Distribution of GyrA intein in non-tuberculous mycobacteria and genomic heterogeneity of *Mycobacterium gastri*

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Abstract To gain further insights into the understanding of the intein invasion process in mycobacteria, intein sequences in the *gyrA* gene of 42 mycobacterial strains were searched and a new gyrA intein was found in *Mycobacterium gastri* (*Mga*). This 1260 bp intein, named *Mga*GyrA, inserted at the GyrA-a site, is highly homologous to the members of the *Mycobacterium leprae* GyrA allelic family. As the *recA* intein, *Mga*GyrA was detected in only one out of six *Mga* strains examined, while the *pps1* intein was a constant character of *Mga*. This data supports the genomic heterogeneity of *Mga* towards intein invasion, a finding that may have phylogenetic implications. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Intein; Protein splicing; Mycobacteria; RecA; GyrA; Pps1

1. Introduction

Inteins are protein introns that are inserted in functionally important domains of essential proteins. Their coding sequences are inserted in frame in host genes that are translated into large polypeptide precursors and the autocatalytic and post-translational splicing of the intein yields to the mature host protein. Intein coding sequences are widely distributed in all three kingdoms [1,2] but, to date, no essential function has yet been ascribed to them. Although the majority of known inteins contain conserved LAGLIDADG motifs of the homing endonucleases DOD family [1,3], this endonuclease activity seems only to be involved in the perpetuation and transfer of the intein [4] and has been experimentally demonstrated for only a few inteins that include the mycobacterial *Mycobacterium gastri* (*Mga*)Pps1, the only eubacterial intein described so far to exhibit this enzyme activity [5]. This fact may be related to the observation that most of the eubacterial inteins (23 out of the 33) are recensed in the *Mycobacterium* genus; these inteins are located in four host proteins, i.e. GyrA, RecA, DnaB and Pps1. In this context, we have recently found new inteins in *recA* and *pps1* genes from various mycobacterial species and showed that both families of inteins may have distinct insertion sites [5,6]. For instance, while the RecA intein invades *Mycobacterium tuberculosis* (*Mtu*) at the RecA-a site, seven RecA inteins interrupt the *recA* sequence at the RecA-b site in *Mycobacterium chitae*, *Mycobacterium fallax*, *Mycobacterium flavescens* (*Mfl*), *Mga*, *Mycobacterium leprae* (*Mle*), *Mycobacterium shimodei* and *Mycobacterium thermoresistibile*, and belong to the same intein allelic family. Similarly, the three inteins found in Pps1 from *Mga*, *Mle* and *Mtu* are located in three distinct insertion sites.

To date, only one intein insertion site has been described in GyrA from the six mycobacterial species, i.e. *Mfl*, *Mycobacterium gordonae* (*Mgo*), *Mycobacterium kansasii* (*Mka*), *Mle*, *Mycobacterium malmoense* (*Mma*) and *Mycobacterium xenopi* (*Mxe*). This observation may be related to the functional importance of this GyrA insertion site or, alternatively, due to the limited number of strains analysed. To discriminate between these two possibilities and to gain further insights into the understanding of the intein invasion process in mycobacteria, we deliberately searched for the presence of inteins in the mycobacterial *gyrA* gene by analysing 42 mycobacterial strains and species. This resulted in the identification of one more intein in the *Mga* genome and, more importantly, to the finding of a genomic heterogeneity among the six strains of *Mga* examined.

2. Materials and methods

2.1. Mycobacterial strains, growth conditions and genomic DNA isolation

A total of 42 non-tuberculous mycobacterial strains were used in this study; these include 37 out of the 39 strains described elsewhere [6] and five additional strains of *Mga* (listed in Table 1); the two strains from the published list that were not analysed were *Mycobacterium aurum* IP 141210005 and *Mycobacterium cooki* ATCC 49103. All these strains were from the Reference Centre for Mycobacteria (Institut Pasteur, Paris) and were kindly provided by Dr V. Vincent Lévy-Frébault. All the strains were identified using the conventional biochemical tests [7] and chemotaxonomic criteria such as mycolic acid and species-specific glycolipids analyses [8]. Moreover, the genetic differentiation between the six *Mga* and the *Mka* strains was performed by amplified ribosomal DNA restriction analysis (ARDRA) (see below).

