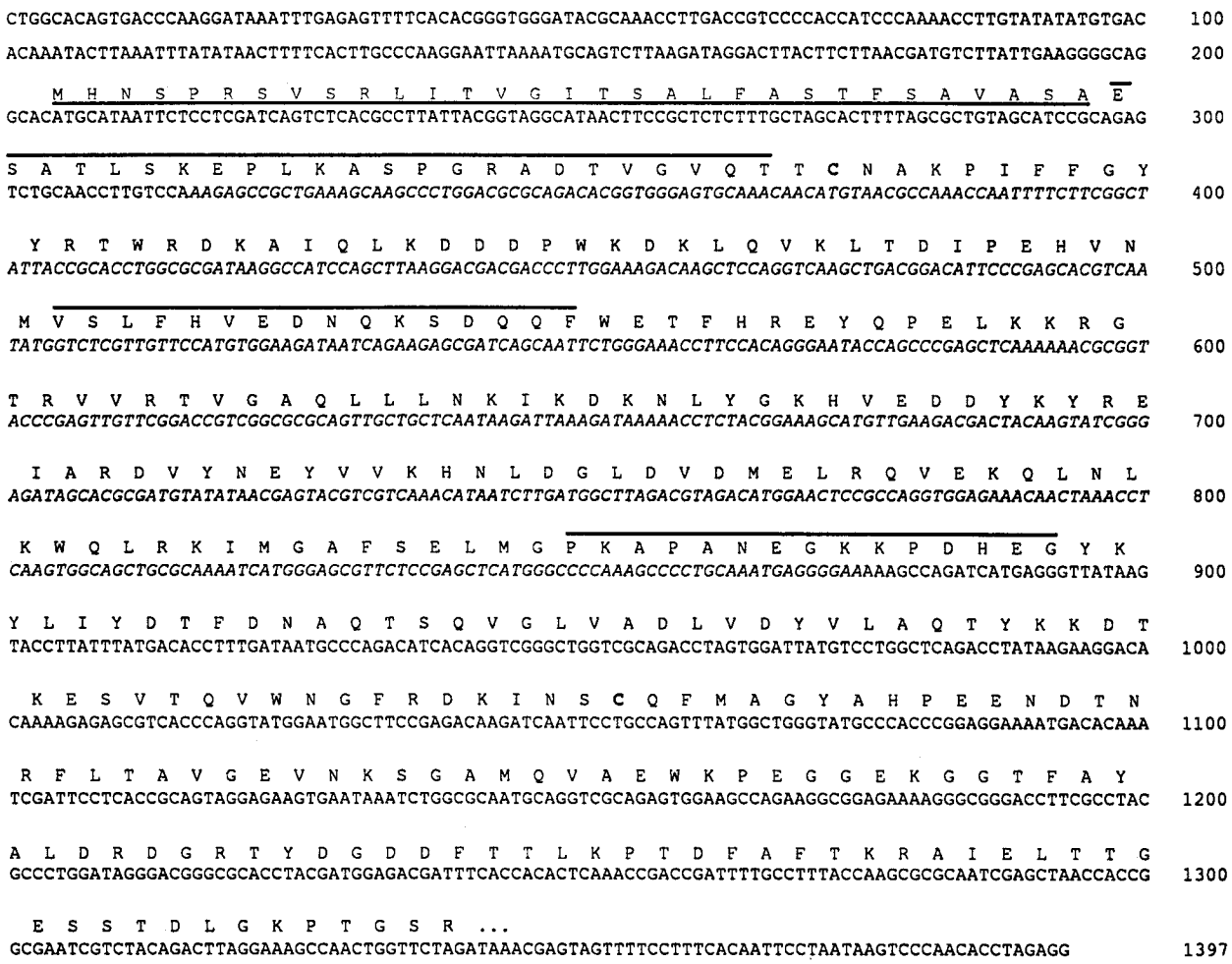

FIG. 1. Nucleotide and deduced amino acid sequences of the plasmid constructs containing the gene encoding the 40-kDa protein from *C. pseudotuberculosis*. The amino acid sequences determined from cyanogen bromide digestion are overlined, and the 31-amino-acid signal sequence is underlined. The two cysteine residues are in boldface, and the 559-bp PCR product nucleotide symbols are in italics.

