Cloning and Expression of the Gene for the Cross- Reactive
α Antigen of Mycobacterium kansasii

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The gene for the extracellular α antigen of Mycobacterium kansasii was cloned by using the α-antigen gene fragments of M. bovis BCG as probes. Gene analysis revealed that this gene encodes 325 amino acid residues, including 40 amino acids for the signal peptide, followed by 285 amino acids for the mature protein. A comparison of the nucleotide sequences of the genes isolated from these two mycobacterial species showed that the levels of DNA and amino acid homology were 84.8 and 89.1%, respectively. The hydropathy profiles were also compared, and two highly changed hydrophilic regions were observed, which might account for the antigenic diversity of this antigen or its acquisition of antigenic specificity.

MATERIALS AND METHODS

Reagents. All enzymes (except BanI), the pUC18 vector, the XhoI linker, the nick translation kit, the 7-deaza sequencing kit, the DNA ligation kit, and the DNA blunting kit were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). Plasmid pKK233-2 (3) was from Pharmacia (Up- sala, Sweden). Restriction endonuclease BanI was from Toyobo Co., Ltd. (Tokyo, Japan). [α-32P]dCTP was purchased from Amersham Japan Co., Ltd. (Tokyo, Japan). Peroxidase-conjugated goat anti-rabbit immunoglobulin G was from Dako Co., Ltd. (Copenhagen, Denmark).

Bacterial strains and culture. M. kansasii ATCC 12478 was grown in Sauton medium (28), and E. coli JM109 was grown in L broth (19).

Preparation of probes. Plasmid pP-1 (18), into which the 0.8-kilobase-pair (kb) PstI fragment containing most of the α-antigen gene of M. bovis BCG was subcloned, was digested with both PstI and XhoI. The resulting 490 base-pair (bp) PstI-XhoI (N-terminal part of the α antigen) and 310-bp XhoI-PstI (C-terminal part of the α antigen) fragments were isolated by the DE81 paper method as described previously (18). About 1 μg of the fragments was nick translated by using a nick translation kit and [α-32P]dCTP (3,000 Ci/mmol) at 15°C for 2 h, followed by phenol extraction and ethanol precipitation with 30 μg of carrier calf thymus DNA. Precipitated probes, designated probe A for the 490-bp fragment and probe B for the 310-bp fragment, were used for the hybridization experiments.

Genomic Southern hybridization. M. kansasii DNA was prepared by the method of Suzuki et al. (28). Southern

Myobacterium tuberculosis and M. leprae are among the most common and important pathogenic mycobacteria in developing countries. In addition, considerable attention has been attracted by atypical mycobacteria, such as M. kansasii, M. avium, and M. intracellulare, which are often encountered in patients with acquired immunodeficiency syndrome (17) and autoimmune diseases (25). Because it is difficult to separate these pulmonary infectious diseases from each other, there is an urgent need for rapid and accurate diagnostic reagents to aid in determining optimum treatment. Recently, efforts have been focused on the search for mycobacterial antigens in view of their potential use in immunodiagnosis and the T-cell proliferative response (20, 22, 24, 33). However, there are few reports of the isolation and characterization of species-specific antigens for atypical mycobacteria which could directly contribute to their immunodiagnosis (1). Accordingly, homologous proteins possessing both species-common and species-specific antigenic determinants would be worth studying.

The α antigen, which produces a strong delayed-type hypersensitivity reaction, is a cross-reacting material which is widely distributed among M. tuberculosis, M. bovis, and nontuberculous mycobacteria (38). It has been reported that a species-specific antigenic determinant is contained in each of the α antigens derived from M. kansasii, M. marinum, M. gordonae, M. scrofulaceum, and M. szulgai (30, 31). A complex-specific antigenic determinant is also contained in the α antigens of the M. tuberculosis-M. bovis-M. microti complex and the M. avium-M. intracellulare complex (29, 31). The species-specific antigenic determinant, therefore, could be a good candidate for an immunodiagnostic reagent to identify these species. In recent studies, we have been concerned with the cloning and characterization of the gene for the α antigen secreted by M. bovis BCG (18). By using the appropriate gene fragments of that gene as probes, it appears possible to clone each α-antigen gene from the various nontuberculous mycobacteria. Furthermore, comparison of the DNA sequence for each α-antigen gene is of particular significance, because it may disclose not only the structural characteristics of the species-specific antigenic site but also the region critical for the physiological and pathological activities of this protein.

