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ESAT-6 and CFP-10 in Clinical versus Environmental Isolates of *Mycobacterium kansasii*

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Mycobacterium kansasii consists of 5 genetically distinct groups, of which 2 are associated with human disease. Determinants of the differences in virulence are unknown. Potential genes of interest are esat-6 and cfp-10, which are associated with virulence of Mycobacterium tuberculosis and Mycobacterium bovis but are lacking in bacille Calmette-Guérin and in most environmental mycobacteria (M. kansasii is an exception). We investigated esat-6 and cfp-10 genes in 22 clinical and 14 environmental isolates of M. kansasii. Both were present in all isolates; each genetic group had its own characteristic Southern-blot pattern corresponding to a highly conserved fingerprint pattern. Nucleotide sequences of the genes differed 12.6% and 10.1%, respectively, from the M. tuberculosis homologues, but the deduced amino acid sequences were <5% different. In vitro, clinical and environmental genotypes of M. kansasii expressed CFP-10 and ESAT-6. Thus, virulence of M. kansasii is not directly related to esat-6 and cfp-10 genes or gene expression.

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Mycobacterium kansasii differs from most other environmental mycobacteria in its ability to cause disease in otherwise healthy and presumably immunocompetent persons, whereas the majority of the environmental mycobacteria are mainly associated with disease in immunocompromised individuals. Recognized risk factors for *M. kansasii* pneumonia are male sex, older age, smoking, and preexisting lung disease, but infection can occur in the absence of such risk factors. However, *M. kansasii* is a genetically heterogeneous species. On the basis of genotyping of the 16S 23S intergenic spacer region, which is located between the genes encoding the 16S and 23S ribosomal RNA genes, *M. kansasii* can be divided into 5 genetically distinct groups (MKA-I through MKA-V). In previous studies, clinical isolates were mainly MKA-I and less often MKA-II, whereas environmental isolates were MKA-II, MKA-III, MKA-IV, or MKA-V [1 3]. The MKA-II genotype has been associated with infection in immunocompromised patients [4, 5], which could reflect a lower pathogenic potential than the MKA-I genotype. This partly overlapping dichotomy suggests the presence of genetically based differences in virulence between clinical and environmental *M. kansasii* strains, but no specific virulence genes have thus far been identified.

M. kansasii and *Mycobacterium tuberculosis* have several characteristics in common. They have been found, on the basis of the 16S–23S rRNA gene internal transcribed spacer (ITS) regions, to be phylogenetically related [6]. *M. kansasii* pneumonia is clinically and radiographically indistinguishable from classical pulmonary tuberculosis, which could reflect similarities in pathogenesis [7, 8]. Thus, to study potential virulence genes in *M. kansasii,* interesting genes would be those that are shared by *M. tuberculosis* and *M. kansasii* but that are lacking in other, less virulent environmental mycobacteria. In previous studies, comparative genomics between *M. tuberculosis* or pathogenic *Mycobacterium bovis* and the attenuated vaccine strain *M. bovis* bacille Calmette-Guérin (BCG) led to the identification of regions of difference, one of which, named "RD1," was found to be present in all *M.*

tuberculosis and pathogenic *M. bovis* strains but is lacking from all BCG strains [9, 10]. Several RD1-encoded proteins, of which ESAT-6 and CFP-10 have been studied most thoroughly, have been identified as potent T cell antigens in both animals and humans [11 13]. These antigens are currently under investigation for diagnostic use, to discriminate infection with *M. tuberculosis* from vaccination with BCG [14–18]. Besides potential diagnostic use, insertion and deletion studies have also demonstrated that RD1-encoded genes are important for mycobacterial virulence in experimental animals [19, 20] and that the loss of RD1 was relevant for the attenuation of BCG [21].

Although the *esat-6* and *cfp-10* genes were lacking in most environmental mycobacteria and all BCG strains, they were demonstrated, by polymerase chain reaction (PCR) and/or Southern-blot analysis, to be in *M. kansasii* and *Mycobacterium marinum,* both of which can cause disease in apparently immunocompetent persons, and in the essentially apathogenic *Mycobacterium szulgai, Mycobacterium flavescens,* and *Mycobacterium gastrii* [11, 22 24]. Thus, *esat-6* and *cfp-10* represent suitable candidates for further investigation with regard to the virulence of *M. kansasii.* The present study aimed to investigate the *esat-6* and *cfp-10* genes and gene expression in a large collection of clinical and environmental isolates of *M. kansasii.*

MATERIALS AND METHODS

Mycobacterial isolates. The *esat-6* gene was studied in 30 different isolates of various mycobacterial species from the available collection at the National Institute of Public Health and the Environment (Bilthoven, The Netherlands) (table 1). For comparison, 1 BCG strain and *M. tuberculosis* H_{37} Rv were used. Next, a total of 36 *M. kansasii* isolates were studied. The origin and source of these isolates are listed in table 2. The isolates from the Czech Republic, Italy, Belgium, and Germany were retrieved from the strain collection of the Mycobacteriology Unit of the Institute of Tropical Medicine in Antwerp, Belgium. The isolates from The Netherlands originated from the strain collection of the Mycobacteria Department at the National Institute of Public Health and the Environment.

Table 1. Isolates of various mycobacterial species tested with esat-6 restriction-fragment length polymorphism.

