Independent Loss of Immunogenic Proteins in *Mycobacterium ulcerans* Suggests Immune Evasion\††

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The highly immunogenic mycobacterial proteins ESAT-6, CFP-10, and HspX represent potential target antigens for the development of subunit vaccines and immunodiagnostic tests. Recently, the complete genome sequence revealed the absence of these coding sequences in *Mycobacterium ulcerans*, the causative agent of the emerging human disease Buruli ulcer. Genome reduction and the acquisition of a cytotoxic and immunosuppressive macrolide toxin plasmid are regarded as crucial for the emergence of this pathogen from its environmental progenitor, *Mycobacterium marinum*. Earlier, we have shown the evolution of *M. ulcerans* into two distinct lineages. Here, we show that while the genome of *M. marinum* M contains two copies of the *esxB-esxA* gene cluster at different loci (designated MURD4 and MURD152), both copies are deleted from the genome of *M. ulcerans* strains belonging to the classical lineage. Members of the ancestral lineage instead retained some but disrupted most functional MURD4 or MURD152 copies, either by newly identified genomic insertion-deletion events or by conversions of functional genes to pseudogenes via point mutations. Thus, the *esxA* (ESAT-6), *esxB* (CFP-10), and *hspX* genes are located in hot-spot regions for genomic variation where functional disruption seems to be favored by selection pressure. Our detailed genomic analyses have identified a variety of independent genomic changes that have led to the loss of expression of functional ESAT-6, CFP-10, and HspX proteins. Loss of these immunodominant proteins helps the bacteria bypass the host’s immunological response and may represent part of an ongoing adaptation of *M. ulcerans* to survival in host environments that are screened by immunological defense mechanisms.

The emerging pathogen *Mycobacterium ulcerans* is the causative agent of Buruli ulcer, a mycobacterial disease of skin and soft tissue with the potential to leave sufferers scarred and disabled. While it is endemic in more than 30 countries (26), the major disease burden falls on children living in poor rural communities of West Africa. Buruli ulcer is prevalent in areas neighboring rivers, slow-flowing waters, and swamps, but the exact mode of transmission has remained elusive. This is partly attributable to a clonal population structure and an associated lack of high-resolution genetic fingerprinting methods for mycoparasitologic studies.

*M. ulcerans* seems to have recently evolved via lateral gene transfer and reductive evolution from the fish disease-causing environmental species *Mycobacterium marinum* (40, 43). Particularly, it has acquired the virulence plasmid, pMUM001, encoding the genes for the synthesis of the macrolide toxin, mycolactone. This toxin has cytotoxic and immunomodulatory properties and plays a decisive role in producing an extracellular infection after an initial phase within macrophages (4, 41, 42, 47). In addition, *M. ulcerans* has undergone extensive gene loss due to DNA deletions, DNA rearrangements, and pseudogene formation, which apparently drives its evolution toward a niche-adapted specialist (27, 34, 39). Previous findings suggest that *M. ulcerans* lineages from different geographic areas reveal variations in virulence (27, 32; also F. Portaels, unpublished data).

The ESX-1 secretion system is required for the virulence of *Mycobacterium tuberculosis* and related pathogenic mycobacteria. It comprises the 6-kDa early secretory antigenic target protein (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10), which are among the strongest T-cell response elicitors in tuberculosis patients (7, 8). The genes encoding these proteins are localized on the region of difference 1 (RD1) locus, which is intact in virulent members of the *M. tuberculosis* complex but absent from the attenuated vaccine strain *Mycobacterium bovis* BCG (RD1\†BCG) (21, 29). Similarly, the vole bacillus, *Mycobacterium microti*, was found to have a natural deletion (ΔRD1\micr) overlapping the deletion ΔRD1\BCG (6, 18). The so-called extended RD1 encompasses most of the genes that form the ESX-1 secretion apparatus (7, 16, 17) or are crucial for both ESAT-6/CFP-10 secretion and virulence (7, 17, 19, 31). This secretion apparatus enhances virulence in *M. tuberculosis* and *M. marinum* by secretion of effector proteins into the cytosol of infected macrophages (37), prevention of phagosomal maturation (28, 45), and cytolytic activity (24). On the other hand, infected individuals develop strong T-cell responses against these proteins, which seem to be relevant for immune protection (8). The 16-kDa heat shock protein HspX, or α-crystallin-like protein Acr, a dominant protein expressed during static growth in *M. tuberculosis*, is required for mycobacterial persistence within the macrophage. HspX is yet another potent immune response elicitor and suitable for detecting *M. tuberculosis* infection (14, 15, 20, 25, 35, 49).

