Polymorphic Nucleotide within the Promoter of Nitrate Reductase (NarGHJI) Is Specific for *Mycobacterium tuberculosis*

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Mycobacterium tuberculosis rapidly reduces nitrate, leading to the accumulation of nitrite. This characteristic served for the past 40 years to differentiate *M. tuberculosis* from other members of the *Mycobacterium tuberculosis* complex (MTBC), such as *Mycobacterium bovis* (non-BCG [referred to here as simply “*M. bovis*”]), *Mycobacterium bovis* BCG, *Mycobacterium africanum*, or *Mycobacterium microti*. Here, a *narG* deletion in *M. tuberculosis* showed that rapid nitrite accumulation of *M. tuberculosis* is mediated by *narGHJI*. Analysis of *narG* mutants of *M. bovis* and *M. bovis* BCG showed that, as in *M. tuberculosis*, nitrite accumulation was mediated by *narGHJI*, and no other nitrate reductase was involved. However, in contrast to *M. tuberculosis*, accumulation was delayed for several days. Comparison of the *narGHJI* promoter revealed that, at nucleotide –215 prior to the start codon of *narG*, *M. tuberculosis* carried a thymine residue, whereas the bovine mycobacteria carried a cytosine residue. Using LightCycler technology we examined 62 strains of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. microti*, and *M. africanum* and demonstrated that this single nucleotide polymorphism was specific for *M. tuberculosis*. For further differentiation within the MTBC, we included, by using LightCycler technology, the previously described analysis of *oxyR* polymorphism, which is specific for the bovine mycobacteria, and the RD1 polymorphism, which is specific for *M. bovis* BCG. Based on these results, we suggest a LightCycler format for rapid and unambiguous diagnosis of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG.

Mycobacterium tuberculosis, the major pathogen of human tuberculosis, predominantly affects the respiratory tract, whereas *M. bovis*, the major pathogen of tuberculosis in cattle, is typically found in extrapulmonary tuberculosis (5, 32). Attenuated *M. bovis* BCG, the only currently available vaccine against tuberculosis, has been administered to more than 3 billion people worldwide (17). It may be isolated from immunocompromised individuals, who might develop disseminated disease with the vaccine strain after vaccination with *M. bovis* BCG. *M. africanum* and *M. microti* are rarely encountered members of the *M. tuberculosis* complex (MTBC) (11, 18, 22). *M. africanum* causes human tuberculosis in certain regions of tropical Africa. *M. microti* causes naturally acquired generalized tuberculosis in voles and produces local lesions in guinea pigs, rabbits, and calves and has been described very occasionally as a cause of infection in human immunodeficiency virus-positive patients.

Nitrate reductase activity is a widely used phenotypic trait to differentiate between *M. tuberculosis*, which rapidly accumulates nitrite from nitrate, and other members of the MTBC (18). In other pathogenic and environmental bacteria, at least three different nitrate reductases have been found, one of which is the respiratory nitrate reductase encoded by *narGHJI* (19). The membrane-bound complex consists of NarG, -H, and -I, with NarG being the catalytic subunit, whereas NarJ is required for the assembly of the enzyme. Expression of *narGHJI* is typically induced under anaerobic conditions (20). Previous studies on mycobacterial nitrate reduction were limited to its role in classification and identification of the genus

Mycobacterium (2, 6, 28, 29). However, recent reports revived interest in a possible role for enzymes involved in nitrate metabolism during infection with *M. tuberculosis* or *M. bovis* BCG (4, 12, 30). We provided evidence that *M. bovis* BCG weakly accumulates nitrite from nitrate under strictly anaerobic conditions, that this activity is mediated by *narGHJI*, and that a mutant of *M. bovis* BCG with a partial deletion of the *narG* gene was attenuated in mice, linking anaerobic nitrate reduction to mycobacterial pathogenesis (7, 31).