Species	RIVM no.	*esat-6* RFLP
M. kansasii gastrii	Myc 4296	Positive, type IV
M. kansasii gastrii	8842	Positive, type IV
M. kansasii	Atyp 889	Positive, type I
M. kansasii	Myc 212	Positive, type I
M. marinum	Myc 1580	Positive
M. marinum	ATCC 927	Negative
M. marinum	Mis 101	Positive
M. marinum	9900037	Negative
M. marinum	9900872	Negative
M. avium paratuberculosis	B8524	Negative
M. avium paratuberculosis	316F	Negative
M. avium paratuberculosis	Jeltema	Negative
M. avium hominissuis	IWGMT 29	Negative
M. avium hominissuis	IWGMT 23	Negative
M. avium avium	IWGMT 17	Negative
M. malmoënse	J10792	Negative
M. malmoënse	S709	Negative
M. xenopi	Myc 527	Negative
M. xenopi	SCS 74/47	Negative
M. gordonae	ATCC 14470	Negative
M. gordonae	Myc 813	Positive
M. gordonae-ureoliticum	Myc 1064	Negative
M. chelonei abscessus	Myc 11303	Negative
M. chelonei abscessus	Myc 4662	Negative
M. chelonei abcsessus	Myc 11303	Negative
M. peregrinum	11539	Negative
M. peregrinum	Myc 235	Negative
M. fortuitum	11401	Negative
M. fortuitum	Hb5794	Negative
M. xenopi	Scs 74/47	Negative
M. bovis BCG (Pasteur)	...	Negative
M. tuberculosis H37Rv	...	Positive

NOTE. BCG, bacille Calmette-Guérin; RIVM no., identification code of the collection of nontuberculous mycobacteria at the National Institute of Public Health and the Environment.

Table 2. Characteristics of 36 isolates of Mycobacterium kansasii.

RIVM no.	Year of isolation	INNO LiPA (MKA) type	*esat-6* RFLP	MPTR RFLP	*esat-6* sequence	*cfp-10* sequence	Source (patient code)[a]	Country of isolation
NLA000014036	1991	I	I	I	I	I	Human (A)	The Netherlands
NLA000014051	1991	I	I	I	I	I	Human (B)	The Netherlands
NLA000016152	1992	I	I	I	I	I	Human (C)	The Netherlands
NLA000016483	1993	I	I	I	I	I	Human (D)	The Netherlands
NLA000016529	1993	I	I	I	I	I	Human (E)	The Netherlands
NLA000017001	1993	I	I	I	I	I	Human (F)	The Netherlands
NLA000017097	1993	I	I	I	I	I	Human (F)	The Netherlands
NLA000017010	1993	I	I	I	I	I	Human (G)	The Netherlands
NLA000017202	1993	I	I	I	I	I	Human (G)	The Netherlands
NLA000017098	1993	I	I	I	I	I	Human (H)	The Netherlands
NLA009501577	1995	I	I	I	I	I	Human (I)	The Netherlands
NLA009600029	1995	I	I	I	I	I	Human (J)	The Netherlands
NLA009700554	1997	I	I	I	I	I	Human (K)	The Netherlands
NLA009700576	1997	I	I	I	I	I	Human (K)	The Netherlands
NLA009800036	1997	I	I	I	I	I	Human (L)	The Netherlands
NLA009702392	1997	I	I	I	I	I	Human (M)	The Netherlands
NLA009802295	1998	I	I	I	ND	ND	Human (N)	The Netherlands
NLA009900419	1999	I	I	I	I	I	Human (O)	The Netherlands
NLA009902193	1999	I	I	I	I	I	Human (P)	The Netherlands
NLA000000893	2000	I	I	I	I	I	Human (Q)	The Netherlands
NLA000101548	2001	I	I	I	I	I	Human (R)	The Netherlands
NLA000001494	1995	I	I	I	I	I	Shower water	Czech Republic
NLA000001495	1995	I	I	I	I	I	Hot tap water	Czech Republic
NLA000001049	2000	II	II	II	II	II	Human (S)	The Netherlands
NLA000001469	1994	II	II	II	II	II	Environment	Italy
NLA000001471	1994	II	II	II	II	II	Environment	Italy
NLA000001454	1991	III	III	III	III	III	Tap water	Belgium
NLA000001493	1995	III	ND	ND	IIIb	IIIb	Tap water	Germany
NLA000001457	1993	IV	IV	IV	IV	IV	Biofilm	Germany
NLA000001458	1993	IV	IV	IV	IV	IV	Tap water	Germany
NLA000001470	1994	IV	ND	ND	IV	IV	Environment	Italy
NLA000001459	1995	V	V	V	V	V	Tap water	Belgium
NLA000001464	1995	V	V	V	V	V	Tap water	Belgium
NLA000001465	1996	V	V	V	V	V	Tap water	Belgium
NLA000001461	1996	V	V	V	V	V	Toilet	Belgium
NLA000001468	1996	V	V	V	V	V	Soil	Belgium

NOTE. ND, not determined; MPTR, major polymorphic tandem repeat; RIVM no., identification code of the collection of nontuberculous mycobacteria at the National Institute of Public Health and the Environment; RFLP, restriction-fragment length polymorphism.

[a] Each capital denotes a different patient; 2 isolates from patients F, G, and K were included.