Strains were grown at their optimal temperature of growth [7] on the Löwenstein–Jensen medium for a few days to several weeks, depending on the growth rate of the mycobacterial species. Genomic DNA isolation from 42 mycobacterial strains was performed using the glass-bead disruption method as described by Saves et al. [6].

2.2. ARDRA

ARDRA was used to establish the identity of the six *Mga* strains, i.e. to discriminate between *Mga* and *Mka* species [9,10]. The 16S

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Abbreviations: Mfl, Mycobacterium flavescens; *Mga, Mycobacterium gastri*; *Mgo, Mycobacterium gordonae*; *Mka, Mycobacterium kansasii*; *Mle, Mycobacterium leprae*; *Mma, Mycobacterium malmoense*; *Mtu, Mycobacterium tuberculosis*; *Mxe, Mycobacterium xenopi*

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RNA gene was amplified using universal primers Bact01 (5′-agagtttgatcctggctcag-3′) and Bact11 (5′-aaggaggtgatccagcc-3′). For this purpose, 5 μl of genomic DNA was incubated with *Taq* DNA polymerase in 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTP and 15 pmol of each oligonucleotide in a 50 μl mix, which was incubated for 5 min at 94°C, 35 times for 1 min at 94°C, 1 min at 54°C and 1.5 min at 72°C and finally for 5 min at 72°C. The 1.5 kb amplified fragments were extracted from a 1% agarose gel in TBE buffer (90 mM Tris–Borate, 2 mM EDTA) using Qiaquick gel extraction kit (Qiagen). Subsequent restriction digestions of these DNA fragments with enzymes *Rsa*I and *Ava*I (New England Biolabs) led to ARDRA patterns which allowed us to discriminate between the mycobacterial species.

2.3. Intein detection by PCR amplification

The primers GyrA-5′ (5′-cggaccgcagccacgc-3′) and GyrA-3′ (5′-gttgtgcggcgggatattggt-3′), used to amplify the *gyrA* gene, were chosen in the most conserved part of the *gyrA* sequence; they correspond to *Mle gyrA* gene sequence or its complementary sequence between positions 200–215 and positions 562–582, respectively. The primer pairs RecA-5′ and RecA-3′ and Pps1-5′ and Pps1-3′, used to amplify *recA* and *pps1* genes, respectively, as well as the primer pair *Mga*-ATG and *Mga*-3′, used to amplify the coding sequence of *Mga*Pps1 intein, were previously described [5,6].

PCR amplifications were performed with *Taq* DNA polymerase in 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTP, using 5 μl of genomic DNA preparation as DNA matrix. Several PCR assays were performed in different reaction conditions with regard to oligonucleotide concentrations and hybridisation temperature to ensure amplification specificity.

2.4. Cloning and sequencing of MgaGyrA intein coding sequence

The *Mga* PCR fragment containing the invading sequence was purified after migration on a 1% agarose gel by the Qiaquick extraction kit and was directly cloned in the PCR2.1-TOPO plasmid using the TOPO-TA cloning kit (Invitrogen). The resulting plasmid was double-strand sequenced (MWG-Biotech) using universal primers M13forward and M13reverse.

2.5. Accession numbers for nucleotide and peptide sequences

GenBank accession numbers for *Mga recA* and *Mga pps1* genes are AJ251715 and AJ276118, respectively. GenBank accession numbers for *Mga*, *Mle*, *Mtu*, *Mxe*, *Mfl*, *Mka*, *Mgo* and *Mma gyrA* genes are AJ251716, Z68206, L27512, U67876, Z68209, Z68207, Z68208 and AJ002066, respectively.