In this report, we describe the cloning and characterization of the α-antigen gene of M. kansasii and discuss the structural similarities and differences between M. bovis BCG and M. kansasii. We also describe the expression of the cloned gene in Escherichia coli to give an unfused protein which reacted with polyclonal antibodies for the α antigen purified from M. kansasii (α-K).
blotting (27) of restriction fragments of M. kansasii DNA fractionated by 0.8% agarose gel electrophoresis was performed as described previously (18). Filters were wetted with hybridization solution (5 × SSC [1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5 × Denhardt solution [16], 0.1% sodium dodecyl sulfate [SDS]) containing 5 × 10^6 cpm of 32P-labeled probes. After hybridization at 58°C for 18 h, the filters were washed once with 2 × SSC-0.1% SDS for 10 min at room temperature and four times with 0.1× SSC-0.1% SDS for 15 min at 60°C. Then they were dried and autoradiographed.

**DNA cloning.** The *KpnI* fragments with lengths of 2.2 to 3.0 and 5.0 to 6.3 kbp were harvested by the DE81 paper method. Ligation of these fragments with *KpnI*-digested pUC18 and transformation of *E. coli* JM109 were carried out as previously described (18). Ampicillin-resistant (Amp'^r') transformants were screened by the colony hybridization technique (10) by using both probes A and B.

**DNA sequencing.** A 2.0-kbp *HincII* fragment from the inserted DNA of pKA-52 was subcloned into the *HincII* site of pUC18 (pKAH20). Similarly, *XhoI-HincII* fragments were subcloned into the *Smal-Sall* site, *HincII-NaeI*, and *Nael* fragments were subcloned into the *HincII* site, *HincII-PstI* fragments were subcloned into the *HincII-PstI* site, and *Sau3AI* fragments were subcloned into the *BamHI* site of pUC18. These plasmids were used for nucleotide sequence determination by the deoxyxy-chain termination method with intact pUC plasmids (11). Moreover, to confirm the continuity of the *Nael* fragments, we synthesized a 17-mer primer which possessed the sequence 5′-TTCTGGGCTGTTGGG A-3′ and used it for sequencing.

**Construction of α-antigen expression vector.** *E. coli* expression vector pKK232-2 was digested with both EcoRI and *HindIII*. An approximately 300-bp EcoRI-*HindIII* fragment was obtained, including the *rc* promoter (3), the Shine-Dalgarno sequence, and the *NcoI* cloning site, and this was recloned into the *EcoRI-HindIII* site of pUC18. The unique *PstI* site of the resulting plasmid (designated pUCK10) was changed to an *XhoI* linker by inserting an *XhoI* linker into the *PstI* site, which was blunted by using a DNA-blunting kit. This plasmid was named pUCK20. In addition, pKAH20, into which the 2.0-kbp *HincII* fragment was subcloned, was digested with either *XhoI* and *HindIII* or *BamHI* and *XhoI*. Digestion generated a 1.1-kbp *XhoI-HindIII* fragment and a 0.5-kbp *BamHI-XhoI* fragment. These, respectively, included the sequence for Ser-183 to the C-terminal Arg-285 and for Val-13 to Pro-182 of the *M. kansasii* α-antigen gene and were similarly isolated by the DE81 paper method. The 1.1-kbp fragment was ligated with *XhoI-HindIII* double-digested pUCK20, giving rise to plasmid pUCK100. This plasmid, however, lacked the sequence for Phe-1 to Pro-182. Then, the 0.5-kbp *BamHI-XhoI* fragment was inserted into the *NcoI-XhoI* site of pUCK100 by using two kinds of synthetic adaptors connected between the *NcoI* and *BamHI* sites. The sequences for adaptor A (wild type) were 5′-CATGTTCCTCC CTTCGCCGCTGCCGTTGGAGTACCCACGAG-3′ for the upper strand and 5′-GCACCTGTGTTGACTCCACCGCA GGGCGGAGCAAGGAGA-3′ for the lower strand, and those for adaptor B (AT-rich type) were 5′-CATGTTCCTCC CTTCGCCGCTGCCGTTGGAGTACCCACGAG-3′ for the upper strand and 5′-GCACCTGTGTTGACTCCACCGCA GGGCGGAGCAAGGAGA-3′ for the lower strand. The resulting plasmids, named pUCK200 and pUCK201, contained adaptors A and B, respectively.