Characterization of the ITS subtypes. ITS subtypes were determined by use of INNO-LiPA Mycobacteria (Innogenetics), a DNA probe test that targets the 16S–23S rDNA ITS sequence, as described elsewhere [27]. This commercially available assay differentiates between *M. kansasii* group I and II and detects group III, IV, and V without discriminating among these ITS types. To differentiate between the latter types, a research LiPA strip (strip K) was used (Innogenetics; data not shown) that contained specific DNA probes for each ITS type. The *M. kansasii* isolates included all 5 genotypes.

Culture condition. *M. kansasii* isolates were kept at -70°C in aliquots in Middlebrook 7H9 supplemented with oleic acid albumin dextrose catalase and 15% glycerol until testing. Cultures were grown on Löwenstein-Jenssen medium at 35.5°C until sufficient growth was obtained for Western blotting and DNA isolation.

Southern-blot analysis. Southern blots of the *M. kansasii* isolates were prepared as described elsewhere [25]. Briefly, DNA isolation was performed by use of the N-cetyl-N,N,N,-

trimethyl ammonium bromide method. The purified DNA was digested with the restriction enzyme *Pvu*II and subjected to electrophoresis. A mixture of a *Pvu*II-digested supercoiled DNA ladder (Invitrogen) and *Hae*III-digested phiX174 DNA was used as an internal DNA size marker. The DNA fragments were transferred to a DNA membrane. The DNA membranes were hybridized with the major polymorphic tandem repeat (MPTR) [26], and complete *esat-6* and *cfp-10* genes were hybridized with the enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection systems (Amersham Biosciences Europe). The probes of the *esat-6* and *cfp-10* genes were obtained by PCR by use of *M. tuberculosis* H_{37}Rv DNA as target and were purified as described below. The primer sets used were Esa-12 CAT GAC AGA GCA GCA GTG and Esa-303 5′-GCC CTA TGC GAA CAT CCC-3′ [11] for *esat-6* and opBR78 5′-GTA GCC CGG GAT GGC AGA GAT GAA GAC CGA TGC C-3′ and opBR103 5′-TCA GAA GCC CAT TTG CGA GGA CAG C-3′ for *cfp-10.* Computer-assisted analysis of the restriction-fragment length polymorphism (RFLP) patterns was done by use of BioNumerics software (version 3.0; Applied Maths).

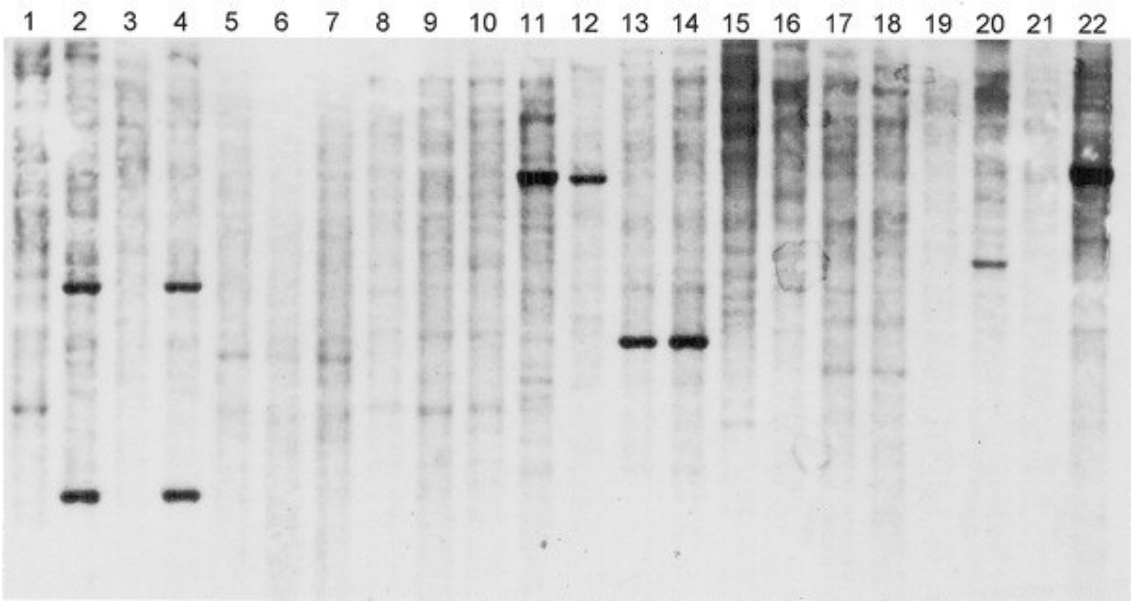
Nucleotide sequencing. Amplification of the *esat-6* and *cfp-10* genes was performed by use of 10 pmol of the primers described above. The primers, together with 50 ng of purified DNA, were added to 12.5 μL of HotStarTaq Master mix (Qiagen), and the reaction volume was adjusted to 25 μL with Milli-Q water (Millipore). The amplification mixture was subjected to initial denaturation for 3 min at 96°C; followed by 30 cycles at 96°C for 1 min, 60°C for 2 min, and 72°C for 3 min; and a final extension period at 72°C for 6 min. The PCR products were purified by use of a PCR purification kit (Qiagen). The nucleotide sequence of the *esat-6* and *cfp-10* genes was determined on both strands by direct sequencing of the PCR products on an automated capillary sequencer (model 310; Applied Biosystems) by use of fluorescence-labeled dideoxynucleotide terminators (ABI PRISM BigDye Terminator cycle sequencing ready reaction kit; Applied Biosystems). Comparison of the sequence data of *esat-6* and *cfp-10* of the environmental and clinical *M. kansasii* isolates with the *M. tuberculosis* sequence of H37Rv was performed by use of DNASTAR software (version 5.0; DNAstar).

