Insertion site and distribution of a genomic island conferring DNA phosphorothioation in the *Mycobacterium abscessus* complex

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Nearly half of US clinical isolates of the emerging pathogen *Mycobacterium abscessus* were reported to exhibit smeared DNA during PFGE. This DNA degradation (Dnd) phenotype results from DNA phosphorothioation, a sulfur modification found in other bacteria and conferred by *dnd* genes located on mobile elements. Putative *dnd* genes are located on a 19.6 kbp genomic island (GI) in the *M. abscessus* type strain ATCC 19977. We confirmed that ATCC 19977T is Dnd-positive by PFGE and we developed a PCR assay to predict Dnd phenotype. Dnd-positive strains generated an amplicon from *dndC* whereas Dnd-negative strains generated a bridge amplicon that spanned the GI insertion site, indicating they lacked the entire ‘Dnd-GI’. Comparative analyses of sequences from the bridge amplicon with ATCC 19977T revealed the Dnd-GI is flanked by 22 bp repeats in *M. abscessus sensu stricto* and between inverted repeats. Regions flanking the Dnd-GI were highly conserved within the *M. abscessus* complex. Bioinformatics studies suggest the Dnd-GI inserted independently into a strain of *Mycobacterium massiliense* and that other species of mycobacteria also have *dnd* genes, supporting reports that the Dnd phenotype is common among actinomycetes. Within the *M. abscessus* complex, Dnd-positive clinical isolates were primarily *M. abscessus sensu stricto*, and tandem repeat typing indicated these isolates were highly related, confirming previous PFGE studies and revealing a widespread family of strains with significance in human disease.

INTRODUCTION

*Mycobacterium abscessus* is a rapidly growing species of mycobacteria and an emerging respiratory pathogen in cystic fibrosis patients and older females with bronchiectasis (Brown-Elliott et al., 2012; Olivier et al., 2003). It is a causative agent of skin and soft tissue infections following surgery, other medical treatments and traumatic injury (Furuya et al., 2008; Medjahed et al., 2010; Villanueva et al., 1997). Classification within the *M. abscessus* complex is in flux (Leao et al., 2011), but three subspecies are currently recognized: *M. abscessus* subsp. *abscessus* (*M. abscessus sensu stricto*), *M. abscessus* subsp. *massiliense* (*Mycobacterium massiliense*) and *M. abscessus* subsp. *bolletii* (*Mycobacterium bolletii*) (Bastian et al., 2011; Macheras et al., 2011). These subspecies are usually differentiated by PCR-restriction analysis of the hsp65 gene, or sequence analysis of hsp65 and other housekeeping genes (Leao et al., 2009; Macheras et al., 2009; Zelazny et al., 2009). More recently, *M. massiliense* has been differentiated from the other two subspecies on the basis of specific deletions in the *erm(41)* gene (Bastian et al., 2011; Kim et al., 2010), although a new report indicates that *erm(41)* is intact in some strains of *M. massiliense* (Shallom et al., 2013). The *erm(41)* gene confers inducible resistance to macrolides (Nash et al., 2009) and deletions in this gene render strains susceptible to macrolides (Bastian et al., 2011; Kim et al., 2010; Koh et al., 2011; Nash et al., 2009).
Until recent advances in typing by variable number tandem repeats (VNTRs) (Harris et al., 2012; Wong et al., 2012), PFGE was the primary method for differentiating strains within the M. abscessus complex and for investigating outbreaks (Jónsson et al., 2007; Sermet-Gaudelus et al., 2003; Wallace et al., 1993; Zhang et al., 2004). However, Wallace et al. (1993) reported that over 40% of clinical isolates of M. abscessus were untypeable by PFGE due to degradation of the DNA, also known as DNA smearing. The phenomenon of DNA degradation (Dnd) during PFGE occurs with other species of bacteria (Corkill et al., 2003; Wallace et al., 1993; Zhou et al., 1988, 2004, 2005). DNA from Streptomyces lividans analyses of natural variants and recombinant strains of 2003; Wallace et al. within the PFGE occurs with other species of bacteria (Corkill degradation of the DNA, also known as DNA smearing. dnd shown to have highly similar PFGE patterns, suggesting a running buffer (Chen electrophoresis, and it was discovered that this technical problem can be resolved by adding thiourea to the gel running buffer (Chen et al., 2010; Ray et al., 1992, 1995; Zhou et al., 1988). Further investigations determined that the genomic DNA of S. lividans is sulfated at specific sequences, and that a cluster of five genes, dndABCDE, encodes a specialized DNA modification system that replaces a non-bridging oxygen in the DNA backbone with sulfur at these sites (Dyson & Evans, 1998; Wang et al., 2007; Xu et al., 2009; You et al., 2007; Zhou et al., 2005). The resulting sulfated (phosphorothioated) DNA is susceptible to oxidative cleavage during electrophoresis, and hence the Dnd phenotype of strains carrying this gene cluster (Chen et al., 2010; Zhou et al., 2005). Using thiourea, 69 isolates of M. abscessus with the Dnd phenotype were shown to have highly similar PFGE patterns, suggesting a clonal relationship (Zhang et al., 2004).