Genomic DNA library construction. Size-selected genomic DNA libraries were constructed in the pBluescript SK$^-$ plasmid vector. Phenol-extracted genomic DNA was further purified on a cesium chloride density gradient, and 20 μg was digested with either *Xba*I or *Cla*I and run on a 0.7% agarose gel. DNA fragments of 1.8 to 4.0 kbp were extracted from the agarose by using a Prep-a-Gene kit (Bio-Rad), cloned into the *Xba*I or *Cla*I site of pBluescript, and transformed into *E. coli* XL1-Blue (Stratagene). Transformants were screened with ^{32}P-labelled 40-kDa protein-specific probes.

Nucleotide sequence analysis. The nucleotide sequences of positive clones and cloned PCR products were determined by using a T7 DNA polymerase sequencing kit (United States Biochemical Corporation). Overlapping sequence was obtained either by subcloning, by exonuclease digestion, or by using oligonucleotide primers designed from previously obtained sequence data.

Overexpression of recombinant 40-kDa protein. The genomic clone of the entire open reading frame (ORF) without the leader sequence was subcloned into a pGEX2T vector (Amrad, Melbourne, Victoria, Australia), using standard ligation techniques. The expression construct was transformed into *E. coli*, and recombinant protein expression was induced by using 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Boehringer Mannheim) according to the manufacturer's instructions. Bacterial cells were pelleted by centrifugation and resuspended in standard SDS-polyacrylamide gel electrophoresis (PAGE) reducing loading buffer, and samples were subjected to SDS-PAGE (12% gel). Recombinant proteins were immunoblotted and probed with antisera raised against the native 40-kDa protein as described previously (34).

Purification of the recombinant protein. The recombinant 40-kDa protein was found to be insoluble and therefore was purified by the method of Frangioni and Neel (13). Briefly, 100 ml of induced bacterial culture was centrifuged, and the cell pellet was resuspended in 6 ml of STE buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA) with lysozyme (Boehringer Mannheim) at a final concentration of 100 mg/ml. Following a 15-min incubation at room temperature, dithiothreitol (Boehringer Mannheim) and Sarkosyl were added to final concentrations of 5 mM and 1.5%, respectively, and the solution was vortexed. Lysed bacteria were sonicated for 20 s, using an Edwards sonicator with a tapered probe, and Triton X-100 (Sigma) was added to a final concentration of 4% to neutralize the Sarkosyl. The solubilized cell pellet was centrifuged at 10,000 × *g* to remove cellular debris, and the supernatant was applied to 1.5 ml of glutathione-agarose (Amrad). The fusion protein was cleaved by incubation with 100 mg human thrombin (Sigma) per mg of bound recombinant protein. Approximately 2 mg of each protein (15 ml of bead slurry) was subjected to minigel SDS-PAGE and electroblotted to a polyvinylidene difluoride sequencing-grade membrane (Millipore), and the N-terminal amino acid sequence determined as described above.

Detection of proteolytic activity by substrate minigel SDS-PAGE. Analysis of proteolytic activity was performed by using an SDS-polyacrylamide minigel copolymerized with either 0.1% gelatin or 0.1% casein as described previously (14). Samples were run under both nonreducing and reducing conditions as described by Laemmli (21) with a 4% stacking gel without gelatin. Minigels were electrophoresed at 15 mA per gel, washed twice in 100 ml of 2.5% Triton X-100 (Sigma) for a total of 2 h to remove SDS, and then incubated in 0.1 M Tris–2.5 mM $CaCl_2$ (pH 8.3) for 16 h at 37°C. Gels were stained in Coomassie brilliant blue and destained, and proteolytic activity was observed as clear bands against a blue background.