Western-blot analysis. Lysates of 2 clinical and 4 environmental *M. kansasii* isolates were obtained after culturing in modified Sauton medium at 37°C on an orbital shaker, as described elsewhere [28]. Bacteria were harvested after centrifugation, and the bacterial pellet was washed once in PBS (pH 7.4) and resuspended in a suspension that contained equal volumes of 0.1-mm glass beads and PBS with 1 mmol/L phenylmethylsulfonyl fluoride. The bacteria were thereafter lysed on a Mini-BeadBeater (Biospec Products), and the protein concentration was determined by the bicinchoninic acid method (Pierce). Ten micrograms of each protein lysate was applied for SDS-PAGE and Western-blot analysis, as described elsewhere [12]. As a reference, a short-term culture filtrate of *M. tuberculosis* was included [28]. ESAT-6 was detected by the monoclonal antibody HYB 76-8 [29], and CFP-10 was detected by the rabbit antibody K8493 raised against recombinant CFP-10, as described elsewhere [30]. The ECL Western-blotting system (Amersham Biosciences) was used for chemiluminescence detection of Western blots.

RESULTS

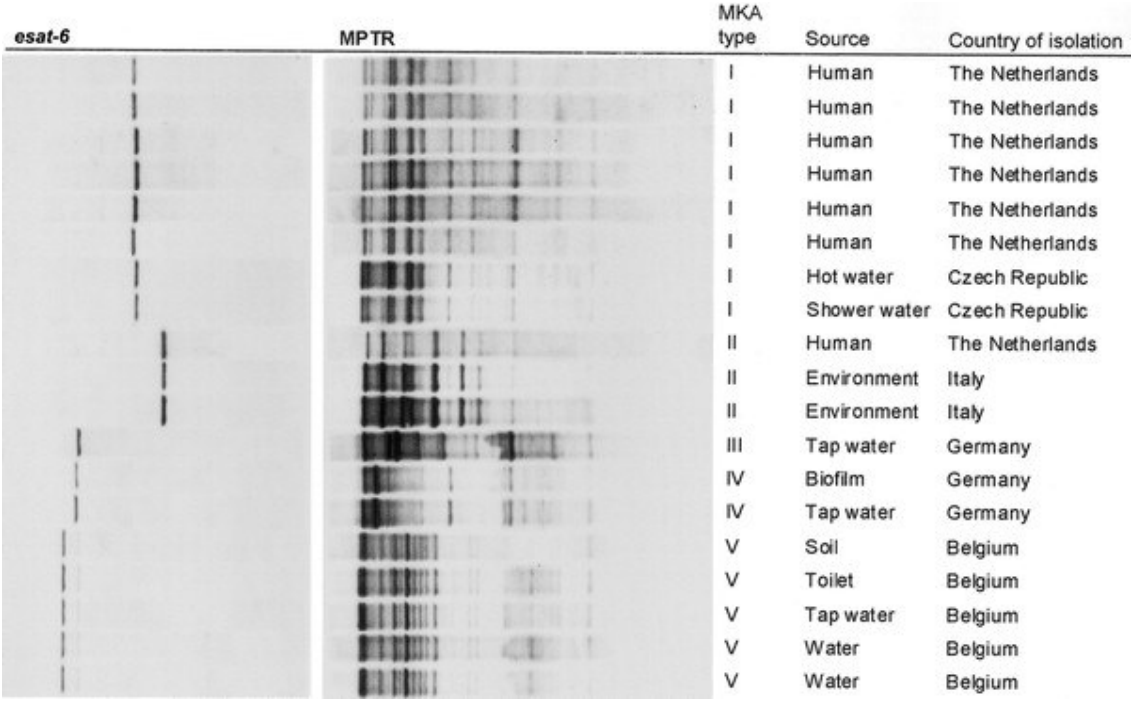
esat-6 ***RFLP in different mycobacterial species.*** Hybridization of a Southern blot containing DNA of a large variety of mycobacterial species with the *esat-6* probe showed that the *esat-6* or "*esat-6*–like" gene was present in *M. tuberculosis, M. kansasii* (MKA-I), *M. kansasii gastrii* (MKA-IV), and 2 of 5 *M. marinum* isolates (table 1). A weak but positive band was observed in 1 of 2 *Mycobacterium gordonae* isolates. The isolates were retested, with identical results. When the *cfp-10* probe was used, all 5 *M. marinum* isolates gave a positive band. No *esat-6* or *esat-6*–like genes were found in *Mycobacterium avium hominissuis* [31], *M. avium avium, M. avium paratuberculosis, Mycobacterium malmoënse, Mycobacterium xenopi, Mycobacterium chelonei abscessus, Mycobacterium peregrinum, Mycobacterium fortuitum,* or *M. bovis* BCG (Pasteur), confirming previous findings [11]. A representative selection of these findings is presented in figure 1.