A broad range of species is now known to contain homologues of dnd genes, with several confirmed to have the Dnd phenotype (Chen et al., 2010; He et al., 2007; Ou et al., 2009; Wang et al., 2011). In species analysed so far, the dnd gene clusters are located on mobile genetic elements, predominantly genomic island (GIs) (Ou et al., 2009; Zhou et al., 2004), which are segments of ‘alien’ DNA acquired through horizontal gene transfer (Langille et al., 2010). ATCC 19977T, the type strain of M. abscessus, is negative for the Dnd phenotype (Zhang et al., 2004), which differs in Dnd phenotype (Chen et al., 2010; Dyson & Evans, 1998; Wang et al., 2007; Xu et al., 2009; You et al., 2007; Zhou et al., 2005). The resulting sulfated (phosphorothioated) DNA is susceptible to oxidative cleavage during electrophoresis, and hence the Dnd phenotype of strains carrying this gene cluster (Chen et al., 2010; Zhou et al., 2005). Using thiourea, 69 isolates of M. abscessus with the Dnd phenotype were shown to have highly similar PFGE patterns, suggesting a clonal relationship (Zhang et al., 2004).

In this study, we examined the correlation between Dnd phenotype, dnd genes and the Dnd-GI, characterized the Dnd-GI insertion site, and investigated whether Dnd-positive strains represent a clonal subgroup within the M. abscessus complex. We also discuss the potential relevance of DNA phosphorothioation in the spread of M. abscessus strains.

**METHODS**

**Strains.** Clinical isolates of the M. abscessus complex and Mycobacterium chelonae were obtained from the strain collection of the Mycobacteria/Nocardia Reference Laboratory at the University of Texas Health Science Center at Tyler and were used with approval of the Institutional Review Board. Subspecies of the M. abscessus complex were differentiated by PCR restriction enzyme analysis of the hsp65 gene (Telenti et al., 1993). Studies included strain ATCC 19977T and CIP 102897T, the latter the type strain of M. massiliense.

**DNA extraction and PCR.** Crude extracts of genomic DNA were used for PCR. Briefly, bacteria from plate cultures were resuspended in Tris-EDTA buffer in microcentrifuge tubes, heat-killed, and then lysed by bead-beating for 5 min using a Disruptor-Genie (Scientific Industries). Samples were centrifuged for 15 min to pellet debris, supernatants were removed and centrifuged again. The final supernatants were used for PCR. Amplicons were generated using PCR Master Mix (Promega) and were normally analysed on 1.2% agarose gels containing 50 μg etidium bromide ml⁻¹ in Tris/acetate buffer. For some VNTR typing, 3% MetaPhor (Lonza) agarose gels were used to achieve greater band separation.