In mycobacterial disease control, highly antigenic proteins serve both as targets for diagnostic tests and as candidate

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proteins for vaccine development (1, 8, 30). While being present in the sequenced *M. marinum* strain M (http://www.sanger.ac.uk/cgi-bin/BLAST/submitblast/m_marinum), genes encoding ESAT-6, CFP-10, and HspX are absent from the genome of the sequenced Ghanaian *M. ulcerans* strain Ag99 (http://genopole.pasteur.fr/Muc/BuruList.html). However, earlier data showed that some *M. ulcerans* isolates and other related mycolactone-producing mycobacteria harbor at least segments of these genes (32, 48). Recently, we have identified two distinct genetic lineages of *M. ulcerans*, with representatives of the ancestral lineage being phylogenetically closer to its progenitor, *M. marinum*, than members of the *M. ulcerans* classical lineage (27). Here, we have analyzed a worldwide collection of *M. ulcerans* strains belonging to these two lineages for the presence of *esxA*, *esxB*, and *hspX* and their surrounding genomic regions.

MATERIALS AND METHODS

Mycobacterial strains and genomic DNA extraction. *M. marinum* strain M was used for interspecies comparison. A worldwide strain collection of *M. ulcerans* had been used earlier for investigation of genomic strain variations (34). Although several attempts to differentiate these strains achieved only low resolution (2, 3, 11, 22, 23, 38, 44), this collection of patient isolates was shown to be divided in two lineages displaying major genomic differences (27). In this study, we used *M. ulcerans* clinical isolates of both lineages as follows. For the classical lineage, the following strains were used: Ghana Ag99, Ghana ITM (Institute of Tropical Medicine, Antwerp, Belgium) 970321, Ghana ITM 970359, Ghana ITM 970483, Ivory Coast ITM 940662, Ivory Coast ITM 940815, Ivory Coast ITM 940851, Benin ITM 960657, Benin ITM 960886, Benin ITM 940512, Benin ITM 970104, Democratic Republic of Congo (DRC) ITM 5150, DRC ITM 5151, DRC ITM 5155, Togo ITM 970680, Angola ITM 960567, Angola ITM 960658, Papua New Guinea (PNG) ITM 941331, PNG ITM 9537, Malaysia ITM 941328, Australia ITM 941324, Australia ITM 941325, Australia ITM 941327, Australia ITM 9549, Australia ITM 9550, Australia ITM 98849, Australia ITM 940339, Australia ITM 5142, and Australia ITM 5147. For the ancestral lineage, the following strains were used: China ITM 980912, Japan ITM 8756, French Guiana ITM 7922, Surinam ITM 842, and Mexico ITM 5143. The presence of the specific genomic regions.

| RD | Locus | Description of PCR product |
|----|-------|----------------------------|
| 13/14 | MURD4/MURD152 | Presence of *esxA* as in Ag99/MURD152 |
| 610 | CH1 | MURD92 deletion as in Ag99 |
| 13 MURD152 Presence of *esxA* as in Ag99 162 | CH3 | Deletion ARD13B |
| 13 MURD4 deletion as in Agy99 1,712 | CH4 | Presence of *esxB*-esxA cluster in MURD92 |
| 13 MURD152 MURD152 deletion as in Agy99 1,712 | CH5 | Presence of *esxB*-esxA cluster in MURD |
| 13 MURD152 MURD152 deletion as in Agy99 162 | CH6 | Presence of *esxA* as in Ag99/MURD152 |
| 13 MURD152 Deletion ARD13B | CH7 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH8 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH9 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH10 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH11 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH12 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH13 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH14 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH15 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH16 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH17 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH18 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH19 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH20 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH21 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH22 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH23 | Deletion ARD13B |
| 13 MURD152 Deletion ARD13B | CH24 | Deletion ARD13B |
products were analyzed on 1 to 2% agarose gels by gel electrophoresis using ethidium bromide staining and the AlphaImager illuminator and AlphaImager software (Alpha Innotech, San Leandro, CA). Primers as summarized in Table 1 were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR fragments produced for analysis of unknown genomic sequences were purified using a NucleoSpin purification kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and subjected to direct sequencing or cloned using a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA), transformed into JM109 (Sigma Aldrich, Buchs, Switzerland) bacterial cells, and sequenced after DNA preparation (Miniprep Kit; Sigma Aldrich, Buchs, Switzerland). Variable-number tandem repeat typing analysis undertaken for confirmation of strain identities was performed according to the method of Stragier et al. (44). Sequencing was performed using a BigDye kit and an ABI Prism 310 genetic sequence analyzer (Perkin-Elmer, Waltham, MA). All gene sequences were reproduced and subjected to alignment and comparison with an ABI Prism Autoassembler, version 1.4.0 (Perkin-Elmer, Waltham, MA).