Figure 1 Southern-blot analysis by use of the esat-6 probe of Mycobacterium tuberculosis on a selection of mycobacterial species (listed in table 1). National Institute of Public Health and the Environment nos. of the isolates are in parentheses after the species name. The apparent vague bands in lane 15 are artifacts. Lane 1, Mycobacterium bovis bacille Calmette-Guérin (P3); lanes 2 4, Mycobacterium marinum (Myc 1580, ATCC 927, Mis101); lanes 5 7, Mycobacterium avium paratuberculosis (B8524, 316F, Jeltema); lanes 8 and 10, M. avium hominissuis (IWGMT 29 and 23); lane 9, M. avium avium (IWGMT 17); lanes 11 and 12, Mycobacterium kansasii gastrii (Myc 4296 and 8842); lanes 13 and 14, M. kansasii (Atyp 889 and Myc 212); lanes 15 and 16, Mycobacterium malmoënse (J10792 and S709); lanes 17 and 18, Mycobacterium xenopi (Myc 527 and SCS 74/47); lanes 19 and 20, Mycobacterium gordonae (ATCC 14470 and Myc 813); lane 21, Mycobacterium chelonei (Myc 11303); and lane 22, Mycobacterium tuberculosis (H37Rv).



Association of MPTR-RFLP,* esat-6 *RFLP, and* cfp-10 *RFLP, respectively, among* M. kansasii *genotypes. Southern-blot analysis of the *esat-6* and *cfp-10* genes demonstrated that a clear distinction could be made between clinical and environmental isolates of *M. kansasii* that corresponded to MPTR pattern distribution among the MKA genotypes. The MPTR-RFLP and *esat-6* RFLP of a representative selection of isolates is shown in figure 2. Within each genotype, the pattern of the remaining isolates listed in table 2 were identical. For all isolates, the results obtained with *cfp-10* RFLP were identical to those of the *esat-6* RFLP.

Figure 2 Southern-blot analysis by use of the esat-6 probe of Mycobacterium tuberculosis and major polymorphic tandem-repeat (MPTR) fingerprinting in relation to the Mycobacterium kansasii genotype, according to the 16S 23S intergenic spacer region. Similar results were obtained with the cfp-10 probe (data not shown).



MKA type	Source	Country of isolation
I	Human	The Netherlands
I	Human	The Netherlands
I	Human	The Netherlands
I	Human	The Netherlands
I	Human	The Netherlands
I	Human	The Netherlands
I	Hot water	Czech Republic
I	Shower water	Czech Republic
II	Human	The Netherlands
II	Environment	Italy
II	Environment	Italy
III	Tap water	Germany
IV	Biofilm	Germany
IV	Tap water	Germany
V	Soil	Belgium
V	Toilet	Belgium
V	Tap water	Belgium
V	Water	Belgium
V	Water	Belgium

Gene sequencing. Of the 5 different genotypes of *M. kansasii,* the nucleotide sequences of the PCR products of the *esat-6* and *cfp-10* genes were determined, including at least 2 isolates per genotype (figure 3*A* and 3*B*). Within each genotype, the sequences of *esat-6* and *cfp-10* genes were always 100% identical, except for MKA-III. Comparison of the 2 MKA-III genotype isolates that were sequenced showed a 1-bp difference in both the *esat-6* and *cfp-10* genes. When genotype MKA-I was used as a reference, the DNA sequences of the *esat-6* gene (285 bp) differed between 0.3% (1 bp in MKA-IV) and 2.1% (6 bp in MKA-III), whereas the *M. tuberculosis* homologue differed by 10.5% (30 bp). For the *cfp-10* gene (300 bp), the observed differences varied between 0.7% (2 bp in MKA-II) and 1.3% (4 bp in genotypes MKA-IIIb, -IV, and -V), whereas the *cfp-10* gene of *M. tuberculosis* differed by 10% (30 bp). The deduced amino acid sequences were aligned with the *M. tuberculosis* homologues, as is shown in figure 4*A* and 4*B*. The putative ESAT-6 and CFP-10 proteins of *M. kansasii* differed by only 2 or 3 and 5 or 7 amino acids, respectively, from the *M. tuberculosis* protein. Importantly, ESAT-6 and CFP-10 of the MKA-I and MKA-II genotypes were either identical to those of the environmental genotypes or differed by 2 aa at most.

Figure 3 Alignment of the nucleotide sequence of esat-6 (A) and cfp-10 (B) of Mycobacterium tuberculosis (used as reference) with the nucleotide sequences of the homologues of the 5 Mycobacterium kansasii genotypes. MKA-III and MKA-IIIb indicate 2 different isolates of MKA-III that differed from each other by 1 bp in both genes. At least 2 isolates per genotype were studied, as specified in figure 4.