To determine the class of protease to which the 40-kDa protein belongs, samples were electrophoresed on 0.1% gelatin SDS-polyacrylamide gel with one of the following protease inhibitors in the sample buffer: 1 mM Pefa-bloc SC (Boehringer Mannheim), 1 mM L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E64; Sigma), 10 mg of pepstatin (Sigma) per ml, 10 mM 1,10-phenanthroline (Sigma), or 10 mM phenylmethylsulfonyl fluoride (Sigma). As the last three inhibitors are reversible, they were also included in the wash buffers at the

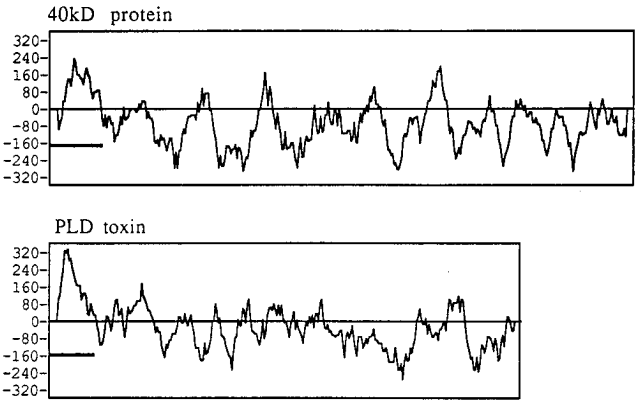

FIG. 2. Kyte-Doolittle hydrophobicity plot (20) of the deduced amino acid sequence of the 40-kDa protein and the PLD protein of *C. pseudotuberculosis*. Increasing hydrophobicity is above the center line and the respective leader sequences are indicated by bold lines.

same concentration, and $CaCl_2$ was omitted from the incubation buffer with the metalloprotease inhibitor 1,10-phenanthroline.

Nucleotide sequence accession number. The reported nucleotide sequence has been submitted to GenBank and assigned accession number U10424.

RESULTS

Protein sequencing and PCR. The 40-kDa protein was purified by ammonium sulfate precipitation, hydrophobic column chromatography, and then electroelution. Purified protein was cyanogen bromide digested, and the N-terminal amino acid sequences of the undigested 40-kDa protein and the peptides were determined (Fig. 1). The N-terminal and internal amino acid sequence data were used to design sets of oligonucleotide PCR primers for the synthesis of a polynucleotide specific for the 40-kDa gene. The expected sizes of the PCR products were not known; however, by comparing the nucleotide sequence of the PCR products with that deduced from the amino acid data 5′ of the N-terminal PCR oligonucleotide, it was possible to determine if a 40-kDa specific product had been obtained. We found that a 559-bp PCR polynucleotide had the expected sequence (Fig. 1), and it was used subsequently as a 40-kDa protein-specific probe.

Library construction and nucleotide sequence analysis. On the basis of Southern blot analysis with the PCR-derived polynucleotide as a probe, an *Xba*I size-selected *C. pseudotuberculosis* genomic DNA library was constructed, from which the genomic clone pX26BS was isolated. As sequence analysis of pX26BS revealed that the ORF of the 40-kDa protein gene was incomplete, a second genomic library was constructed by using *Cla*I, for which a site was identified 250 bp 5′ of the 3′ *Xba*I site. Southern blot analysis of *Cla*I-digested genomic DNA showed that the 250-bp *Xba*I-*Cla*I polynucleotide from pX26BS hybridized to a 2.0-kbp *Cla*I fragment of DNA. The genomic clone, pC20BS, was isolated from a *Cla*I size-selected library, and the nucleotide sequence was determined. Combining the sequence data from these two clones revealed an ORF of 1,137 bp encoding a protein of 379 amino acids, corresponding to a molecular weight of 42,909. Comparison of the N-terminal amino acid sequence data of the mature 40-kDa protein with the ORF indicated a predicted leader sequence of 31 amino acids (Fig. 1). This putative leader sequence is hydrophobic, while the mature protein is predominantly hydrophilic, a structure which is similar to that of the *C. pseudotuberculosis* PLD toxin (15), as can be seen by a Kyte-Doolittle hydrophobicity plot in Fig. 2. Furthermore, both the 40-kDa protein and PLD have an alanine-x-alanine cleavage motif at the C terminus of the leader sequence which is typical of other gram-positive secreted proteins (32). This result agrees with previous observations that the majority of the native 40-kDa protein is found in *C. pseudotuberculosis* culture supernatant (34). The mature protein has a predicted molecular weight of 39,751 with 348 amino acids. A database search using GenBank and Swissprot at the nucleotide and amino acid levels, respectively, revealed no significant homology to any protein previously described.