Data analyses and bioinformatics. Retrieved sequences were compared to the BuruList (http://genopole.pasteur.fr/Muc/BuruList.html) and the M. marinum (http://www.sanger.ac.uk/ cgi-bin/BLAST/submitblast/m_marinum) BLAST servers and analyzed using the Sequence Manipulation Suite (http://bioinformatics.org/sms/index.html), the sequence alignment tool BLAST 2 sequences (http://www.ncbi.nlm.nih.gov/BLAST2/secqu/blast2.cgi), the multiple sequence alignment website Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html), and the Artemis software program, release 9 (The Wellcome Trust Sanger Institute, Hinxton, United Kingdom) (36). The sequences for M. tuberculosis were retrieved from the following Web page: http://www.sanger.ac.uk/Projects/M_tuberculosis. Linear genomic comparison was performed using the Artemis comparison tool software, release 6 (9).

Nucleotide sequence accession numbers. The sequences of the indicated genes from M. ulcerans strains have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under the following accession numbers (the associated protein is shown in parentheses): for hspX (HspX), accession numbers EU257156, EU257157, EU257158, EU257159, and EU257160; for esxA (ESAT-6), accession numbers EU257151, EU257152, EU257153, EU257154, and EU257155; and for esxB (CFP-10), accession numbers EU257146, EU257147, EU257148, EU257149, and EU257150. Accession numbers correspond to genes from the Japan 8756, China 980912, Surinam 842, French Guiana 9722, and Mexico 5143 strains, in respective order. Note that the annotated hspX gene in M. ulcerans Agy99 is an orthologue of M. tuberculosis htpX and that the M. tuberculosis hspX orthologue is not present in strain Agy99.

RESULTS

Presence of esxB-esxA in M. ulcerans strains of the ancestral lineage. Blast searches of the partially annotated genome of M. marinum M (http://www.sanger.ac.uk/cgi-bin/BLAST/submitblast/m_marinum) showed that this strain contains two copies of the esxB-esxA (CFP-10–ESAT-6) gene cluster. Both copies are deleted from the genome of the African M. ulcerans isolate Agy99 (43). The corresponding two RDs between the genome

FIG. 1. Confirmation of the MURD-specific deletions affecting esxB (CFP-10) and esxA (ESAT-6) in an M. ulcerans worldwide strain collection. (A) Schematic view of an alignment of M. marinum M and M. ulcerans Agy99 genomic sequences displayed by the Artemis comparison tool (9). Regions of conformity are shown in parallel gray plains, an inverted DNA segment is depicted as an inverted surface, and white areas represent unique sequences like MURD152, which is present only in M. marinum M and is deleted from M. ulcerans Agy99. Indicated are the genes esxB and esxA and the PCR primers (CH1 through CH4) used for this experiment. (B) PCR products of 162 bp or 1,712 bp proved the MURD152 deletion of 2.8 kb and the MURD4 deletion of 12 kb, respectively.
sequences of the two mycobacterial species have been designated MURD152 (M. marinum genome position 6489253 to 6592034) and MURD4 (M. marinum genome position 218302 to 230285) (43).