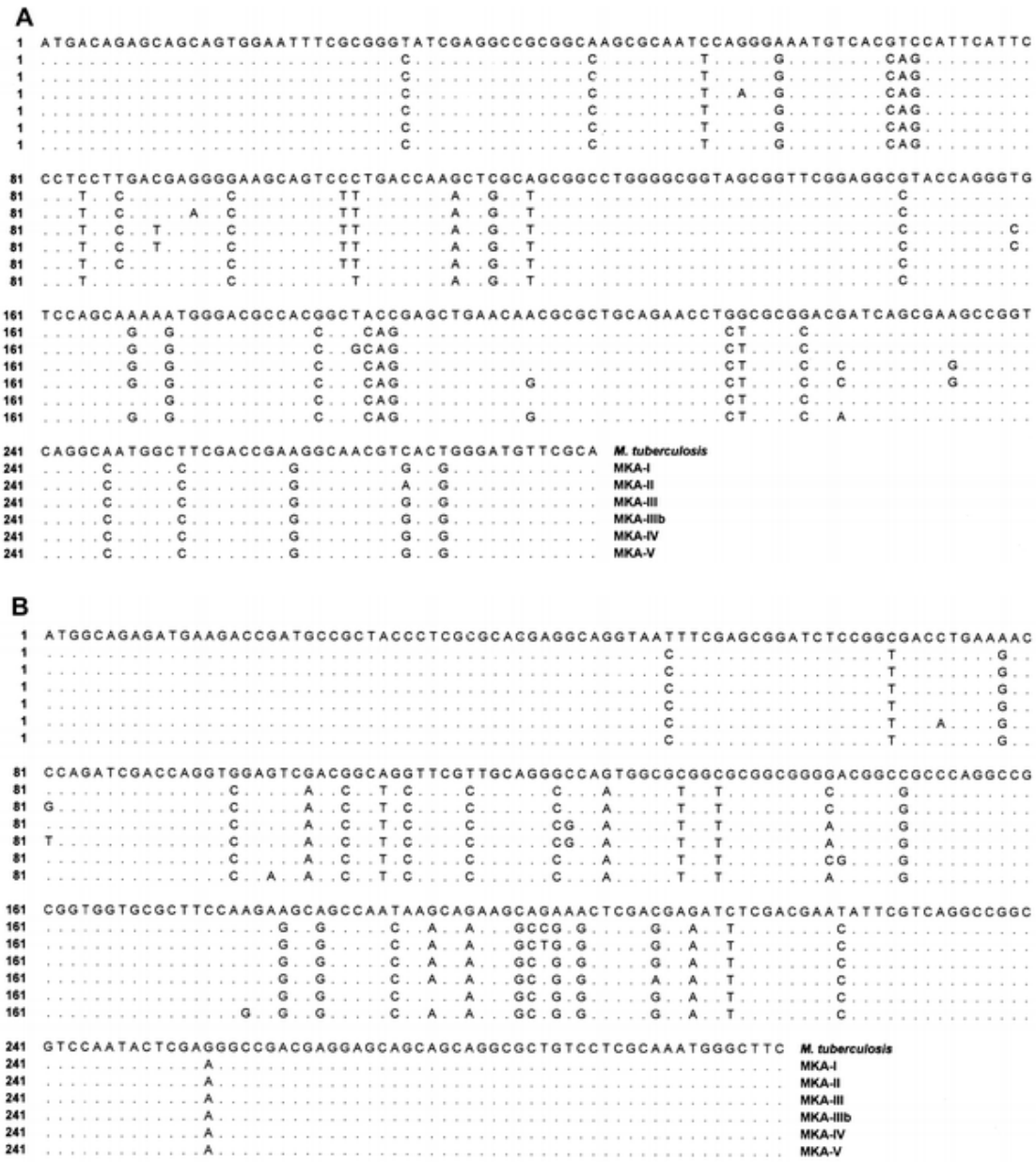
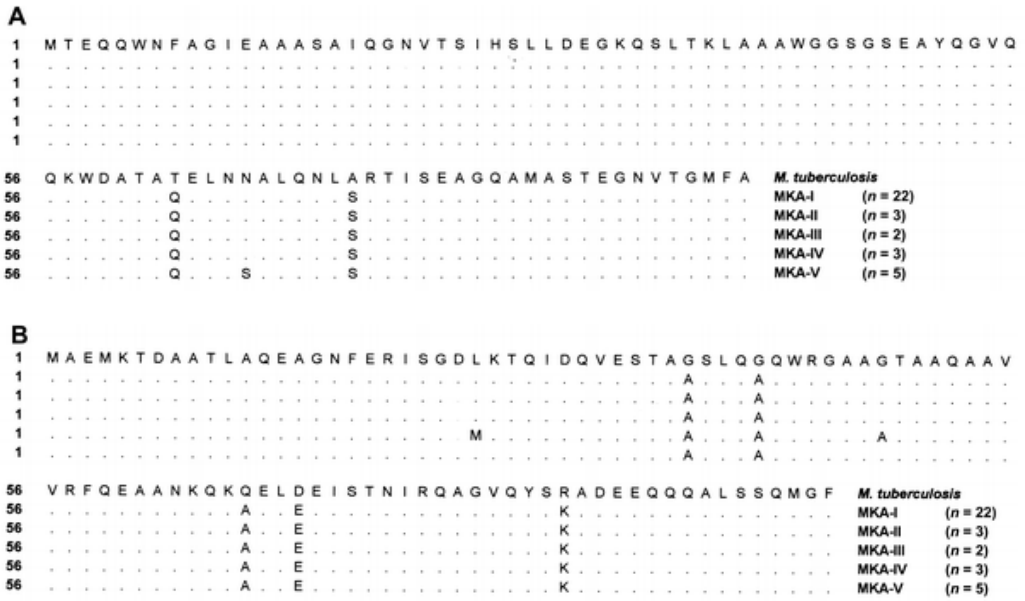


Figure 4 Alignment of the amino acid sequence of ESAT-6 (A) and CFP-10 (B) of Mycobacterium tuberculosis with the deduced amino acid sequences of the homologues in Mycobacterium kansasii isolates. Nos. in parentheses after the MKA genotype indicate the no. of isolates that was studied.



