Characterization of an IS-like element from *Mycobacterium tuberculosis*

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A DNA sequence, present in members of the *Mycobacterium tuberculosis* complex, has been identified and characterized. The distribution of this DNA sequence among mycobacterial species was analysed by DNA hybridization and PCR experiments. As the sequence was detected only in bacteria belonging to the *M. tuberculosis* complex, it may be useful for the rapid discrimination of mycobacteria. Interestingly, the sequence has some characteristics of an insertion element (IS) and codes for a hypothetical protein with significant homologies to proteins encoded by several IS elements of other organisms, namely IS427 and IS869 from *Agrobacterium tumefaciens*, IS402 from *Pseudomonas cepacia*, Tn4811 from *Streptomyces lividans* and ISRm4 from *Rhizobium meliloti*. Together, these elements form a previously unrecognized family of transposable elements. This finding suggests the possibility of horizontal gene transfer between pathogenic mycobacteria and other organisms including Gram-negative plant-pathogenic bacteria.

Introduction

During the recent genetic analysis of several mycobacterial species, various repetitive DNA elements have been found; many of them have features in common with typical IS elements (Cirillo *et al.*, 1991; Eisenach *et al.*, 1990; Green *et al.*, 1989; Kunze *et al.*, 1991; Martin *et al.*, 1990; McAdam *et al.*, 1990; Moss *et al.*, 1992; Patel *et al.*, 1990; Thierry *et al.*, 1990; Woods & Cole, 1990). Transposable elements can be of value as genetic tools. For example, in many other pathogens, insertion mutants have contributed substantially to our understanding of the respective virulence mechanisms. Furthermore, there exist many reports which demonstrate the value of repetitive elements for epidemiological and diagnostic purposes, as the presence or number and distribution of these elements in the bacterial genome can be specific for a species or even for strains within a species (Rappuoli & Gross, 1990). The use of mycobacterial transposable

elements and repetitive DNA sequences for diagnostic and epidemiological purposes has already been established by several groups (Clark-Curtiss & Docherty, 1991; Eisenach *et al.*, 1990; Grosskinsky *et al.*, 1989; McFadden *et al.*, 1987; Patel *et al.*, 1990; van Embden *et al.*, 1993).

Here, we report on the identification of a DNA sequence that appears to be specific for species of the *Mycobacterium tuberculosis* complex, including the vaccine strain *M. bovis* BCG. Interestingly, the sequence has similarities with IS elements of other organisms such as *Agrobacterium tumefaciens*, *Rhizobium meliloti*, *Pseudomonas cepacia* and *Streptomyces lividans*. Together these elements form a novel family of insertion elements. This finding raises the question of the possibility of horizontal gene transfer between pathogens of plants and mammals.

Methods

Mycobacterial strains. Table 1 (Results) shows the mycobacterial isolates used in this study.

DNA manipulations. Mycobacterial DNA was isolated as recently described (Vismara *et al.*, 1990). All other DNA manipulations and DNA sequencing were carried out according to standard procedures (Maniatis *et al.*, 1982). For cloning and sequencing experiments the vector pBluescript (Stratagene) was used. Oligonucleotide primers for

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sequencing were synthesized on a 380A DNA synthesizer (Applied Biosystems).

DNA probes and hybridization experiments. Radioactive labelling of a 451 bp *Pst*I/*Bam*HI restriction fragment containing about half of the IS-like element was carried out using a random priming kit (Gibco-BRL). Hybridization experiments were carried out under stringent conditions as previously described (Vismara *et al.*, 1990). Briefly, hybridization was carried out at 65 °C in 5×SSC buffer overnight. Then the filter was washed three times for 30 min at 65 °C in a buffer containing 0.1×SSC and 0.1% SDS.

PCR techniques. The oligodeoxyribonucleotide primers with the sequence 5'-GTGGTCGAGCCGTTGATGCCG-3' and 5'-AGCCGCGTTCGACGGTGTTCG-3' specific for the coding region of the mycobacterial ORF at sequence positions 101–121 and 831–851, respectively, were used in PCR amplification experiments. Amplification was performed in 0.5 ml microfuge tubes (Perkin-Elmer) in a final volume of 50 µl. The reaction mixture contained 10 mM-Tris/HCl (pH 8.3), 50 mM-KCl, 1.5 mM-MgCl₂, 50 µM-dNTP, 0.1 µM-primer, 100 ng chromosomal DNA and 1.25 U Taq polymerase (Promega). The reaction was performed using an automated Thermal Cycler (Perkin-Elmer). The samples were amplified in 30 cycles consisting of denaturation at 94 °C for 1 min, annealing of primers at 65 °C for 1 min and primer extension at 72 °C for 1 min.