Compared to M. marinum M, the M. ulcerans Agy99 genome has a 2.8-kb deletion in MURD152, which is associated with a large inversion at the 5′ end of the deletion (Fig. 1A). To test whether all M. ulcerans lineages share this genome constellation in MURD152, we screened a comprehensive M. ulcerans strain collection of worldwide origin by PCR analysis using a primer pair (CH3 and CH4) that yields a PCR product of 162 bp only when MURD152 is deleted and flanked by the inverted sequence (Fig. 1A and B). Whereas members of the ancestral lineage (strains from Asia, South America, and Mexico) were negative, members of the classical lineage (strains from Africa, Papua New Guinea, Malaysia, and Australia) were positive, except for strain Australia 9549, which has a larger deletion in this region (see below). Likewise, a PCR using a primer pair (CH8 and CH9) specific for the sequence constellation of strain Agy99 in MURD4 revealed a PCR product of 1,712 bp only for representatives of the classical and not for members of the ancestral lineage (Fig. 1B), demonstrating genomic diversity between the two M. ulcerans lineages in this locus.

A PCR with primers (CH1 and CH2) corresponding to the 5′ end of the exsB coding sequence and the 3′ end of the exsA coding sequence (Fig. 1A) yielded a PCR product of the expected size of 610 bp with genomic DNA from the M. marinum control and in all M. ulcerans strains belonging to the ancestral lineage (Fig. 2). Primers corresponding to the flanking regions of either the MURD4- or the MURD152-associated exsB-exsA gene cassette were used to determine the localization of this cluster in the genomes of these M. ulcerans strains (Fig. 2). Results indicated that exsB-exsA of the Asian and South American strains is located in MURD152, whereas in the Mexican strain the gene cluster is located in MURD4 (Fig. 2). These localizations were verified by PCR analyses extending several kilobases further into the flanking regions. While in the Asian and South American haplotypes the respective M. marinum MURD152 genome constellations were found, the cluster was flanked in the case of the Mexican haplotype by the MURD4-associated sequences of M. marinum.

Unique deletions in MURD152 in strains 5143 from Mexico and 9549 from Australia. While MURD152 exsB-exsA is deleted from Mexican strain 5143 (Fig. 2), no PCR product specific for the MURD152 constellation of the strains belonging to the classical lineage was obtained with primers CH3 and CH4 (Fig. 1B), giving evidence for a larger deletion. A PCR analysis with primers corresponding to different positions of the genomic sequences flanking MURD152 demonstrated that the Mexico 5143 strain has a deletion (Fig. 3, ΔRD13A) that is replaced by an IS2404 element. This insertion-deletion (indel) event can have occurred either from an M. marinum M-like genome constellation or from an M. ulcerans Agy99-like constellation (loss of 41.8 kb or of 8 kb, respectively). The DNA sequences flanking ΔRD13A in the Mexican strain have a slightly higher identity to the corresponding sequence stretches of M. ulcerans Agy99 than to those of M. marinum M (98% versus 94% over 986 bp).

Failure to obtain a PCR product with both the CH1/CH2 and the CH3/CH4 PCR primers for the Australian strain 9549 (Fig. 1) provided evidence for yet another deletion type within the MURD152 region. PCR analysis using primers located in the sequences flanking the corresponding region in the M. ulcerans Agy99 genome led to the characterization of a deletion of 13,662 bp including an IS2404 element on each of the ends of the deleted DNA segment (Fig. 3, ΔRD13B). The deleted DNA stretch was, in strain Australia 9549, replaced by an IS2404 element that, according to sequence analysis, differed from both versions of IS2404 in Agy99 that were deleted in the ΔRD13B deletions.

Sequence variation in ESAT-6 and CFP-10. PCR products obtained with primers corresponding to MURD locus-specific flanking regions and comprising the respective exsB-exsA clusters (Fig. 2) were sequenced. Deduced amino acid
sequences of all versions of *M. ulcerans* ESAT-6 and CFP-10 encoded in MURD4 (Mexico 5143) or MURD152 (South American and Asian strains) were compared with the *M. marinum* M sequences in the two loci (Fig. 4; see also the supplemental material). As expected, the translated ESAT-6 amino acid sequence of the Mexican strain clustered to and was identical with the MURD4-associated *M. marinum* M sequence (Fig. 4B). While the four MURD152-associated *M. ulcerans* ESAT-6 sequences of the Asian and the South American strains were identical to each other, their amino acid sequences differed at six positions from the MURD152-associated *M. marinum* sequence but only at two positions from the MURD4-associated *M. marinum* sequence (Fig. 4B). At the nucleotide level, the esxA genes of the Asian and South American strains appear as hybrids composed of an *M. marinum* MURD4 sequence stretch at the 5′/H11032 end and a MURD152 stretch at the 3′/H11032 end.