Sequences for primers located within, or flanking, the Dnd-GI are detailed in Table 1. In the duplex PCR, dndC was amplified by primers Mab1095PrF2 and Mab1095PrR2, and the bridge amplicon was amplified using primer dndFL5 with either dndRF3A or dndRF3B. For PCR conditions, we used an initial denaturation step of 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 20 s and extension at 72 °C for 1 min, followed by a final step of 2 min at 72 °C. The high annealing temperature was used to avoid the spurious amplicons we observed at annealing temperatures below the Tm of the primers. To amplify erm(41), we used primer ermR1 (5'-GACTTCCCCGACCCGATTC) (Kim et al., 2010), and the in-house primer ermPrF (5'-GCTGCGTCCGCAACGACG), which begins 189 bp upstream of the erm(41) (MAB_2296) start codon. For VNTR typing, we used published primers and conditions (Harris et al., 2012; Wong et al., 2012).

**Sequence analysis and bioinformatics.** Amplicons were sequenced on an ABI 3500 Genetic Analyzer. Sequence comparisons with the National Center for Biotechnology Information (NCBI) nucleotide and protein databases (http://www.ncbi.nlm.nih.gov/) were conducted using the Basic Local Alignment Search Tool (BLAST) programs (Altschul et al., 1997). Genomic sequences for ATCC 19977T (Ripoll et al., 2009) (www.ncbi.nlm.nih.gov/nuccore/ CU458896.1) and M. massiliense strain GO-06 (Raiol et al., 2012) (www.ncbi.nlm.nih.gov/nuccore/CP003699.1) were used in some comparisons. Comparative analyses with the genome of Mycobacterium tuberculosis H37Rv 1 were conducted using Tuberculist version 2.6 (tuberculist.epfl.ch/index.html) (Lew et al., 2011). Additional sequence alignments for M. abscessus were generated by CLUSTAL W using MacVector 10.0. Resources available on the Dnd database dndDB (Ou et al., 2009) (http://db-mml.sjtu.edu.cn/dndDB/) were also utilized.

**PFGE.** Bacteria were lysed in agarose plugs and genomic DNA was digested with AseI, as described (Zhang et al., 2004). Digested DNA
was electrophoresed alongside a bacteriophage lambda DNA size marker (Bio-Rad Laboratories) on 1% agarose gels for 20 h on a CHEF Mapper system (Bio-Rad Laboratories), using a switch time of 5–20 s in 0.5× Tris/borate-EDTA buffer, with or without 50 μM thiourea. Restriction fragments were visualized by staining the gels with ethidium bromide after electrophoresis.

RESULTS

In an early PFGE study, strain ATCC 19977\(^T\) was described as negative for the Dnd phenotype (Zhang et al., 2004). However, subsequent and repeated analyses have determined that DNA from this strain usually degrades during PFGE (Fig. 1a, and S. McNulty and R. J. Wallace, unpublished data), consistent with the presence of the dnd gene cluster (Ripoll et al., 2009). As demonstrated (Fig. 1a), ATCC 19977\(^T\) and a clinical isolate show DNA smearing in the absence of thiourea, whereas addition of thiourea to the electrophoresis buffer negates this degradation.

For strains of M. abscessus lacking the Dnd phenotype, we considered that the genes responsible for DNA degradation could be either missing or inactive. However, studies on Streptomyces (He et al., 2007; Zhou et al., 2004) suggested that absence of the genes was the more likely explanation. Therefore, we designed primers P1 and P2 to amplify a 484 bp region of dndC (MAB_1095) (Fig. 1b), the central of the five genes predicted to compose the dnd locus (Ripoll et al., 2009). ATCC 19977\(^T\) and clinical isolates known to be positive or negative for the Dnd phenotype were then tested, and representative results are shown (Fig. 1c). Strains with the Dnd phenotype (Dnd-positive) were amplified by the dndC primers (Fig. 1c), and sequence analysis of this amplicon from four isolates revealed they had 100% identity to dndC of ATCC 19977\(^T\). In contrast, Dnd-negative strains were not amplified (Fig. 1c), suggesting that dndC is missing. The erm(41) control gene was detected in all strains (Fig. 1c).