In the present study, we generated targeted deletion of *narG* to compare the role of *narGHJI* in nitrite accumulation by *M. tuberculosis*, *M. bovis* (non-BCG [referred to here as simply “*M. bovis*”]), and *M. bovis* BCG. Analysis of the promoter region of *narGHJI* from either species revealed a single nucleotide polymorphism that separated *M. tuberculosis* from the bovine mycobacteria. We tested a variety of different strains within the MTBC by using LightCycler technology and showed that this single nucleotide polymorphism was specific for *M. tuberculosis*, thus allowing rapid identification of *M. tuberculosis*. For further differentiation within the MTBC based on LightCycler technology, we adapted analysis of the *oxyR* polymorphism, which is specific for the bovine mycobacteria, and the region of differences 1 (RD1) polymorphism, which is specific for *M. bovis* BCG, to a LightCycler format.

MATERIALS AND METHODS

Strains and cultures. To test nitrate reductase activity and the generation of *narG* mutants, we used *M. tuberculosis* H37Rv ATCC 25618, a clinical strain of *M. bovis*, and *M. bovis* BCG Pasteur ATCC 35734. All strains were cultured in 7H9 broth or on 7H10 plates (Difco Laboratories, Inc., Detroit, Mich.) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% ADS (0.5% bovine albumin fraction V, 0.2% glucose, 140 mM NaCl) unless indicated otherwise.

For LightCycler-based analysis, strains were grown in the MGIT 960 automated culture system (Becton Dickinson, Sparks, Md.). A pellet from 0.5 ml of a MGIT culture was resuspended in 10 mM Tris (pH 8.0) and 1 mM EDTA,

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TABLE 1. Melting temperatures for *Mycobacterium* strains in this study

Species	Strain(s) ^a (n)	Mean melting temp (°C) of oligonucleotide specific for ^b :		
		<i>M. tuberculosis</i> <i>narGHJI</i> (SD)	Bovine mycobacteria <i>oxyR</i> (SD)	<i>M. bovis</i> BCG RD1 (SD)
<i>M. tuberculosis</i>	H37Rv ATCC 25618	56.8	68.0	62.6
	Erdmann ATCC 35801	57.0	68.3	62.1
	Clinical strains (33)	56.9 (0.36)	68.2 (0.15)	61.9 (0.47)
<i>M. africanum</i>	Clinical strains (3)	63.4 (0.21)	68.0 (0.28)	62.4 (0.49)
<i>M. microti</i>	Clinical strains (2)	63.2 (0.28)	68.2 (0.07)	61.7 (0.07)
<i>M. bovis</i>	ATCC 19210	63.1	59.3	62.5
	Clinical strains (12)	63.2 (0.30)	59.2 (0.13)	61.7 (0.26)
<i>M. bovis</i> BCG	Pasteur ATCC 35734	63.6	59.7	49.4
	Copenhagen ATCC 27290	63.5	59.0	49.6
	Moreau ATCC 35736	63.7	59.2	49.5
	Tice ATCC 35743	63.5	59.0	49.5
	Connaught ATCC 35745	63.7	59.1	49.4
	Clinical strains (4)	63.2 (0.26)	59.2 (0.09)	49.6 (0.14)

^a Strains were obtained from the ATCC or various sources as indicated in Materials and Methods. Bacilli were cultured in the MGIT automated culture system, and DNA was prepared by mechanical disruption and used directly for LightCycler amplification.

^b For ATCC strains, individual melting temperatures are shown. For all clinical strains, the standard deviations are given in parentheses for each group of strains. Melting temperatures specific for each *Mycobacterium* group are indicated in boldface in the respective columns.

supplemented with glass beads (106 µm in diameter), and vortexed for 2 min, and the supernatant was used for the LightCycler reaction. As indicated in Table 1, 62 strains were tested. Eight strains were obtained from the American Tissue Culture Collection (ATCC). A total of 54 strains were recovered from clinical sources and either identified in our laboratory between 2000 and 2002 ($n = 41$, including 33 strains of *M. tuberculosis*, 1 strain of *M. africanum*, 3 strains of *M. bovis*, and 4 strains of *M. bovis* BCG), or they were provided by the National Reference Center for Mycobacteria in Borstel, Germany ($n = 4$, including 2 strains of *M. africanum*, and 2 strains of *M. microti*) or by the Federal Research Centre for Virus Diseases of Animals in Jena, Germany ($n = 9$, all of which were *M. bovis* strains). Clinical isolates from the Medical School of Hannover were identified by partial sequencing of 16S rRNA gene (13), phenotypic testing of niacin/nitrate (18), and analysis of RD1 as described by Talbot et al. (27). Strains rendering doubtful results were sent to the National Reference Center for Mycobacteria in Borstel for further testing. Patients from whom mycobacteria were isolated at the Medical School of Hannover originated from Germany, from other European countries, and from Africa and Asia (8, 9). Human and animal isolates of *M. bovis* strains provided by the Federal Research Centre for Virus Diseases of Animals in Jena were recovered from sources in Germany and other European countries. ATCC strains originated from sources worldwide.