The two *M. ulcerans* esxB genes differed only at three nucleotide positions at the 5′ end (Fig. 4A), encoding CFP-10 proteins with identical deduced amino acid sequences (Fig. 4B). The esxB gene of the Mexican strain differed at four positions from the *M. marinum* M MURD4 locus but at only one position from the MURD152 locus. While the esxB gene sequences of the South American *M. ulcerans* strains were identical to the MURD152-associated sequence, a frameshift mutation has converted esxB of the Asian strains to a pseudogene (Fig. 4B).

**Lack of the immunodominant HspX/Acr protein in the classical lineage of *M. ulcerans*.** Next, we screened the worldwide *M. ulcerans* strain collection for the presence of the coding sequence (CDS) encoding the immunogenic protein HspX (Acr) located in MURD92 (*M. marinum* genome position 4271366 to 4313737) (43). Using primers (CH14 and CH15) corresponding to the hspX flanking regions, a PCR product of 791 bp comprising the complete hspX gene was obtained for all members of the ancestral lineage but for none of the strains belonging to the classical lineage (not shown). Instead, amplification of a 469-bp PCR product (primers CH16 and CH17) again demonstrated the presence of the Agy99 genome constellation (related to the MURD92 deletion) in all members of the classical lineage. While strains coming from the same geographical area had identical gene sequences, Asian and South American sequences differed slightly from each other and from the *M. marinum* sequence (Fig. 5A; see also the supplemental material). In the case of the Mexican strain, nucleotide insertions...
resulted in a frameshift mutation leading to a truncated translation product (Fig. 5B).

**DISCUSSION**

The *M. tuberculosis* proteins ESAT-6, CFP-10, and HspX are strong T- and B-cell immunogens. This makes them suitable targets for immunodiagnostic tests (7, 8, 14, 15, 20) and potentially also for subunit vaccine development (1, 30, 35). These approaches cannot be duplicated for Buruli ulcer, since these proteins are not expressed by classical-lineage *M. ulcerans* strains that are found in areas of endemicity in Africa and Australia and are responsible for the vast majority of clinical cases worldwide.

The genome of the *M. marinum* strain M harbors two *esxB-* *esxA* gene clusters at distant chromosomal locations, one in MURD4 and the other in MURD152. Such duplications are common for proteins of the Esx protein family (46). In this report we demonstrate that all analyzed *M. ulcerans* strains belonging to the ancestral lineage have lost only one copy of the *esxB-* *esxA* cassette: the Asian and South American strains have lost the MURD4 copy and the Mexican strain has lost the MURD152 copy. Furthermore, a frameshift mutation has converted the remaining *esxB* gene of the Asian strains to a pseudogene. The basis for the high degree of identity of the N-terminal *esxA* nucleotide sequence located in the MURD152 locus in the South American and Asian haplotypes with the *M. marinum* MURD4 sequence is unclear, but the finding implies a history of homologous recombination between the two copies of the *esxB* and *esxA* genes before loss of the MURD4 region. Members of the classical lineage have lost both copies, probably in a bottleneck situation that forged this lineage.

Since MURD152, MURD92, and MURD4 show genomic differences not only between *M. marinum* and *M. ulcerans* but also within *M. ulcerans* strains, we designated these RDs.
RD13, RD14, and RD15, respectively, in continuation of the previously assigned RDs within the species *M. ulcerans* (34). A detailed alignment of the chromosomal organization in RD13, which corresponds to RD1 in *M. tuberculosis*, is shown in Fig. 3. These RDs represent hot spots of genetic variation potentially suitable for performing genetic fingerprinting of *M. ulcerans*.