We next investigated whether the absence of dndC correlated with the absence of the entire Dnd-GI, using primers that flank the Dnd-GI insertion site of ATCC 19977\(^T\) (Ripoll et al., 2009). Unless deletions had occurred in these regions, all strains should generate amplicons from the left and right flanks with, respectively, primer pairs P3/P4 and P6/P7 (Fig. 1b). However, only strains missing the Dnd-GI should generate the 610 bp ‘bridge’ amplicon (Fig. 1b, dashed line) using primer P3/P5. If the Dnd-GI were present, no amplicon would be generated with P3/P5 under the conditions used due to the large size (19.6 kb) of the island. Strains analysed with the dndC primers (Fig. 1c) were retested with the three primer pairs (Fig. 1d). All strains generated amplicons corresponding to the left and right flanks, whereas only Dnd-negative strains produced the bridge amplicon (Fig. 1d). The results with P3/P5 contrasted directly with results for dndC (Fig. 1c). Overall, these findings suggested that Dnd-negative strains lack the entire GI.

The dndC and bridge primers were retested in duplex PCRs with the Dnd-positive strain ATCC 19977\(^T\) and Dnd-negative strain MC2308 (Fig. 1e). Amplicons of the same size were produced using the primer pairs separately or together. Both amplicons were generated with pooled DNA samples (Fig. 1e, lane 9), indicating that mixed infections with the two genotypes may be detectable, although the amount of the larger bridge amplicon was reduced. Duplex PCR testing of an additional 20 clinical isolates of M. abscessus sensu stricto with known Dnd phenotype indicated the method was suitable for predicting Dnd phenotype (data not shown).

Analysis of M. massiliense for the Dnd-GI

Next, we screened seven clinical isolates of M. massiliense (Fig. 2a). DNA degradation has not been reported for this subspecies, so we were surprised that strain MC4716 (Fig. 2a, lane 3) produced a band of similar size to the dndC amplicon. Furthermore, sequence analysis revealed the amplicon had 100% identity at the DNA level to dndC of ATCC 19977\(^T\). Therefore, we conducted PFGE analysis to test this strain for DNA degradation. In the absence of thiourea, MC4716 exhibited the characteristic smeared DNA of Dnd-positive strains (Fig. 2b, lane 1). To confirm this isolate represented M. massiliense, we analysed the erm(41) gene by PCR. Most strains of this subspecies have a partially deleted erm(41) gene (Bastian et al., 2011; Kim

Table 1. Primers used for PCR analysis of the Dnd-GI

<table>
<thead>
<tr>
<th>Region</th>
<th>Primer*</th>
<th>Position†</th>
<th>Sequence (5’–3’).</th>
</tr>
</thead>
<tbody>
<tr>
<td>dndC</td>
<td>P1 (Mab1095PrF2)</td>
<td>1106 971–1106 993</td>
<td>GGGTTCCGCCCTTTGATGAGTTGG</td>
</tr>
<tr>
<td></td>
<td>P2 (Mab1095PrR2)</td>
<td>1107 454–1107 435</td>
<td>GGTACCGGACCCAGGATCG</td>
</tr>
<tr>
<td>Dnd-GI, left flank</td>
<td>P3 (dndLF5)</td>
<td>1102 363–1102 383</td>
<td>GCTCTGGTGCCCTTACCTTGG</td>
</tr>
<tr>
<td></td>
<td>P4 (dndLF3)</td>
<td>1102 653–1102 633</td>
<td>GTCTGGGCTCTACCCAGATCG</td>
</tr>
<tr>
<td>Dnd-GI, right flank</td>
<td>P5 (dndRF3A)</td>
<td>1122 615–1122 591</td>
<td>CGTGGGCAATGACCTCTATAGTAC</td>
</tr>
<tr>
<td></td>
<td>P6 (dndRF5)</td>
<td>1122 495–1122 515</td>
<td>GCGGTAGTGCCCGATAC</td>
</tr>
<tr>
<td></td>
<td>P7 (dndRF3B)</td>
<td>1122 858–1122 840</td>
<td>GGCAGCCGCTTGCTGTGTT</td>
</tr>
</tbody>
</table>

*For simplicity, the designations P1–P7 were used for these primers in Fig. 1.
†Genomic position in M. abscessus strain ATCC 19977\(^T\) (GenBank accession no. CU458896.1) (Ripoll et al., 2009).