3. Results

3.1. The survey of GyrA inteins revealed a new intein in Mga

A total of 42 non-tuberculous mycobacterial strains belonging to 31 species ([6] and Table 1), 21 non-pathogenic and 10 opportunistic pathogens [7], were tested for the presence of intein in the *gyrA* gene. The oligonucleotide pair chosen to detect the presence of an invading sequence in a 383 bp fragment of the *gyrA* gene limited the screening to the 5′-end of the gene, which contains the GyrA-a insertion site. Among the 42 strains examined, only three strains showed an invading sequence in *gyrA* (Table 1) as attested by the presence of a fragment of approximately 1600 bp that was amplified from genomic DNA of these strains, namely *Mfl*, *Mka* and *Mga*, while a 383 bp fragment was amplified from the other genomic DNA preparations. While the occurrence of GyrA inteins in *Mfl* and *Mka* was expected from previous reports [11,12], the absence of the intein in the type-strain *Mgo* was surprising since some strains of this species were reported to contain GyrA intein [12]. This observation may be related to a heterogeneity of strains belonging to the same mycobacterial species towards GyrA intein. This assumption was supported by the presence of the intein in only one out of the six strains of *Mga* examined (Fig. 1). However, based on the close phylogenetical relationship between *Mga* and *Mka* [7], which contains an intein in *gyrA*, it was important to ascertain that the presence of a GyrA intein in only one strain of *Mga* did not arise from a misidentification of this strain.

3.2. Genomic heterogeneity of Mga towards GyrA and RecA inteins

Because the amplification and digestion of ARN 16S genes, i.e. ARDRA, is the most reliable technique currently used to clearly discriminate between two mycobacterial species, the six *Mga* strains labelled were analysed by this method using an authentic strain of *Mka* as a standard. The restriction patterns obtained after digestion of PCR products by *Rsa*I and *Ava*I were analysed and were found to be identical for the six *Mga* strains and differed from those obtained with *Mka* (not shown). Thus, it was concluded that strain HB 4389 that harbours *Mga*GyrA intein belongs to the species *Mga* which is heterogeneous in terms of GyrA intein content. Since our previous studies have revealed that this particular strain of *Mga* (strain HB 4389) contains also inteins in *recA* and *pps1* [5,6], we checked for the presence of intein sequence in these two genes in the five other strains of *Mga*. PCR amplification was performed with oligonucleotide pairs allowing the detection of an intein in a 229 bp fragment of the *recA* gene, spanning both intein insertion sites RecA-a and RecA-b, and in a 513 bp fragment of *pps1* gene, spanning the three intein insertion sites known in this gene, Pps1-a, Pps1-b and Pps1-c. The presence of inteins in *recA* and *pps1* genes from *Mga* was revealed by the amplification of a 1333 bp and a 1657 bp fragment, respectively. The amplification of the *recA* gene from the five additional *Mga* genomic DNA preparations generated five small fragments of 229 bp (Fig. 1). Hence, as in the case of the *gyrA* gene, only strain HB 4389 of *Mga* possesses an intein in the *recA* gene; its sequence and insertion

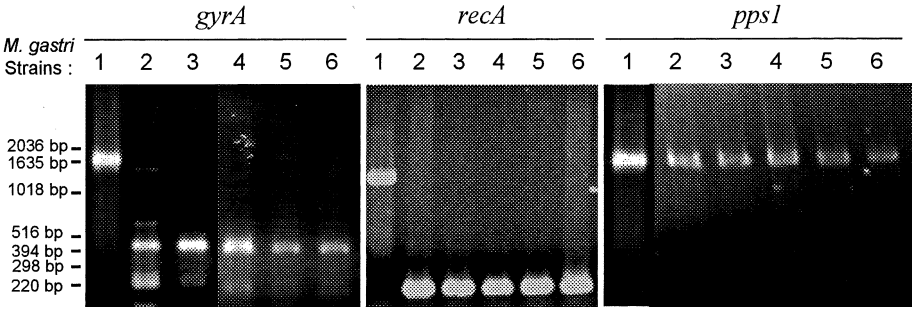

Fig. 1. PCR Amplification of fragments of the *gyrA*, *recA* and *pps1* genes of six strains of *Mga*. The references of strains numbers 1–6 are HB4389, W471, IP140340001, IP140340003, IP140340010 and IP140340011, respectively.