**Immunological techniques.** Polyclonal antibodies raised against α-K were prepared as described by Tasaka and Matsuo (30). During induction, culture of *E. coli* JM109 containing the α-antigen expression vector was performed in 1 mM isopropyl-β-D-thiogalactopyranoside at 30°C for 4.5 h. Except for the variation in induction conditions, preparation of cell lysates, SDS-polyacrylamide gel electrophoresis, and Western blot (immunoblot) assays were performed as described in our previous report (18).

**RESULTS**

**Southern hybridization analysis.** Restriction fragments of *M. kansasii* total cellular DNA were fractionated by agarose gel electrophoresis, and genomic Southern hybridization was performed with either probe A or probe B. The results are presented in Fig. 1. In both cases, two radioactive bands were observed with probes A and B on *BamHI*- and *KpnI*-digested gels. Since the 5.5- and 2.7-kbp *KpnI* fragments seemed to be convenient for cloning, we attempted to clone both into the *KpnI* site of pUC18 and screened colonies by the colony hybridization technique. One positive colony was found after the screening of 200 Amp'' white colonies for the 5.5-kbp fragment. The recombinant plasmid extracted from this colony was designated pKA-52 and was used for further study.

**Nucleotide sequence.** To elucidate the location of the α-antigen gene, we carried out Southern hybridization experiments on restriction fragments of pKA-52. The α-antigen gene appeared to be located on a 2.0-kbp *HincII* fragment which was derived from the 5.5-kbp *KpnI* fragment (data not shown), so this 2.0-kbp *HincII* fragment was subcloned into pUC18 and sequenced. The nucleotide sequence of the 1.396 bp and the amino acid sequence deduced are shown in Fig. 2 in comparison with the *M. bovis* BCG gene. The nucleotide

![Fig. 1](http://iai.asm.org)
sequence contained an open reading frame which could encode 325 amino acid residues from Met at position 1 to Arg at position 973. To determine the N-terminal amino acid sequence of the mature protein, we performed protein sequencing of the purified α-K by using a 470A protein sequencer (Applied Biosystems Inc.). The sequence of the five N-terminal amino acids was determined to be Phe-Ser-Arg-Pro-Gly, which was identical to that of the M. bovis BCG antigen. This sequence was deduced from the nucleotide sequence, beginning with TTC codon 41. Therefore, the nucleotide sequence for the first 40 codons was expected to encode a signal peptide, and the mature protein must consist of 285 amino acids. Its theoretical molecular weight was calculated as 30,620, which was consistent with the reported value. The G+C content was 65.8% in the open reading frame, and that for position 3 of the codon was 85.0%. This

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the M. kansasii α antigen. Sequences similar to the E. coli consensus sequences are labeled −35 and −10. A likely ribosome-binding site is marked SD. The different amino acid residues in the M. bovis BCG α antigen are shown below the M. kansasii sequence.
pattern was similar to those of other mycobacterial genes isolated from *M. tuberculosis* and *M. bovis* species (18, 24, 32, 33, 36, 37). The promoter sequences (marked −35 and −10 in Fig. 3) and the Shine-Dalgarno sequence were found to be upstream of the open reading frames. The hydrophathy profile is shown in Fig. 3 in comparison with that of the *M. bovis* BCG antigen.