http://mic.sgmjournals.org
et al., 2010; Shallom et al., 2013), and MC4716 generated the short \textit{erm}(41) amplicon associated with \textit{M. massiliense} (Fig. 2c, lane 5). A BLASTN (Altschul et al., 1997) search of the NCBI nucleotide database with the \textit{dndC} amplicon sequence revealed a 100\% match to an unidentified ORF in \textit{M. massiliense} GO-06, a recently sequenced clinical isolate from Brazil (Raiol et al., 2012). Although the Dnd phenotype of this strain has not been reported, this finding suggests \textit{M. massiliense} GO-06 is Dnd-positive.

\textbf{Analysis of \textit{M. bolletii} and \textit{M. chelonae} for the Dnd-GI}

We examined six isolates of \textit{M. bolletii} and eight isolates of the rapid-grower \textit{M. chelonae}. \textit{M. abscessus} was previously
considered a subspecies of *M. chelonae* (Kusunoki & Ezaki, 1992) and some genomic regions are highly similar between the two species (Ripoll et al., 2007). For this duplex PCR test, we used primer P7 (Fig. 1b) instead of P5 to generate a larger bridge amplicon (bridge-2) that could be more easily separated from the *dndC* amplicon. As expected, ATCC 19977*^T* and the Dnd-negative strain MC2308 produced, respectively, the 480 bp *dndC* amplicon and 850 bp bridge-2 amplicon (Fig. 2d, lanes 1 and 2). The *dndC* gene was not detected in any isolate of *M. bolletii* (Fig. 2d). However, an amplicon consistent with the *dndC* gene was not detected in any isolate of *M. massiliense* (Fig. 2d, lane 11), and sequence analysis confirmed it had 100% identity to *dndC* of ATCC 19977*^T*. Four *M. bolletii* isolates and *M. chelonae* isolate MC6036 produced an amplicon of similar size to the bridge-2 amplicon (Fig. 2d, lanes 4, 6–8 and 14), suggesting they are Dnd-negative. The identity of several bridge-2 amplicons was confirmed by sequence analysis (see next section). *M. bolletii* isolate 11-823 produced a band of intermediate size (Fig. 2d, lane 3). Sequence analysis indicated this was an aberrant amplicon resulting from mispriming (data not shown).

Both the *dndC* and the bridge-2 amplicons were absent from one isolate of *M. bolletii* (Fig. 2d, lane 5) and six isolates of *M. chelonae* (Fig. 2d, lanes 9, 10, 12, 13, 15 and 16). These findings suggest that broader genetic variation occurs in this region, potentially ranging from nucleotide substitutions to large deletions in the primer-binding regions, especially with the more distantly related *M. chelonae*. Faint non-specific bands were also observed for several isolates of *M. chelonae*, so PCR conditions may need further optimization for screening this species. However, these strains are probably Dnd-negative as the bridge-2 amplicon was detectable in an isolate of *M. chelonae* using the same primers (Fig. 2d).

### Sequence features at the Dnd-GI insertion site

To aid in characterizing the features associated with insertion of the Dnd-GI, bridge amplicons were sequenced for four Dnd-negative isolates of *M. abscessus sensu stricto*, two isolates of *M. bolletii*, and one isolate each of *M. massiliense* and *M. chelonae* (Fig. 3a). Sequence comparisons revealed this genomic region is highly conserved (Fig. S1, available in *Microbiology* Online), and that sections immediately flanking the GI insertion site in Dnd-negative strains are identical to each other and to the corresponding sections of ATCC 19977*^T* (Fig. 3a). Direct repeats (DRs)
and tRNA genes are frequently associated with GIs (Hacker et al., 1997; Liu & Zhu, 2010; Ou et al., 2006; Reiter et al., 1989; Williams, 2002). The Dnd-GI insertion site (Fig. 3a, vertical arrow) is located immediately after a 22 bp sequence that is duplicated at the end of the island in ATCC 19977\(^T\), and a tRNA-Ala gene upstream of the Dnd-GI in ATCC 19977\(^T\). There is a short inverted repeat within the tRNA gene and another inverted repeat is located downstream, within the region that becomes the right flank of the Dnd-GI. This downstream repeat consists of nine paired G/C nucleotides with the potential to form a highly stable hairpin structure.