Computer analysis. Sequence data were analysed with the GCG package run on a VAX/VMS computer (Devereux *et al.*, 1984). The TFasta and Pileup programs were used under standard conditions for database searching and alignment of predicted protein sequences.

Results and Discussion

Features of the nucleotide sequence

During the molecular characterization of clones from a λ gt11 library of *M. tuberculosis*, part of an open reading frame (ORF) coding for a hypothetical protein with sequence homologies to proteins of known IS elements was identified (see below). The entire ORF was cloned by screening a *M. tuberculosis* library in λ 2001 (Vismara *et al.*, 1990) using the cloned *Eco*RI fragment of the λ gt11 library containing the ORF as a hybridization probe. The sequence analysis revealed that the complete ORF is located on a 2.1 kb *Pst*I/*Eco*RI fragment. The ORF shows a codon usage which is similar to other mycobacterial genes sequenced so far (data not shown) and comprises 924 bp, corresponding to a coding capacity of 308 amino acids (Fig. 1). A putative Shine–Dalgarno sequence is present, which is nearly identical to that of the 65 kDa heat shock protein of *M. tuberculosis* (data not shown), but no obvious transcriptional signals can be found. The further analysis of the nucleotide sequence revealed some interesting features such as long inverted repeats in front of the ORF and at its 3'-end. These repeated sequences are 17 bp in length and contain only a single mismatch (Fig. 1). The inverted repeats are flanked by short 4 bp direct repeats with the sequence 5'-CTAG-3' (Fig. 1). The presence of such a structure, long inverted repeats flanked by direct repeats, is a typical feature of many insertion elements.

Comparison of the mycobacterial sequence with the sequences of other IS elements

Using the TFasta program (Devereux *et al.*, 1984), a homology search in databases was performed, which revealed interesting homologies of the putative mycobacterial protein to proteins from IS427 and IS869 of *A. tumefaciens*, to IS402 of *P. cepacia*, to Tn4811 of *S. lividans* and to ISRm4 of *R. meliloti* (Chen *et al.*, 1992; De Meirsmen *et al.*, 1990; Ferrante & Lessie, 1991; Paulus *et al.*, 1991; Soto *et al.*, 1992). Fig. 2 shows the alignment of the various polypeptides and demonstrates that the N-terminal part of the mycobacterial protein shows several regions of similarity in its amino acid sequence to the proteins encoded by the ORFs 3 and 4 of IS869 and IS427, respectively, to one ORF of IS402, to ORF 3 of Tn4811 and to the unique ORF of ISRm4. Interestingly, the C-terminal part of the mycobacterial protein has significant homologies to ORF1 of IS869, the second ORF of IS402 and ORF_x of IS427. Apparently, two different proteins encoded by the elements IS869, IS402 and IS427 are unified into one protein in the case of *M. tuberculosis*.