Accumulation of nitrite under anaerobic conditions. The presence of nitrite can be demonstrated by naphthylamide and sulfanilic acid reagents, which form a red diazonium dye when they react with nitrite (18). Prior to testing for accumulation of nitrite, bacteria were grown under aerobic conditions in 7H9 broth to an optical density at 600 nm of between 0.7 and 1.0. For the experiment shown in Fig. 1, a dense culture was washed three times with MB medium without added nitrate. MB medium is a minimal medium based on various salts, buffers, and trace elements. Fully supplemented MB medium contains albumin, dextrose, and saline (i.e., ADS) and also nitrate. The addition of Tween 80 is necessary to prevent mycobacteria from clumping in liquid culture. The exact formula of 1 liter fully supplemented MB medium is as follows: 1 g of KH_2PO_4 , 2.5 g of Na_2HPO_4 , 2.0 g of K_2SO_4 , 0.5 mM MgCl_2 , 0.5 mM CaCl_2 , 0.2% glycerol, 0.05% Tween 80, 10% ADS, 10 mM nitrate, and 2 ml of trace elements. One liter of trace elements contained 40 mg of ZnCl_2 , 200 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 10 mg of $\text{CuCl}_2 \cdot 4\text{H}_2\text{O}$, 10 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 10 mg of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, and 10 mg of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$. After the culture was washed three times, the pellet was resuspended in fully supplemented MB medium reaching an optical density at 600 nm of ca. 0.2 corresponding to ca. 10^7 bacilli/ml. An anaerobic environment was achieved with the AnaeroGen anaerobic system (Oxoid, Ltd., Basingstoke, Hampshire, England) in a standard anaerobic jar. As recommended by the manufacturer, an indicator strip was used to confirm anaerobic conditions. At the indicated time points, sulfanilic acid and *N,N*-dimethyl-1-naphthylamine (both reagents were taken from the API system [bioMérieux, Marcy l'Étoile, France])

were added to aliquots of culture, followed by centrifugation at $15,000 \times g$ for 15 min at room temperature. The absorbency of supernatant was measured at 530 nm and compared to a known standard of nitrite (ranging from 10^{-6} to 10^{-3} mol/liter). Testing was always performed in duplicates.

Diagnostic nitrate reductase activity. According to the recommendations of the American Society for Microbiology, the diagnostic nitrate reductase activity test must be performed with actively growing cultures that are inoculated directly into phosphate buffer supplemented with nitrate, followed by incubation for 2 h at 37°C. Culture prior to testing should be done on solid medium (15, 18). Thus, to test for diagnostic nitrate reductase activity, mycobacteria were cultured on 7H10 agar (Difco) supplemented with 0.2% glycerol and 10% ADS. For the experiment shown in Fig. 3, bacilli were inoculated into phosphate buffer supplemented with 10 mM nitrate. After 2 h of incubation at 37°C, naphthylamide and sulfanilic acid reagents were added, and a photograph was obtained.

FIG. 1. Anaerobic nitrate reductase activity of wild-type and *narG* mutant strains of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG. A total of 10^7 wild-type (closed symbols) and *narG* mutant (open symbols) *M. tuberculosis* (squares), *M. bovis* (triangles), and *M. bovis* BCG (circles) bacteria/ml were cultured in MB medium supplemented with 10 mM nitrate, and aliquots were tested for production of nitrite after 1, 2, 3, 5, and 7 days.