While conducting tblastn (Altschul et al., 1997) searches with the sequences surrounding the Dnd-GI insertion site, we found matches to M. massiliense strain GO-06 (Raio et al., 2012). However, comparative analyses of flanking sequences revealed that tRNA-Ala is fully duplicated on the right side of the Dnd-GI in strain GO-06 (Fig. S2). Additionally, a short stretch of nucleotides from the right flank of the Dnd-GI in ATCC 19977\(^T\) is present in the left and right flanks of M. massiliense strain GO-06 (Figs 3a, b, and S2). Nucleotide ambiguities complicated the analyses, but blast comparisons and sequence alignments of the flanks indicate the DR of M. massiliense strain GO-06 is approximately 180 bp (Fig. S2), and that the Dnd-GI insertion point is approximately 55 bp further downstream than in ATCC 19977\(^T\) (Fig. 3b). Therefore, we conclude that insertion of the Dnd-GI into ATCC 19977 and M. massiliense strain GO-06 occurred independently. Based on the locations of the repeats (Fig. S2), the Dnd-GI of M. massiliense strain GO-06 appears to be of similar length to the Dnd-GI of ATCC 19977\(^T\).

**Genetic similarity of Dnd-positive isolates of M. abscessus sensu stricto**

Published (Zhang et al., 2004) and unpublished PFGE analyses (S. McNulty, R. J. Wallace) of Dnd-positive strains of M. abscessus sensu stricto with thiourea indicate they have highly similar restriction fragment patterns, suggesting the strains are closely related. Recent developments in VNTR typing of the M. abscessus complex provided an alternative method to evaluate strain relatedness. Wong et al. (2012) identified 18 tandem repeat (TR) regions for VNTR typing, but noted that strains could be efficiently differentiated by just six of these regions (TR45, TR109, TR116, TR150, TR155 and TR172), which we refer to as TR subset-6. We tested 15 Dnd-positive isolates from different patients for TR subset-6. Four VNTR patterns were detected and 12 strains (80%), including ATCC 19977\(^T\),
had VNTR pattern 1 (Table 2 and data not shown). Three isolates had the different copy number at TR172, with two of the isolates also varying from the main pattern at TR116 or TR150. Examination of over 20 Dnd-negative isolates demonstrated that they had a greater range of copy numbers at each locus and that none shared a VNTR pattern found in Dnd-positive isolates (Table 2 and data not shown).

Preliminary analyses of several Dnd-positive isolates using other VNTR loci (Harris et al., 2012) indicated they had the same pattern as that reported for ATCC 19977T, and that these patterns differed from those of Dnd-negative isolates (data not shown). Overall, these data support a clonal relationship among Dnd-positive isolates of M. abscessus sensu stricto, and suggest they spread subsequent to insertion of the Dnd-GI.

**DISCUSSION**

In the first paper describing a complete genome sequence for a member of the M. abscessus complex, Ripoll et al. (2009) reported that the gene cluster MAB_1093c–MAB_1098 of ATCC 19977T closely resembled the dndABCDE genes of S. lividans and Geobacter uraniireducens Rf4T. Our results strongly support the role of this gene cluster in the Dnd phenotype of M. abscessus. We developed a duplex PCR to screen strains for dndC and the bridge amplicon that spans the Dnd-GI insertion site. The dndC gene was amplified in Dnd-positive strains whereas the bridge amplicon was amplified only in Dnd-negative strains, with the latter amplicon indicating the entire Dnd-GI was absent. This finding is consistent with Streptomyces strains wherein a Dnd-negative phenotype usually correlates with the absence of the mobile element containing the dnd genes (He et al., 2007; Zhou et al., 2004). Although our group initially reported that ATCC 19977T was Dnd-negative (Zhang et al., 2004), additional PFGE analyses showed it exhibits DNA degradation, consistent with the presence of the Dnd-GI. We suspect our early studies with the strain were performed with ‘good’ Tris buffer (Zhou et al., 1988), thus avoiding DNA degradation. Indeed, a report by another laboratory also shows interpretable PFGE patterns for ATCC 19977T in the absence of thiourea (Matsumoto et al., 2011).