1	CGGCTGCAGTACTAGGGCGTCTCCCAATTTTATGTAAGTCTGGCCAGGAGGATGGCC	60
61	GGTGACGCGAGTGGGTGTGATTTCGGACGAGTTCGGCCGCTGGTGGAGCCGTTGATGCC	120
	V T R V G V I S D E F W A V V E P L M P	
121	GTGCGATGAGGGCAAGCCCGGACAGCGTTTACGATCACCGGCTTATCCTGGAAGGGAT	180
	S H E G K P G R R F S D H R L I L E G I	
181	CGCGTGGCGGTTCCGTACGGGAAGTCCGTCGGCGGACCTGCCCGTGAAGTTCGGCCGCTG	240
	A W R F R T G S P W R D L P A E F G D P F	
241	GCAACGCTGTGGGAAGCCGATCACCGTGGTCTGGATGGTACCTGCGACGAGGTGTT	300
	Q T V W K R H H R W S L D G T C D E V F	
301	CGCCCAAGTTCGCGCGGTGTTCCGGGTGGACGCTGAGGTGGCCGAGGATATCGAGAAGCT	360
	A H V A A V F G V D A E V A E D I E K L	
361	GCTGTGGTGGATTCCACGAACGTCGGGACACAGCATTCCGGGGCGCTGCTCGGAC	420
	L S V D S T N V R A H Q H S A G A A R T	
421	ACGCTGCCACAGGGGGCACTGTCGGATTACGAAGTTCGCGGATGAACCGGACATCA	480
	R S P Q G A L S D Y F K K S A D E F D D H	
481	TGCGATCGCGCGTCCGCGCGGGGTGACACCAAGATCCATGCCCTGACCGATCAGCG	540
	A I G R S R G G L T T K I H A L T D Q R	
541	CGAAGCCCGGTTCCGATCCGCTTACCGCCAGGCCCGCCGACACCCGCAACTGCT	600
	E A P V R I R L T A G Q A G D N P Q L L	
601	GCCCCGTGCTCGACGATATCGCCATGCCAGCACCGAATACGCCCTGGGCGACCGGATTT	660
	P L L D D Y R H A S T E Y A L G S T D F	
661	CGGTTACTCGCGACAGGCGCTACTCACACCAAGTACCGGTCGCGCATACGGTCTAA	720
	R L L A D K A Y S H P S T R A A L R S K	
721	GAGATCAAGCACACCATCCCCAAGCCAGCATCAGATCGACCGGCGAAGGCCAAGGG	780
	K I K H T I P E R H D Q I D R R K A K G	
781	GTCTGCGCGGGGGGCCACGACATTCGACCGCGCGCTCTACGGCTACGCAACACCGT	840
	S A G G R P P A F D A A L Y G L R N T V	
841	CGAAGCGGCTTCATCGATCAAGCAGTGGCTGCGCAACCGCTACGACAAATA	900
	E R G F H R L K Q W R G I A T R Y D K Y	
901	CGCCCTGACCTACCTCGCGCGCTCTGCTGGCGTCCGCGTCACTCCAGCGCGAGTGGG	960
	A L T Y L G G V L L A C A V I H A R V G	
961	AACTCGAATTTGGGAGACACGCGCTAGCCGCGAGACCGGG	1000
	T P K L G D T P *	

Fig. 1. General organization of the putative IS element found in *M. tuberculosis* H37RV. Below the nucleotide sequence, the amino acid sequence of the 308-amino-acid protein is shown starting with a GTG codon close to a possible Shine–Dalgarno sequence (marked by asterisks, *). The inverted repeats at the termini of the element are underlined and the duplicated host sequence is in bold.

Myco	VTRVGVISD.EFHAVVEPLNPSEHGE....P
IS427orf4	msrydLTDFE.WrVIEPLLP.nkPR....G
IS869orf3	mtrRrIdLTDFE.WTVIQPLLP.nkPR....G
IS402orf1	makpIdDdE1WTLIEPLLPpkPRreknP
ISRm4	mettmawtdftrpqyarralryaSdLTDrE.WSLIaPfMPPp....rrIG
Tn4811orf3	mhrhlypsdMTDaE.WALVEPLLPpacdtarg
Myco	GRFSDERLILEGIAWRFTGSPWRDLFAE..FGFWQTWVKKRWSLQD
IS427orf4	vpRvDDR.VLNGIIFWVLRSGAPWRDLPer..YGPrTcYnrRfRWRkaG
IS869orf3	vpRvDDR.VLNGIIFWVLRSGAPWRDLPer..YGPrTcYnrRfRWRkaG
IS402orf1	GRlpvsnRaaLTGILFvIKTGLrWRDLFAEmgoGSGvTcWRRlrdWqaaG
ISRm4	rpRkTDmReVVNALlYiasTGoqWRmLPkD..FPPFstVqgyfyfWrate
Tn4811orf3	rpekhrReIVDAIRYvvdTGckWRALPAD..FPPWrtVWgmfaRWAavG
Myco	TCDEVFAHVAAPGVDAEVAEDIEKLLSVDSVYRAHQSSAGAARTSPQ
IS427orf4	vwDrMdaITaAYdgD.....IqmIDSTsVRAHQqAATAKRGleii...
IS869orf3	vwDhVlGeISkaFdgD.....IVmIDSScVRvHQHAATGKRGlnTm...
IS402orf1	vwDrLhelLLlAkiraadQIdfser...aAVDSSSIRA..vGAGqklGqTpp...
ISRm4	lWnrInhHLvmetrelegrq.asptagvIDSqsVKTtEsGG..iRGdAg...
Tn4811orf3	vigQLrdaLAgrrirDmGrG.pravatiIDSqsVKAastvGkdSRGyAg...
Myco	GALSDYKKSADFPDDHAIGRSRGGGLTTKIHALTQDRAPVRIETAGQAG
IS427orfX	...PAGcDgKKG...Dr.DHcLGRSRGGGLTTKIHAVvDgqglPIRLsLTAGQSh
IS869orf1	...STcGhgKKG...DkhDgCMGRSRGGGLTTKIHAVvDadgrPIRLaLTAGQSh
IS402orf2	...fiIdSRRRGrpkTgpnPtdRARPG..SKhHlVTangTPLaaiLTGANVn
Myco	DNPQLPLLDYRHAETAYALG.STDFRLLADKAYSEPTAAALRSKKIX
IS427orfX	DgqaadGLLDhv.....GAgTiVLADKAYdadriRASLRKgaF
IS869orf1	DgrmaePLlQti.....SKgaiLLADKAYdtnAIRafaKqRqaw
IS402orf2	DvTQLPLLDaippirGIRghPlqrprvVyADRGYdsrzhRrALRdRgIe
Myco	HTIPERHQIDRRKAKSGAGGRPPAFDAALYG.LRMTVERGPTERLKQWRG
IS427orfX	anIPpKaN....RRSK.....PyFstWLYr.eRNlIERffsKlKhFRr
IS869orf1	anIPaKaN....RKGs.....fPFsqvVYrsqRNlIVERffsKlKqFRG
IS402orf2	pviAKRRTEhgsLgK.....Y....RwvVERThawLHhFRr
Myco	IATRYDKALTYLGGVLLCAVIRAR...
IS427orfX	VATRYDKIAenFLAmVqLasrIwlr...
IS869orf1	IATRYDKAPLnFLAAVKLAavrtiwr...
IS402orf2	LrIRPERRAdihgAflkLGCCLicwn...