Table 1
A survey of inteins present in non-tuberculous *gyrA* genes

Species	Strain reference	Intein presence and size	Insertion site
Mfl	ATCC 14474^T	+1263 (421)[a]	GyrA-a
Mga	HB 4389	+1260 (420)	GyrA-a
Mga	W 471^T	–	
Mga	IP 14 034 0001	–	
Mga	IP 14 034 0003	–	
Mga	IP 14 034 0010	–	
Mga	IP 14 034 0011	–	
Mgo	ATCC 14470^T	–[b]	
Mka	ATCC 12478^T	+1260 (420)[a]	GyrA-a

42 strains were examined; these include the 37 out of 39 strains listed earlier [6], except *M. aurum* IP 141210005 and *M. cooki* ATCC 49103, and five additional strains of *Mga*. Type-strain of species where intein has been detected by other workers are included. The exact size in bp (in amino acids) of inteins is indicated.
[a]This intein was cloned and only partially sequenced. Its size and sequence were determined by Fsihi et al. [12].
[b]No intein was detected in this strain, while one was detected in other strains of the same species [12].
^{T}Means type-strain.

site has been described earlier [6]. In the case of the *pps1* gene, fragments of approximately 1600 bp were amplified from all six genomic DNA (Fig. 1), meaning that all the strains possess an intervening sequence in the *pps1* gene. To ascertain that, in all the strains, this intervening sequence corresponds to the *Mga*Pps1 intein described previously [5], we amplified its coding sequence with the specific oligonucleotides formerly used to clone it in an expression vector; in all cases, we were able to detect the expected 1134 bp sequence (not shown). Thus, in sharp contrast with *recA* and *gyrA* host genes, the *pps1* gene harbours the intein sequence inserted at the Pps1-c site in all six strains of *Mga*.

3.3. Analysis of the GyrA intein of Mga

Cloning and sequencing of the *Mga* amplified DNA revealed that the intein coding sequence consists in 1260 bp inserted at the GyrA-a site (Table 1). Thus, this new GyrA intein from *Mga*, named *Mga*GyrA according to the current nomenclature, also belongs to the large allelic family of the *Mle*GyrA intein. Comparison of the nucleotide sequence of the GyrA-a insertion site from *Mga* with those of the six other known GyrA-a sites containing intein showed that these sequences are slightly divergent (Fig. 2). Nevertheless, alignment of the peptide sequence of the *Mga*GyrA intein with those of GyrA inteins of the same allelic family showed that the sequence of the N- and C-terminal junctions between GyrA and inteins are well conserved (Fig. 3). In particular, only two residues of these segments of GyrA sequence are variable. In addition, except for the mini-intein *Mxe*GyrA that lacks 222 residues in its central domain [11], these inteins are 49% identical; furthermore, the comparison of intein sequences by pairs revealed an identity of 64–89% (Table 2). The strong homology among this intein family allowed us to locate the eight intein motifs, blocks A–H, in the peptide sequence of the six full-size inteins (Fig. 3). It is noteworthy that, as expected from the close taxonomical relationship between *Mga* and *Mka* [7], the newly discovered *Mga*GyrA intein is 89% identical to the *Mka*GyrA intein.