The clinical *M. kansasii* isolates of MKA-I were isolated during the period between 1991 and 2001. With regard to the nucleotide sequences of *esat-6* and *cfp-10,* no difference was observed between isolates over time. Interestingly, the 2 water isolates from the Czech Republic that had previously been found to be MKA-I [32] showed identical MPTR RFLP and *esat-6* and *cfp-10* sequences as the clinical MKA-I isolates originating from The Netherlands. The clinical MKA-I strains that were isolated from patients residing in houses with the MKA-I water isolates were not included in the present study.

Expression of ESAT-6 and CFP-10 by clinical and environmental* M. kansasii *isolates. Having demonstrated that there was no important difference between clinical and environmental isolates with regard to the presence of the *esat-6* and *cfp-10* genes or the deduced amino acid sequence of ESAT-6 and CFP-10, we next studied the expression of these genes during in vitro growth. Immunoblots of strain lysates by use of specific antibodies indicated that the ESAT-6 and CFP-10 proteins were expressed by clinical as well as environmental genotypes of *M. kansasii.* However, semiquantitative measurement by ECL indicated that the level of expression of ESAT-6 and CFP-10 was of a similar and high level in MKA-I and MKA-II isolates, whereas the expression of CFP-10 was relatively lower in MKA-III and MKA-IV isolates (table 3).

Table 3. Western blot analysis of mycobacterial lysates.

	MKA genotype	Source of isolate	ESAT-6 (HYB76 8[a])	CFP-10 (K8493[b])
MTB STCF[c]		C	+++	+++
1995-01577	I	C	+++	+++
1999-00419	I	C	+++	+++
2000-1494	I	E	+++	+++
2000-1469	II	E	+++	+++
2000-1454	III	E	+++	+
2000-1457	IV	E	++	(+)

NOTE. Results are expressed semiquantitatively: +++, strong band after 30 s; ++, strong band after 1 min; +, strong band after 3 min; (+), weak band after 3 min; -, no band after 5 min; C, clinical; E, environmental.

[a] Monoclonal mouse anti ESAT-6 antibody.

[b] Polyclonal rabbit anti CFP-10 antibody.

[c] Short-term culture filtrate of *M. tuberculosis*.

DISCUSSION

In the present study, 36 clinical and environmental isolates of *M. kansasii* were compared with regard to the *esat-6* and *cfp-10* genes and gene expression. The results indicate that homologues of the *esat-6* and *cfp-10* genes are present in clinical and environmental isolates of *M. kansasii.* Southern blots showed a characteristic pattern for each genotypically defined group of isolates, which suggests either that these genes reside on different segments of the genome or that differences exist between genotypes that are associated with changes in the target site for the restriction enzyme, causing variation in fragment size. During in vitro culture, clinical isolates produced roughly equivalent amounts of ESAT-6 and CFP-10, whereas the production of ESAT-6 exceeded that of CFP-10 for some of the environmental isolates. The nucleotide sequences of *esat-6* and *cfp-10* of *M. kansasii* were determined, including a number of isolates of each genotype, to assess inter- and intragenotypic polymorphisms. Within each genotype, the sequences were identical, with the exception of a single nucleotide difference between 2 MKA-III isolates. Compared with those of the *M. tuberculosis* homologue, the *esat-6* sequences of the *M. kansasii* isolates were between 10.1% and 12.6% different, but the deduced amino acid sequences of ESAT-6 were nearly identical. The *cfp-10* gene and the predicted protein of *M. kansasii* differed slightly more from the *M. tuberculosis* homologue, with a difference of 10%–10.3% at the nucleotide level and 5 or 7 aa at the protein level. The deduced amino acid sequences of both ESAT-6 and CFP-10 were identical in 4 of 5 MKA genotypes, with only ESAT-6 of MKA-V and CFP-10 of MKA-IV differing from the other genotypes. Thus, on the basis of the ESAT-6 and CFP-10 sequences, no definite distinction could be made between genotypes.

The notion that genes residing within the RD1 region are required for virulence has been strengthened by the results of a number of recent studies. Deletion of the *esat-6* gene in *M. bovis* [19] or the deletion of RD1 in *M. tuberculosis* has been shown to decrease virulence in experimental animal models [20], whereas the reinsertion of RD1 into BCG or *M. microti* increases virulence [21]. Another study found no difference in virulence between BCG and BCG:*esat-6* in mice [33]. Although the latter finding seems to contradict the results of the above-mentioned studies, the lack of virulence could be explained if both ESAT-6 and CFP-10 are required to confer virulence. The results of our study indicate that, if ESAT-6 and CFP-10 do play a role in the virulence of *M. kansasii,* it does not seem to be directly related to the presence of the genes, given that clinical isolates did not differ from environmental isolates in this respect and differed only partially with regard to in vitro protein expression. The functions of ESAT-6 and CFP-10 have only been partially elucidated. Two in vitro studies have found immunomodulating properties of CFP-10 on mouse mononuclear cells [34, 35]. Another study demonstrated a tight 1 : 1 molecular interaction between ESAT-6 and CFP-10, which indicates that the precise level of production of ESAT-6 relative to that of CFP-10 may be relevant for