In addition to the previously identified five *M. ulcerans* indel

### TABLE 2. Genomic deletions and amino acid changes in CDSs of immunogenic proteins

| Strain, lineage, haplotype | Characteristics of the indicated gene (protein) by RD and locusa | exxA (ESAT-6) | exxA-1 (ESAT-6) | exxB (CFP-10) | exxB-1 (CFP-10) | hspX (EspX) |
|---------------------------|---------------------------------------------------------------|-----------------|-----------------|---------------|----------------|-------------|
| *M. marinum* M            |                                                               | CDS             | CDS             | CDS           | CDS            | CDS         |
| *M. ulcerans* ancestral lineage strains |
| South America             |                                                               | CDS             | Deletionb       | Deletionb     | CDS            | A105S       |
| Asia                      |                                                               | CDS             | Frameshift mutation (pseudogene) | <2.8-kb deletionc | CDS            | V139F       |
| Mexico                    |                                                               | ΔRD13A          | ΔRD13A          | ΔRD13A        | Frameshift mutation (pseudogene); D64A, L74R |
| *M. ulcerans* classical lineage strains |
| Agy99, Africa, Australia  |                                                               | MURD152 deletion | MURD152 deletion | MURD4 deletion | CDSd           | MURD92 deletion |
| Australia 954B            |                                                               | ΔRD13B          | ΔRD13B          | MURD4 deletion | MURD4 deletion | MURD4 deletion |

* a Amino acid changes are in comparison to the sequence of the *M. marinum* protein.

b The lack of PCR products as shown in Fig. 1B suggests a deletion that differs from the MURD4 deletion in the classical lineage.

c The deletion in the Asian strains is less than 2.8 kb and hence differs from both the *M. ulcerans* Agy99 MURD4 and South American haplotype deletions.

d A screen using outwardly directed primers that bind in the exxA-exxB cluster and in IS2404 and IS2606 and subsequent tests with nested PCR gave evidence for the presence of both IS2404 and IS2606 in the vicinity in strain Mexico 5143 only, indicating yet further genomic changes in this region.
haplotypes (27, 34), strain Australia 9549 was identified as representing a sixth indel haplotype, which is defined by $\Delta$RD13B.

In MURD152 alone, at least three different deletion events are responsible for the indel diversity within M. ulcerans (Table 2). When this region was analyzed for variations among a collection of mycolactone-producing mycobacteria, an unclear situation was suggested for a Mexican strain (48). Here, we show that the deletion of 8 kb replaced by an IS2404 element ($\Delta$RD13A) in the Mexican strain (or 41.8 kb with respect to the M. marinum backbone) differs from the MURD152 deletion in Ag99. This deletion is independent of yet another extended deletion of 13.7 kb ($\Delta$RD13B) in this genomic region in the Australia 9549 strain. The latter deletion is also replaced by an IS2404 element and displays a second, large sequence polymorphism within Australian isolates in addition to the previously described RD3 (27, 34). It will be worth investigating the distribution of this indel polymorphism within a collection of Australian M. ulcerans isolates using the primer pair combination CH10/CH11, demonstrating the presence of the $\Delta$RD13B deletion, and both CH10/CH12 and CH13/CH11, displaying positive results for strains with the sequence configuration of Ag99 (Fig. 3).

The described deletions also encompass CDSs surrounding the $esxA, esxB$, and $hspX$ genes, indicating loss or modification of molecular apparatuses or pathways. First, PE35, essential for secretion (7), was lost in both MURD152 and $\Delta$RD13A and is also commonly deleted in $\Delta$RD$^{HCG}$ and $\Delta$RD$^{mic}$ (Fig. 3). Second, many of the genes of the ESX-1 secretion system (the genes Rv3866/MMAR_5441 through Rv3881/MMAR_5483) are responsible for the indel diversity within M. ulcerans isolates in addition to the previously described RD3 (27, 34). It will be worth investigating the distribution of this indel polymorphism within a collection of Australian M. ulcerans isolates using the primer pair combination CH10/CH11, demonstrating the presence of the $\Delta$RD13B deletion, and both CH10/CH12 and CH13/CH11, displaying positive results for strains with the sequence configuration of Ag99 (Fig. 3).

Hence, our data suggest that functional disruption or complete loss of major targets of the immune response may confer a selective advantage to this emerging pathogen. Still, it is currently unclear whether pathogenicity for mammalian hosts, i.e., shedding into the environment from chronic wounds, contributes significantly to the survival of the species M. ulcerans. However, the observed loss of expression of highly immunogenic proteins caused by a variety of genomic changes may represent an indication that immune selection plays a role in the adaptation of M. ulcerans to a more stable environment.

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