Fig. 2. Alignment of the amino acid sequences of proteins encoded by IS402, IS427, IS869, Tn4811 and ISRm4 homologous to the mycobacterial protein. On the top, the mycobacterial sequence is shown in **bold**. According to the sequence from the GenEMBL database, ORF_x of IS427 does not contain a start codon; obviously this ORF has lost its function in the sequenced copy of IS427. Capitalized letters symbolize sequence similarity or identity with the mycobacterial sequence. Homologous amino acids were grouped as follows: I, L, V, M; F, Y, W; H, K, R; E, D, N, Q; A, G, S, T, P; C.

Further comparison of the four sequences revealed some other common features such as inverted repeats at the ends of the elements. Moreover, comparison of the insertion point flanking regions revealed in all cases a

small duplication of a host sequence, which in several cases has an interesting similarity, as the dinucleotide 5'-TA-3' can be found in the centre of this sequence (De Meirsmen *et al.*, 1990; Ferrante & Lessie, 1991; Paulus *et al.*, 1991; Scordilis *et al.*, 1987; Vanderleyden *et al.*, 1986).

Therefore, it was tempting to speculate that we have identified an IS-like element, which should have a length of 968 bp and may have caused a duplication of four base pairs during its insertion. Interestingly, the putative element does not itself contain a stop codon for the 308-amino-acid ORF. Instead, the duplicated host sequence 5'-CTAG-3' at the putative insertion point provides the translational stop signal.

Detection of the sequence in mycobacterial species

In most cases, IS elements are found in several copies on bacterial chromosomes. In order to determine whether the mycobacterial sequence is also present several times on the chromosome, we performed hybridization experiments with a labelled probe comprising about half of the sequence up to the internal *Bam*HI site in the element and *Bam*HI-digested chromosomal DNA of *M. tuberculosis* H37RV, *M. tuberculosis* Johnston and *M. bovis* BCG. Surprisingly, as shown in Fig. 3, only a single hybridizing band could be detected. As this band has the same molecular mass in all three strains, it is likely that the position of the sequence is the same in the three genomes. It is therefore possible that the mycobacterial sequence is of IS origin with site-specific integration, or it may be defective. So far, we have been unable to demonstrate transposition of the mycobacterial sequence (data not shown). As there are no obvious transcriptional signals in front of the ORF, it might be that expression of the ORF and therefore the transposition characteristics of the putative element depend on the transcription

Fig. 3. Southern blot analysis of *Bam*HI digests of chromosomal DNA of various mycobacterial species. A labelled probe containing about half of the mycobacterial IS-like element up to its internal *Bam*HI site was used for hybridization.

of genes located nearby at the insertion point. It is also possible that the fusion of the two genes present in the functional IS elements into one ORF in *M. tuberculosis* has caused the inactivation of the respective gene product(s), which are likely to be involved in transposition of the element. On the other hand, several other cases are known in which only a single IS copy is present, e.g. IS1 in *Klebsiella aerogenes*, IS2 in *E. coli* B, IS30 in *E. coli* C (Iida *et al.*, 1983), and IS6110/IS986 in some strains of *M. bovis* BCG and *M. tuberculosis* (Cave *et al.*, 1991).