We chose dndC (MAB_1095) as the target in our screen for Dnd-positive strains because it is present in every dnd gene cluster (Ou et al., 2009) and is essential for phosphorothioation (Xu et al., 2009). The dndC gene encodes phospho-adenosine phosphosulfate reductase, an iron–sulfur protein with ATP pyrophosphatase activity (Chen et al., 2010; You et al., 2007; Zhou et al., 2005), which is activated by the cysteine desulfurase-transerase DndA (You et al., 2007). In M. abscessus, dndA (MAB_1093c) is in reverse orientation to the other four genes (Ou et al., 2009). The dndC amplicon correlated directly with the Dnd phenotype, although it does not confirm the Dnd phenotype, as deletions of other dnd genes would not be detected by our analyses. Although we only examined six strains, we did not detect dndC in M. bolletii, and only one of seven strains of M. massiliense was Dnd-positive. In contrast, a previous study from our group (Wallace et al., 1993) and our unpublished data indicate that 40–50% of M. abscessus sensu stricto strains are Dnd-positive. The Dnd phenotype has also been observed in a strain of Mycobacterium smegnatis (Zhou et al., 2005), and using tBLASTn (Altschul et al., 1997), we identified an ORF identical to DndC/MAB_1095 in M. massiliense GO-06. Using Blastp, we also discovered orthologues of DndC in Mycobacterium phlei strain RIVM601174 (79% identity, 88% similarity), Mycobacterium parascrofulaceum strain ATCC BAA-614T (78% identity, 88% similarity) and Gordonia sputi NBRC 100414T (76% identity, 85% similarity), suggesting these strains are Dnd-positive. During completion of this manuscript, a new annotated database of genomic sequences for the M. abscessus complex was reported (Heydari et al., 2013) and our analyses indicate that at least 13 strains of M. abscessus sensu stricto had dndC. Overall, these data support the

**Table 2. VNTR typing of 15 Dnd-positive isolates of M. abscessus sensu stricto with TR subset-6**

<table>
<thead>
<tr>
<th>Pattern</th>
<th>TR45*</th>
<th>TR109</th>
<th>TR116</th>
<th>TR150</th>
<th>TR155</th>
<th>TR172</th>
<th>No. of isolates†</th>
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<tr>
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<td>3</td>
<td>3</td>
<td>3</td>
<td>4</td>
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<td>Most frequent copy number (per cent)‡</td>
<td>3 (100)</td>
<td>3 (100)</td>
<td>3 (93)</td>
<td>4 (93)</td>
<td>3 (100)</td>
<td>4 (80)</td>
<td></td>
</tr>
<tr>
<td>Range of copy number in subspecies§</td>
<td>1–4</td>
<td>1–4</td>
<td>0–5</td>
<td>1–7</td>
<td>3–5</td>
<td>2–7</td>
<td></td>
</tr>
</tbody>
</table>

*Tandem repeat locus (Wong et al., 2012). Values are the number of copies of the TR in the specified pattern; underlined copy numbers differ from those found in pattern 1.

†Number of Dnd-positive isolates with the specified pattern.

‡Percentage of Dnd-positive isolates (of 15 isolates) with the most frequent copy number for that locus.

§Ranges are based on analysis of 12 isolates by Wong et al. (2012), and preliminary analyses of additional Dnd-negative and Dnd-positive clinical isolates of M. abscessus sensu stricto (our unpublished data).
finding that the Dnd phenotype is common in actinomycetes (He et al., 2007).