Expression of the cloned gene in *E. coli*. Using an *E. coli* expression vector, we examined whether the cloned gene product reacted with an antibody raised against the purified α-K. In the construction of the α-antigen expression vector, we made two attempts to improve the level of expression. The first was to increase the copy number of the expression vector by exchanging the replication origin from pKK233-2 to pUC18. The second was to use AT-rich synonymous codons for the adaptor connecting the α-antigen gene fragment with the *NcoI* site of pKK233-2, to weaken the possibly stable secondary structure of the mRNA. The strategy for construction of the α-antigen expression vector is shown in Fig. 4. *E. coli* JM109 was transformed with pUCK200 and pUCK201, and the transformants were designated AJK200 and AJK201, respectively. Crude lysates of the cultured cells were analyzed by SDS-polyacrylamide gel electrophoresis and Western blot assays. The results are presented in Fig. 5. Although no protein was detected in the control lysate (*E. coli* JM109 harboring pUCK20, lane 9) by polyclonal antibodies to α-K, a single reactive protein migrating with a molecular weight similar to that of α-K was detected in the AJK200 and AJK201 transformants (lanes 7 and 8). The level of expression in the AJK201 transformant harboring pUCK201 was slightly improved in comparison with AJK200, which had the wild-type sequence.

**DISCUSSION**

We have described the successful gene cloning and nucleotide sequencing of the α-antigen of *M. kansasii* in this paper. This is the first report on the sequencing of an atypical mycobacterial gene. Gene analysis revealed that the mature α-antigen from *M. kansasii* consisted of 285 amino acids, which was two residues longer than that of *M. bovis* BCG (18). The gap of two amino acids can be ascribed to the absence of Pro-161–Ser-162 at positions 601 to 606 (Fig. 2) in the α-antigen gene of *M. bovis* BCG. By reexamination of the nucleotide sequence, we found a sequencing error which indicated that the *M. bovis* BCG α-antigen has an additional two amino acids, Pro-161 and Ser-162, as deduced from CCTAGC. It was therefore shown that the *M. bovis* BCG α-antigen actually consists of 285 amino acids and its deduced molecular weight was calculated as 30,666.

Comparison of the nucleotide sequences of the α-antigen isolated from *M. bovis* BCG and *M. kansasii* showed that the nucleotide and amino acid homologies were 84.8 and 89.1%, respectively. The mutation rates at positions 1 to 3 of the codon were 12.3, 3.4, and 29.8%, respectively, indicating that nucleotide substitution at the silent position was predominant. This is in accord with the neutral theory of molecular evolution advanced by Kimura (14). In the amino acid substitutions, although the dominant changes were between hydrophobic amino acids or structurally analogous amino acids, some drastic alterations to amino acids with different properties were also observed, which indicated that the surface fine structure of this protein was likely to be changeable. In the comparison of hydrophathy profiles (Fig. 3), two remarkably different regions were detected at amino acid residues 10 to 30 and 180 to 200. In the former region, there was a substitution of Ala-16 for Pro, which predicted occasional involvement of a β-turn potential in an antigenic site (7). The latter region contained a hydrophilic fragment with five amino acid exchanges, which might account for the antigenic diversity of the α-antigen or its acquirement of antigenic specificity. The evolutionary substitutions (Gln→Glu, Ser→Thr, Leu→Gln, His→Gln, and Glu→Lys) might cause local conformational changes. The species-specific linear epitope, when successfully deduced, would enable us to perform a variety of diagnostic studies. Work is in progress to define the antigenic determinants by using an absorbed antibody specific for *M. kansasii*. Interestingly, the 5′ upstream region of the open reading frame, including the putative promoter and the Shine-Dalgarno sequences, was highly conserved, whereas the 3′ downstream region after the stop codon possessed no homology, just like the *nlyB* and *nlyB′* genes that encode a nylon oligomer-degrading enzyme (21). Therefore, this region might not be functionally important.

It should be noted that the α-antigen of *M. kansasii* has a characteristic signal peptide sequence. The core region,
FIG. 4. Schematic outline of the construction of an α-antigen expression vector by use of an E. coli expression vector and cloned mycobacterial DNA. The inserted DNA, which contained the sequence for the C-terminal part of the α antigen, is shown by a thick black line. The cross-hatched region shows DNA that was inserted later and contains the sequence for Val-13 to Pro-182 of the α antigen. Details are described in Materials and Methods. kb, Kilobases.