Several IS and transposable elements have been described in various mycobacterial species, e.g. IS900, IS901, IS902, IS1096, IS6110, TN610 and others (Cave *et al.*, 1991; Cirillo *et al.*, 1991; Green *et al.*, 1989; Kunze *et al.*, 1991; Martin *et al.*, 1990; McAdam *et al.*, 1990; Moss *et al.*, 1992; Thierry *et al.*, 1990). By computer analysis, no homology between them and the sequence described here could be found, at either the DNA or protein level. Accordingly, a Southern blot analysis with a probe internal to the gene and PCR amplifications with primers specific for the IS-like element did not reveal the presence of any similar sequence in other mycobacterial species. For this analysis, we chose several species belonging to each of the four Runyon groups (Runyon, 1959) (see Table 1). In Southern hybridization experiments, a signal could be detected only in the *M. tuberculosis* strains and in *M. bovis* BCG (Fig. 3). As shown in Fig. 4, similar results were obtained with the PCR amplification technique. A PCR product of the predicted size appeared only in the case of *M. tuberculosis*, but not in DNA preparations of the other species (Table 1). This indicates that the sequence is specific for the members of the *M. tuberculosis* complex.

Table 1. Distribution of the IS-like sequence in mycobacterial species according to Southern hybridizations and/or PCR amplification

Species*		Presence of the IS-like sequence
<i>M. tuberculosis</i> complex	<i>M. tuberculosis</i> H37RV ^a	+
	<i>M. tuberculosis</i> Johnston ^a	+
	<i>M. bovis</i> BCG ^c	+
Runyon I	<i>M. kansasii</i> ^a	—
	<i>M. simiae</i> ^b	—
Runyon II	<i>M. scrofulaceum</i> ^a	—
	<i>M. szulgai</i> ^b	—
Runyon III	<i>M. avium</i> ^a	—
	<i>M. intracellulare</i> ^a	—
	<i>M. terrae</i> ^b	—
Runyon IV	<i>M. fortuitum</i> ^a	—
	<i>M. phlei</i> ^b	—
	<i>M. smegmatis</i> ATCC 607	—

* Source: a, Vismara *et al.* (1990); b, Culture Collection, University of Rome, Italy; c, vaccine strain Sclavo, S.p.A., Siena, Italy.

Therefore, the PCR primers described in this study may be useful for the rapid discrimination of *M. tuberculosis* and *M. bovis* from atypical mycobacteria of the four major Runyon groups.

Evolutionary considerations

How could these IS-like sequences spread among organisms of genera which are evolutionarily very distant from each other? According to the results of the

Fig. 4. PCR analysis with primers specific for the mycobacterial ORF. Lanes 1 and 13, DNA size standard (λ HindIII); lane 2, *M. tuberculosis* H37RV; lane 3, *M. kansasii*; lane 4, *M. avium*; lane 5, *M. scrofulaceum*; lane 6, *M. phlei*; lane 7, *M. terrae*; lane 8, *M. simiae*; lane 9, *M. szulgai*; lane 10, *M. fortuitum*; lane 11, *M. smegmatis* ATCC 607; lane 12, DNA of IS-like element cloned in pBluescript. According to the primer pair used for this experiment, a 740 bp band should be amplified.

hybridization and PCR experiments, bacteria of mycobacterial species that are found in the environment do not contain the sequence here described, and therefore cannot have been the carrier. However, one can speculate that *Pseudomonas cepacia*, believed to be very actively involved in horizontal exchange of genetic information, may have played a role. Several broad host range plasmids are found in this species, on one of which IS402 was originally identified (Ferrante & Lessie, 1991; Kuehn *et al.*, 1992; Scordilis *et al.*, 1987). Indeed, conjugational transfer of broad host range plasmids from Gram-negative bacteria into a mycobacterial species was recently demonstrated (Gormley & Davies, 1991). As *P. cepacia* strains are associated with plant and human disease, especially pulmonary disorders, this organism would have had the opportunity to come into close contact with both the mammalian pathogens in the lung of humans and the environmental organisms. It is possible that an exchange of genetic material between mycobacterial species and other genera has occurred several times as there exists another example of an IS-like element, IS900 of *M. paratuberculosis*, which might be reminiscent of such a horizontal gene transfer event (Green *et al.*, 1989; Hoover *et al.*, 1992).

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