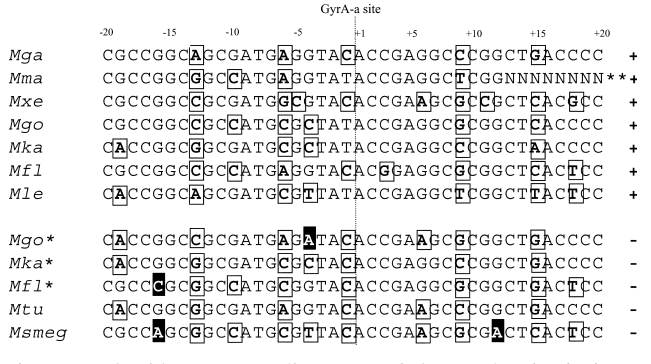

Fig. 2. Nucleotide sequence alignments of the GyrA-a intein insertion sites in *gyrA* genes from different mycobacterial strains and species. The + or −, at the right of the sequences, means that an intein is present or not, respectively, at this insertion site. Nucleotides divergent from the most commonly found nucleotides in inteincontaining genes appear in bold in white boxes; nucleotides divergent from nucleotides found in intein-containing genes appear in black boxes. *: The sequence comes from the inteinless strains of the species described by Fsihi et al. [12]. **: The partial sequence of the *Mma* gene ends at nucleotide +13 downstream of the GyrA-a site.

4. Discussion

The GyrA protein of mycobacteria is a privileged host for protein introns since a seventh intein was discovered in the present work, namely *Mga*GyrA, an intein in the GyrA protein of *Mga* (Table 1). The new intein is located at the same insertion site and is highly homologous to the other members of the allelic family that comprises *Mle*GyrA, *Mfl*GyrA, *Mgo*GyrA, *Mka*GyrA, *Mma*GyrA and *Mxe*GyrA inteins (Table 2). Our study, which screened 42 strains belonging to 31 mycobacterial species, and previous works [11–13] did not find potential intein insertion sites in the 3′-part of the *gyrA* gene. This may be due to the observed fact that the presence of inteins is limited so far to the functionally important domains of the host proteins. Indeed, the seven known inteins are located immediately downstream the tyrosine residue of the active site of the GyrA subunit, suggesting that the intein splicing is absolutely required for the gyrase activity.

Although the partial *gyrA* nucleotide sequences revealed some divergences (Fig. 2), the host protein sequences were more conserved between species and exhibit a high level of identity between the seven known GyrA proteins whose coding genes harbour an intein (Fig. 3). It should be noticed that although the intein sequences are overall well conserved within the same allelic family, the degree of homology between inteins varies. Nevertheless, the newly discovered *Mga*GyrA protein is highly homologous to the inteins of the same allelic family, with 64–89% identity with these inteins (Table 2), ex-