The Dnd-GI is inserted between tRNA-Ala and a G/C-rich inverted repeat that potentially forms a hairpin stem of eight to nine G–C base pairs, and that appears to be part of a Rho-independent termination site for the tRNA transcript (Lesnik et al., 2001; Mitra et al., 2008). Rho-independent terminators usually consist of a hairpin followed by a T-rich region (Lesnik et al., 2001; Miotti et al., 2012; Mitra et al., 2008), and there are seven thymines within the stretch of 10 bases immediately downstream of the inverted repeat in the strains we examined (Fig. S1). tRNA genes are preferred integration sites for mobile elements (Liu & Zhu, 2010; Reiter et al., 1989; Williams, 2002), and as we reported in this study, sequences surrounding these integration sites are often highly conserved (Ou et al., 2006). GIs are also frequently flanked by DRs (Hacker et al., 1997; Ou et al., 2006), and a 22 bp DR flanks the Dnd-GI in ATCC 19977T. We found that the sequenced strain M. massiliense GO-06 (Raiol et al., 2012) also contains a Dnd-GI, but our analyses suggest it inserted independently from the one in ATCC 19977T. This section of the mycobacterial genome may be a hotspot for integration of foreign DNA. Comparative analysis (data not shown) indicates there is extensive synteny between the genomes of ATCC 19977T (Ripoll et al., 2009) and M. tuberculosis H37RvT (Cole et al., 1998; Lew et al., 2011) upstream of the tRNA-Ala gene, a region believed to contain horizontally acquired genes in M. tuberculosis (Becq et al., 2007; Rosas-Magallanes et al., 2006; Talaat et al., 2004).

The presence of dnd genes in a wide array of species (Ou et al., 2009; Wang et al., 2011) raises the question of what advantage DNA phosphoryation might provide. In Salmonella enterica, dnd genes are part of a host-specific restriction system that degrades DNA not modified by phosphoryation (Xu et al., 2010). The genes responsible for restricting unmodified DNA are adjacent to the dnd gene cluster, and homologues of the restriction genes were found in the Dnd-GIs of some but not all species examined (Xu et al., 2010), suggesting this function of phosphoryation is not ubiquitous. In a BLAST search of sequenced strains of the M. abscessus complex, we did not find homologues to DptF and DptG, two proteins involved in the host restriction system of S. enterica (Xu et al., 2010). Other Gram-positive species contain recognizable homologues of these genes (Xu et al., 2010), so our findings suggest that M. abscessus does not contain this restriction system.

Intriguingly, a recent paper indicates that DNA phosphoryation protects DNA from oxidative damage and a Dnd-positive strain of Salmonella showed greater resistance than Dnd-negative mutants to oxidizing agents (Xic et al., 2012). M. abscessus is potentially exposed to reactive oxygen species in macrophages and in the environment. The bacterium is found in biofilms and water distribution systems (Esteban et al., 2008; Fraser et al., 1992; Greendyke & Byrd, 2008; Howard et al., 2006; Vaerejojik et al., 2005), and reactive oxygen species are generated in biofilms (Cáp et al., 2012; Das & Manefield, 2012; Zhu & Kreth, 2012), and the chlorine used in municipal water purification is also an oxidant. We conducted preliminary experiments comparing the resistance of Dnd-positive and Dnd-negative isolates of M. abscessus sensu stricto to hydrogen peroxide. One Dnd-negative isolate had low resistance whereas another Dnd-negative isolate had high resistance, similar to ATCC 19977T (data not shown), suggesting other factors, perhaps catalases, contribute to strain variation in resistance. Mutagenesis experiments will be required to confirm the role of dnd genes in the M. abscessus complex.

Most Dnd-positive strains of M. abscessus sensu stricto have highly similar PFGE patterns (Zhang et al., 2004; S. McNulty, R. J. Wallace, unpublished data), and our VNTR typing revealed only minor pattern variations. A study of respiratory isolates from Malaysia (Wong et al., 2012) detected two isolates that were identical to ATCC 19977T at 18 VNTR loci, including the TR subset-6 loci. In a UK study using different VNTR targets (Harris et al., 2012), nearly half of the isolates of M. abscessus sensu stricto from cystic fibrosis patients had the same VNTR pattern as ATCC 19977T. We predict that isolates with the ATCC 19977T VNTR patterns are Dnd-positive. It will be important to identify additional genetic markers that will distinguish between strains of this family, because their widespread nature could potentially complicate epidemiological studies and lead to misinterpretation of data from putative outbreaks.

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