FIG. 5. Analysis of proteins from recombinant clones by SDS-polyacrylamide gel electrophoresis and Western blot assay. (A) Staining with Coomassie brilliant blue for visualization of all proteins. (B) Protein which reacted with an antibody to α-K. Lanes: 1, size markers; 2 and 6, 2 μg of α-K; 3 and 7, lysate of clone AJK200; 4 and 8, lysate of clone AJK201; 5 and 9, lysate of E. coli JM109 harboring pUCK20 (control). Molecular size standards (Pharmacia) were as follows: α-lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30.0 kDa), ovalbumin (43.0 kDa), bovine serum albumin (68.0 kDa), and phosphorylase b (94.0 kDa).

signal peptides were completely coincident with the −3, −1 rule, as observed in this work. This indicates that the mycobacterial signal peptidase should have high substrate specificity.

It has been reported by Abou-Zeid et al. (2) that the BCG85 complex or MPB59 (35), which is probably identical to the α antigen, has the unique property of binding to fibronectin (FN). In this context, FN-binding protein has also been found on the cell surface of Staphylococcus aureus (8). The gene for this protein was recently cloned, and a sequence essential for binding to FN has been reported (26). The same sequence was not found in the amino acid sequences of the α antigens from M. bovis BCG (18) and M. kansasii. Presumably, these α antigens play the same role as the S. aureus FN-binding protein, although their mode of binding is not clear. To determine the sequence or residues essential for binding to FN, FN-binding activity could be examined with either fragments of α antigen or synthetic peptides. Such studies could clarify the physiological and pathological roles of this protein in mycobacteria but remain to be performed.

The cloned gene for the α antigen was expressed as an unfused protein in E. coli. The expression vector was constructed by using a high-copy-number plasmid, pUC18, with a highly expressed trc promoter. Since the alteration of a GC-rich sequence to an AT-rich sequence in the codons has been reported to improve expression yield (6, 12, 15), we used AT-rich synonymous codons around the initiation codon but found little improvement in the yield. The low expression level in E. coli is a common phenomenon in mycobacterial secreted antigens, such as the α antigen of M. bovis BCG (18) and MPB64 (36). In contrast, MPB57 (37) and the 65-kilodalton (kDa) antigen (24, 33), which are cytoplasmic proteins, have shown high expression in E. coli. It may be that some foreign proteins expressed in the E. coli cytoplasm are easily attacked by proteases, such as ATP-dependent protease La (5). Further attempts to secrete gene
products into the *E. coli* periplasmic space or to use other host-vector systems are under consideration.

Interest in the development of a useful vaccine vehicle from living *M. bovis* BCG has led to the suggestion that the *M. kansasii* α antigen could be used as a carrier protein to secrete foreign antigens. The strategy of using the α antigen has several advantages. (i) It has excellent membrane permeability because of its structural similarity to the *M. bovis* BCG α antigen. (ii) The antigen has the *M. kansasii*-specific marker (30) which can be easily checked for with the absorbed anti-α serum. Therefore, the species-specific epitope could be used as a label (9) for confirmation of chimera protein expression. (iii) The antigen itself is highly immunogenic and elicits a delayed-type hypersensitivity reaction, suggesting that the use of such a carrier protein to induce cell-mediated immunity might well lead to an efficacious vaccine (23). Recently, it has been reported that spleen cells from *M. bovis* BCG-sensitized mice produced significant amounts of gamma interferon in response to the P32 antigen (13), which probably corresponds to our α antigen. Thus, it is reasonable to expect that humoral and cell-mediated immune responses to foreign antigens could be induced in the presence of *M. bovis* BCG. Such studies are in progress.

**ADDENDUM**

After we submitted this report, Borremans et al. (4) described the cloning, sequence determination, and expression of a 32-kDa-protein gene of *M. tuberculosis*. This protein was found to be highly homologous to the *M. bovis* BCG α antigen.

**LITERATURE CITED**