the formation of an active complex [36]. ESAT-6 and CFP-10 do not possess typical signal sequences, and the availability of a mechanism for effective secretion could be a critical factor for pathogenicity. A recent study of *M. tuberculosis* mutants lacking RD1, the *esat-6/cfp-10* operon, or other specific RD1-encoded genes has led to a better understanding of the role played by RD1-encoded proteins in the pathogenesis of tuberculosis [37]. Secreted, but not intracellular, ESAT-6 was shown to be an important virulence factor, in that it causes cytolysis of pneumocytes. Interestingly, *cfp-10,* as well as several other genes in the RD1 region, were found to be involved with the secretion of ESAT-6, and a chaperone-like function would be consistent with the observed 1 : 1 molecular interaction of ESAT-6 and CFP-10. Another argument in favor of the notion that the functions of ESAT-6 and CFP-10 are closely related is that, at least in *M. tuberculosis,* the *esat-6* and *cfp-10* genes reside in the same operon and are regulated under the same promoter [13], which would be expected to result in the expression of equal amounts of both proteins. In that regard, our observation of lower secretion of CFP-10 relative to ESAT-6 in environmental isolates of genotype MKA-III and MKA-IV, but not of MKA-II, could be relevant for the lack of virulence of the former genotypes, which are considered to be apathogenic. If our finding of differences between clinical and environmental isolates of *M. kansasii* with regard to protein expression can be confirmed, more extended sequencing data will be required to evaluate whether the organization of these genes in *M. kansasii* is different from that in *M. tuberculosis.*

At the start of the study, *esat-6* RFLP was performed on different mycobacterial species, to validate the method. Although the results mostly confirmed previously reported data on the species distribution of *esat-6,* several findings were new. In 3 previous reports, the *esat-6* and/or *cfp-10* gene was found to be present in *M. marinum,* the negative isolate originating from cattle [11, 22, 23]. In our study, *esat-6* was lacking in 3 of 5 *M. marinum* isolates, whereas *cfp-10* was present in all of them. This finding could either indicate that *esat-6* may not be essential for the virulence of *M. marinum* or, alternatively, that the *esat-6* gene of some *M. marinum* isolates differs from the *M. tuberculosis* homologue to such an extent that it prevents hybridization of the probe, which will be the subject of further study. One previous study reported the absence of *esat-6* in *M. gordonae* isolated from cattle [22], a species that is considered to be essentially apathogenic, whereas we found positive hybridization with *esat-6* in 1 of 2 isolates. This finding needs to be confirmed by PCR, to provide certainty that an *esat-6*–like gene is indeed present in this species. Taking these findings together, virulence does not seem to be directly related to the presence of *esat-6* or *cfp-10.*

Although the clinical and radiological presentations of pulmonary infection with *M. kansasii* and *M. tuberculosis* are indistinguishable, clinical and epidemiological differences exist between these infections. First, extrapulmonary infection with *M. tuberculosis* occurs in approximately one-sixth of all patients; it results from hematogenous spread early during the course of infection, whereas extrapulmonary localization of infection with *M. kansasii* is rare and occurs either as localized disease after traumatic inoculation or as part of disseminated disease in immunocompromised persons. Second, there is no proof of human transmission of *M. kansasii,* even though patients often excrete large numbers of *M. kansasii* in their sputum. Another difference is that infection with *M. tuberculosis* is characterized by an initial latent stage in the large majority of infected persons, the duration of which varies from weeks to many decades. The lifetime risk of progression to active tuberculosis disease is estimated to be 10%. In the absence of decreased cellular immunity due to HIV infection or immunosuppressive therapy, the factors underlying reactivation are often unknown. Whether a similar latent stage of infection with *M. kansasii* exists has not been studied, because no validated diagnostic test is available for that purpose. For the same reason, the proportion of infected persons who develop clinical illness caused by *M. kansasii* is not known.

The incidence of infection with *M. kansasii* differs by geographic region, but, in general, it is much lower than that with *M. tuberculosis.* Pulmonary infection can occur in apparently healthy persons but is diagnosed most frequently in persons with structural or functional lung abnormalities, whereas disseminated infection is seen almost exclusively in immunocompromised hosts. Thus, together with the lack of human transmission, *M. kansasii* does not seem to be as important a threat to individual and public health as is *M. tuberculosis. M. kansasii* is, nevertheless, an interesting pathogen to study, because only some of the genotypes of *M. kansasii* are associated with clinical illness, which indicates that both virulent

and avirulent strains are represented within the same species—a rare characteristic in human pathogens. Detailed knowledge of the virulence factors of *M. kansasii* could be valuable to the understanding of the pathogenesis of mycobacterial infections in general and of tuberculosis in particular. This may aid the development of novel drugs and of an improved vaccine to target specific virulence mechanisms. In this regard, available data have indicated that vaccination with the immunodominant peptide of ESAT-6 as a subunit vaccine and with DNA containing the *esat-6* gene confers partial protection against tuberculosis in mice [38, 39]. Immunization with a hybrid molecule of ESAT-6 and the immunodominant antigen 85B conferred protection against aerogenic infection with *M. tuberculosis* in mice at a level at least as high as BCG [40]. A recent study in guinea pigs confirmed and extended the findings in mice [41], and effective protection was found in a nonhuman primate model [42]. Further studies of the virulence factors of *M. kansasii* may provide additional important data to support the ongoing battle against tuberculosis.

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