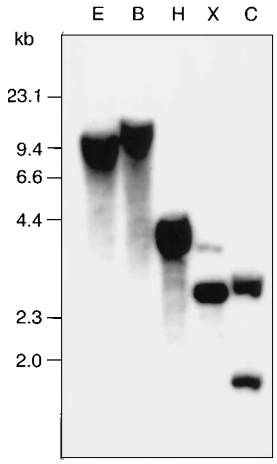

FIG. 3. Southern blot analysis of *C. pseudotuberculosis* genomic DNA digested with *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H), *Xba*I (X), and *Cla*I (C) and probed with a polynucleotide spanning the entire ORF of the 40-kDa protein gene.

Southern blot analysis of *C. pseudotuberculosis* genomic DNA. Southern blot analysis using the 40-kDa protein ORF to probe *C. pseudotuberculosis* genomic DNA digested with five restriction enzymes revealed that the 40-kDa protein was encoded by a single-copy gene (Fig. 3). In accordance with nucleotide sequence analysis, two bands were observed in the lanes digested with *Xba*I and *Cla*I.

Overexpression of recombinant 40-kDa protein. Overexpression of the recombinant 40-kDa protein was achieved in the pGEX2 plasmid vector. The expression construct was made by a series of ligations and subclonings (Fig. 4) so that the ORF without the leader sequence was in frame with the glutathione *S*-transferase (GST) protein.

(i) Construction of the recombinant expression plasmid. Plasmid pX26BS was digested with *Hin*dIII and *Cla*I, and the 1.4-kbp fragment gel purified was then digested with *Hin*fI. The resultant 800-bp fragment was ligated with a *Pst*I-*Hin*fI-specific linker and subcloned into the *Pst*I-*Cla*I sites of pBluescript SK^- to give plasmid pPC700BS. A *Bst*XI-digested, blunt-ended, *Cla*I-digested fragment from pC20BS was ligated with a *Bam*HI-*Cla*I fragment from pPC700BS into a *Bam*HI-*Sma*I-digested vector to give the full ORF without the leader sequence. This plasmid was digested with *Bgl*II-*Eco*RI, and the resultant 1.2-kbp fragment was ligated into a *Bam*HI-*Eco*RI-digested pGEX2T vector, resulting in the expression construct pBE12GEX2. It was expected that the N terminus of the thrombin-cleaved protein would have three extra amino acids, glutamic acid, serine, and methionine, preceding the glutamic acid of the 40-kDa protein.

(ii) Expression of recombinant 40-kDa protein. The construct pBE12GEX2 was transformed into *E. coli*, and overexpression of a fusion protein was induced with IPTG, giving