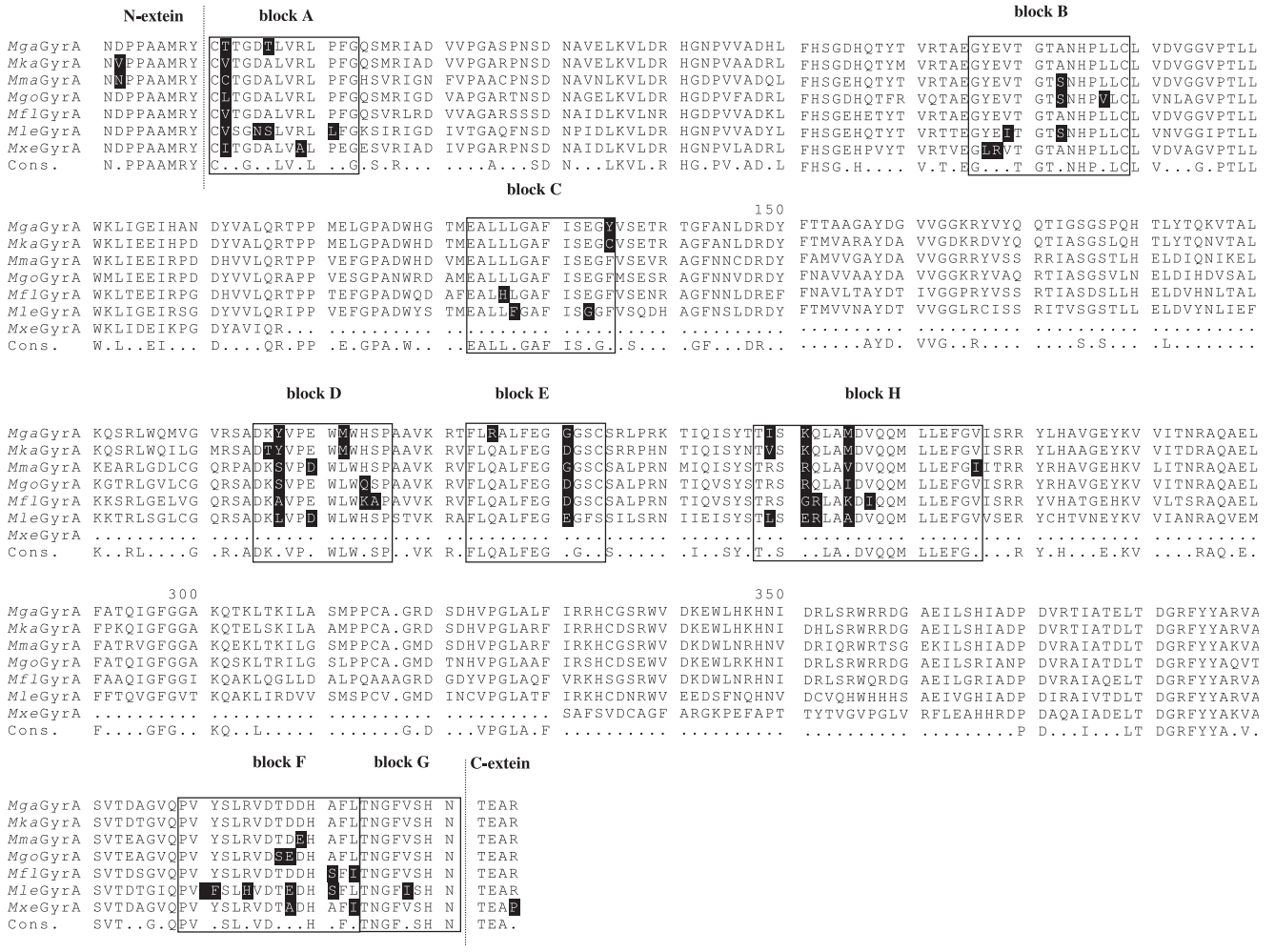

Fig. 3. Sequence alignment of the seven inteins of the *Mle*GyrA allelic family. The partial sequences of N- and C-exteins are also indicated. Intein residues are numbered and conserved motifs are boxed. The variable residues within these motifs and within N- and C-exteins appear in black boxes. The conserved residues (Cons.) are indicated below the seven sequences.

cluding the *Mxe*GyrA mini-intein because of its atypical size and sequence. If the high sequence identity between the *Mga*GyrA and *Mka*GyrA inteins can be associated with the close taxonomic relatedness between *Mga* and *Mka* species [7], there is no obvious rational in the other cases. In other words, no correlation between the percentages of identity between intein sequences and phylogenetical characteristics of the strains such as growth rate or pathogenicity between invaded species.

Thanks to the high sequence conservation between these inteins, the intein signature can be readily located in all inteins, except for *Mxe*GyrA (Fig. 3). The percentage of identity is again higher within specific intein motifs (64%) than the whole sequences (49%) and LAGLIDADG motifs C and E are particularly well conserved between species. The sequence of the former block is atypical in that the 13 residues constituting block C differ greatly from those most commonly found in this block as compiled by Pietrokovski [3]. The two glycine residues at positions 6 and 12 are nonetheless conserved and the GDG triad is replaced by SEG in five inteins, as found in a few archaeal inteins. In *Mle*GyrA, the glutamic acid residue of this triad is replaced by a glycine residue, as in *Mfa*RecA. These so-called splicing blocks are also well conserved, although little is known about the splicing of these inteins; in particular, the block G sequence is identical in all inteins except in *Mle*GyrA where the valine residue is replaced by an isoleucine residue. Since GyrA inteins contain the conserved intein blocks C, D, E and H, which are characteristic of endonucleases, the six full-size GyrA inteins are putative site-specific endonucleases. Consequently, it would be interesting to check for their endonuclease activities and compare the cleavage efficiency on the various GyrA-a nucleotide sequences.