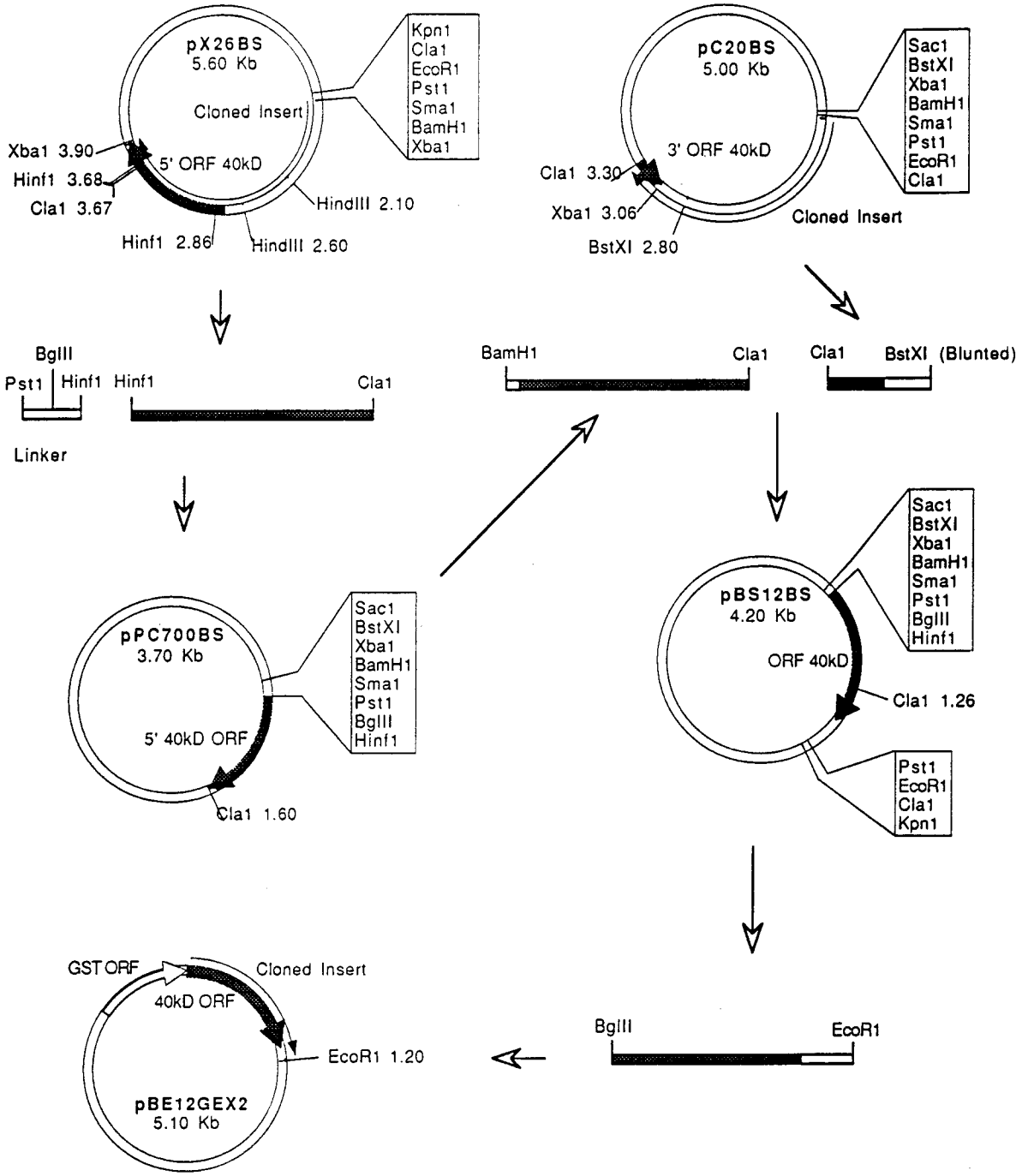

FIG. 4. Cloning strategy to construct the expression plasmid pBE12GEX2 for the overexpression of recombinant GST–40-kDa fusion protein.

high levels of recombinant protein with up to 15% of total cellular proteins, as estimated by an integrated scan of a Coomassie brilliant blue-stained SDS-polyacrylamide gel. Using a solubilization method based on the detergent Sarkosyl, the fusion protein was purified on glutathione-agarose, and the 40-kDa protein was cleaved from the bound GST with thrombin (Fig. 5). Amino acid sequence analysis of the 26- and 40-kDa proteins resulting from the cleavage gave the expected N termini for both GST and the 40-kDa protein. Furthermore, the fusion protein was immunoblotted and probed with antisera raised against the native 40-kDa protein, which bound to both the 66-kDa recombinant fusion and 40-kDa thrombin-cleaved proteins but not to 26-kDa GST (Fig. 5). Breakdown products were also detected, especially a protein of 55-kDa recognized by the 40-kDa protein antisera whose identity was verified by N-terminal amino acid sequencing, as this protein had lost approximately 10 kDa from the N terminus of the fusion protein. Antiserum made to the recombinant 40-kDa protein also reacts with native 40-kDa protein (data not shown).

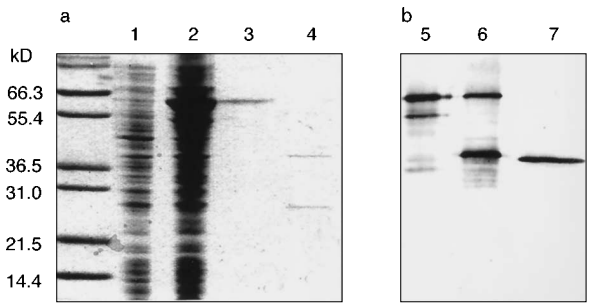

FIG. 5. Coomassie brilliant blue-stained SDS-polyacrylamide minigel (a) and immunoblot probed with antisera against the native 40-kDa protein (b). Lane 1, uninduced, and lanes 2 and 5, IPTG-induced *E. coli* cells transformed with the pBE12GEX2 expression construct; lane 3, glutathione-agarose-purified GST–40-kDa fusion protein; lane 4, fusion protein digested with thrombin giving the expected 40-kDa and 26-kDa products. Since not all fusion protein was cleaved, the more sensitive immunoblot detected uncleaved 66-kDa as well as thrombin-cleaved recombinant 40-kDa protein (lane 6). Lane 7 shows *C. pseudotuberculosis* culture supernatant containing native 40-kDa protein.

Detection of proteolytic activity. The native and recombinant proteins were subjected to gelatin and casein minigel SDS-PAGE to determine the presence of proteolytic activity. Under nonreducing conditions, both the purified native and the thrombin-cleaved recombinant 40-kDa proteins had proteolytic activity, the strongest activity being seen with gelatin as a substrate (Fig. 6). Neither the recombinant GST–40-kDa fusion protein nor unfractionated native protein from *C. pseudotuberculosis* culture supernatants showed any proteolytic activity. Unsuccessful attempts were made to transpose this experiment into a spectrophotometric tube assay with gelatin and casein as substrates.

The proteolytic nature of the protease was determined by using class-specific inhibitors with the gelatin SDS-PAGE detection method. The irreversible serine protease inhibitor Pefa-bloc abolished all, and phenylmethylsulfonyl fluoride abolished most observable proteolytic activity, while the other three inhibitors had no detectable effect (Table 1). A motif search on Prosite and Blocks did not reveal any active site homologies with other serine proteases.

DISCUSSION

DNA sequence analysis of the 40-kDa protein structural gene revealed an ORF of 1,137 bp. Comparison of this gene

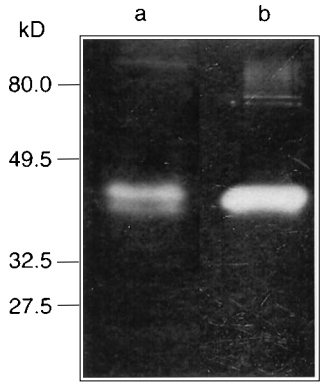

FIG. 6. Gelatin minigel SDS-PAGE demonstrating proteolytic activities of native (a) and recombinant (b) 40-kDa proteins as clear bands against the dark background of the Coomassie brilliant blue-stained gelatin gel.