An important observation of the present study resides in the genomic heterogeneity of *Mga* towards intein invasion; both *Mga*RecA and *Mga*GyrA inteins were observed in only one out of the six strains of *Mga* examined, while *Mga*Pps1 intein was present in all *Mga* strains (Fig. 1). Hence, the presence or the absence of the inteins allows to differentiate between *Mga* strains at a subspecies level, as it was the case for *Mka*. This genomic heterogeneity may also concern other mycobacterial species, as earlier studies [12,13] have also failed to detect a GyrA intein in some strains of *Mgo* and *Mfl*; consistent with these earlier observations is the fact that we could not detect an intein sequence in the type-strain of *Mgo* studied herein.

Based on sequence divergences at the GyrA-a site observed between the *gyrA* genes containing the intein and inteinless

Table 2
Percentage identity between mycobacterial GyrA inteins peptide sequences

	*Mga*GyrA	*Mka*GyrA	*Mma*GyrA	*Mgo*GyrA	*Mfl*GyrA	*Mle*GyrA
*Mle*GyrA	64.3	63.8	69.0	65.2	64.9	100
*Mfl*GyrA	73.2	73.9	75.1	74.6	100	
*Mgo*GyrA	76.9	77.6	79.3	100		
*Mma*GyrA	77.1	76.4	100			
*Mka*GyrA	89.3	100				
*Mga*GyrA	100					

gyrA genes from *Mgo*, *Mka* and *Mfl*, Fsihi and collaborators [12] had formerly suggested that the absence of intein in particular strains could be due to a defective homing event attributable to the homing endonuclease specificity. The alignment of the GyrA-a site sequences presently known, including the seven interrupted sites and five inteinless *gyrA* sequences (Fig. 2) showed, however, that this insertion site is not highly conserved between species. Thus, assuming that the six full-size inteins of this allelic family correspond to a family of isoschizomers endonucleases, as it is the case of the archaeal *Tli*Pol-2 intein family [14], the cleavage specificity of these enzymes should be quite large. Moreover, no major divergences between invaded and inteinless sites are detected. Only the two strictly conserved G at positions −16 and +12 are mutated in *Mfl* and *Mycobacterium smegmatis* inteinless sequences; since the divergent nucleotides also vary in some of the invaded GyrA-a sites, it is doubtful that these nucleotides belong to the minimal DNA sequence cleavable by the intein and that the substitutions found around this site are responsible for the absence of intein in the *gyrA* gene. Furthermore, sequencing of the inteinless *recA* gene from the type-strain of *Mga* revealed only one nucleotide substitution around the RecA-b site compared to the *recA* intein-containing gene (not shown). This point mutation concerns the 13th residue downstream the RecA-b insertion site, an hypervariable position of the sequence [6]. Hence, on the assumption that the intein coding sequences had been disseminated among mycobacteria by homing events consecutive to the specific cleavage of the intein insertion sites, the absence of intein in some mycobacterial strains and species can not be explained by sequence divergences of the target gene.

In contrast with the heterogeneous distribution of RecA and GyrA inteins among *Mga*, *Mgo*, *Mfl* and *Mka* strains, the inteins *Mga*Pps1 (Fig. 3), *Mle*GyrA [12], *Mma*GyrA and *Mxe*GyrA [11,13] are constantly found in strains of *Mga*, *Mle*, *Mma* and *Mxe*, respectively. This raises again the question of whether the intein coding sequences have been early acquired by an ancestral mycobacterial strain and independently lost by some species and strains or if these insertion sequences had been individually acquired by each species and strain during the evolution. The relatively high conservation of the intein insertion site sequences in non-invaded strains suggests that these sites are potential still-unoccupied targets, implying that the invasion of essential proteins by inteins is still in progress in mycobacteria.

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