TABLE 1. Determination of the proteolytic class of the 40-kDa protease, using specific inhibitors on samples run on 0.1% gelatin substrate gels

Inhibitor	Class inhibited	40-kDa protease activity
Pefa-bloc SC	Serine	−
E64	Cysteine	+++
Pepstatin	Aspartic	+++
1,10-Phenanthroline	Metallo	+++
Phenylmethylsulfonyl fluoride	Serine/Cysteine	+
None		+++

with that of the PLD toxin shows that they both have typical bacterial leader sequences containing an Ala-x-Ala cleavage motif at the C terminus and are highly hydrophobic (32, 33). Furthermore, both mature proteins are hydrophilic, as seen by a Kyte-Doolittle hydrophobicity plot (Fig. 2). These similarities are not surprising since, as for PLD, the majority of native 40-kDa protein is found in *C. pseudotuberculosis* culture supernatants (34), indicating that they are both secreted proteins. The lack of homology with nucleotide and protein databases confirms that the 40-kDa protein is a novel protein.

Overexpression of recombinant 40-kDa protein was performed in the well-documented pGEX system. Expression levels were found to be up to 15% of cellular proteins, which is comparable with data for other proteins expressed in this system (29). Different strains of *E. coli* were tried (data not shown), but expression levels in XL1-Blue were among the highest. Breakdown products were also detected by immunoblotting, which is not surprising since in our hands this protein is susceptible to proteolytic degradation which maybe due to its own protease activity or that of its foreign host. Purification of the protein was achieved in a two-step process involving detergent solubilization of *E. coli* followed by affinity purification. While this is a facile technique, the yield of purified protein was only 1% of the yield of solubilized protein, which is poor compared with levels of affinity-purified soluble fusion proteins (29).

Preliminary characterization of the 40-kDa protein revealed that it has serine protease-type activity; however, database searches did not identify any homology with active-site motifs of other serine proteases. This lack of homology could indicate that the 40-kDa protein is an uncharacterized subclass of serine proteases. Protease activity could not be detected in *C. pseudotuberculosis* culture supernatants, despite the presence of microgram quantities of the 40-kDa protein in the gel assay. Protease activity could be detected only after purification of the native protein.

Currently, it is not known whether in vivo-produced 40-kDa protein has proteolytic activity. Conversion of the inactive to the active form of the protein may require conformational change, posttranslational modification, or a combination of these two processes. Conformational change may have been induced during purification, and posttranslational modification may be an autocatalytic self-cleavage process. Other serine proteases such as proteinase K (1) and the subtilisin family of proteases (2) require conformational and cleavage modifications for maximal activity. Further characterization of this protein is required to determine the exact mechanism of its proteolytic activity, conditions for maximal proteolysis, amino acid sequence specificity, and possible target molecules in vivo.

Immunity against CLA in animals vaccinated with the 40-kDa antigen has been attributed to cell-mediated immunity (34), which is an important mediator of protection against a

range of intracellular pathogens (18). The immune response to vaccination with the 40-kDa antigen has not been characterized; however, as it gives protection, the response is assumed to affect the function of the 40-kDa protein and indirectly the growth of the bacterium or result in the direct killing of *C. pseudotuberculosis*. The function of the 40-kDa protein is not known. Other proteases have been shown to act either by aiding intracellular survival, being involved in tissue destruction, or helping the bacterium evade the immune system, thus aiding survival of the bacteria in a host animal (reviewed in reference 22). For example, the intracellular bacterium *Legionella pneumophila* secretes a metalloprotease that has been shown to be highly protective (4), has hemolytic and cytotoxic activities (19, 26), and has also been shown to interfere with neutrophil and monocyte function in vitro. The latter of these actions has been attributed to cleavage of the cytokine interleukin-2 and the T-cell surface protein CD4 (24).

Further studies will involve the use of the recombinant 40-kDa protein in protection studies against CLA and trying to understand its role in vivo and contribution to the pathogenesis of the disease. We suggest that this protein be termed corynebacterial protease 40 (CP40).

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