Generation of *narG* mutants in *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG. The vector pYUB657 does not replicate or integrate in mycobacteria and contains a hygromycin resistance cassette for positive selection and *sacB* for negative selection, thus allowing generation of unmarked mutants by targeting gene deletion (24). To generate *narG* mutants of *M. bovis* and *M. bovis* BCG, a 736-bp *Cla*I fragment within the *narG* gene was deleted. To generate a *narG* mutant of *M. tuberculosis*, a 684-bp *Bsm*I/*Msc*I fragment within the *narG* gene was deleted. The mutated fragments were cloned into the *Pac*I site of pYUB657. *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG were transformed and screened for clones resistant to hygromycin. We performed PCR to screen for the presence of the mutated *narG* gene with ACT GGT TCC ACA GCG ATG as a forward primer, targeting a region just upstream of the first *Cla*I site and the *Msc*I site, respectively. AGG GAT GGA CGG TAT ATC was used as a reverse primer, targeting a region just downstream of the second *Cla*I site and the *Bsm*I site, respectively. A 438-bp fragment was amplified in the presence of the mutated *narG* in *M. tuberculosis*, and a 488 bp in the presence of the mutated *narG* fragment in *M. bovis* and *M. bovis* BCG. A 1,122-bp fragment was amplified in the presence of wild-type *narG* in all three species. Cointegration of the plasmid carrying the mutated *narG* gene next to the *narG* wild-type locus within the mycobacterial genome was confirmed by using Southern blot analysis. Genomic DNAs from potential clones were digested with *Bss*HI, and a DNA probe, targeting a 527-bp region downstream from the *narG* deletion, was constructed by using TCG GAC TTT GAC GCA TTC GC as a forward primer and GTA TCG GCG TAG GTG ATG CG as a reverse primer. A 4,723-bp fragment indicated wild-type *M. tuberculosis*, *M. bovis*, or *M. bovis* BCG, whereas a 17,458-bp fragment indicated cointegration in *M. tuberculosis* and a 17,406-bp fragment indicated cointegration in *M. bovis* and *M. bovis* BCG.

By using hygromycin for selection and sucrose for counterselection as described previously (24), clones were chosen in which the wild-type *narG* was replaced by the mutated *narG*. Chromosomal DNA was digested with *Sma*I restriction enzyme, since the deleted fragment of the *narG* site contained a *Sma*I site. Wild-type *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG showed a 1,114-bp fragment. The *narG* mutant of *M. tuberculosis* carried a 2,302-bp fragment, and the *narG* mutants of *M. bovis* and *M. bovis* BCG contained a 2,250-bp fragment. Eventually, clones were chosen and named MS1 for the *narG* mutant of *M. tuberculosis*, SM118 for the *narG* mutant of *M. bovis*, and SR106 for the *narG* mutant of *M. bovis* BCG.

Standard LightCycler protocol. Using LightCycler model II (Roche, Mannheim, Germany), a commercially available ready-to-use hot-start reaction mixture (LightCycler FastStart DNA master hybridization probes; catalog no. 239272; Roche) containing FastStart *Taq* polymerase, reaction buffer, deoxynucleoside triphosphates, and 1 mM MgCl₂ was supplemented with 2 mM MgCl₂. Primers were supplied with 18 pmol (1.1 μ M final concentration) per reaction and DNA probes with 2 pmol (100 nM final concentration). The amplification program began with denaturation at 95°C for 10 min, followed by 50 cycles of 95°C for 3 s (denaturation), a temperature range of 62 to 68°C for 2 s ("touch-down" annealing), and 72°C for 40 s (extension). For the first five cycles, annealing was performed at 68°C (step delay) and then reduced to 62°C with 1°C per cycle (step size). The temperature transition rate for all cycling steps was 20°C per s. The amplification program was followed by a melting program of 95°C for 30 s (denaturation), 38°C for 30 s (annealing), and then 38 to 80°C at a transition rate of 0.2°C/s with continuing monitoring of fluorescence. The 3.5.3 version of LightCycler profile software automatically adjusted the gain of the F2/F3 channel photometric detector.

Detection of T/C transition within the promoter of *narGHJI*. For DNA amplification, LC66 (AAC CGA CGG TGT GGT TGA C) was used as a forward primer, and LC67 (ATC TCG ATG GAT GGG CGT C) was used as a reverse primer. The polymorphic nucleotide was targeted by using LC63 (GTC GCC ACG CGT CCA GAA AAC C; antisense) as an anchor probe and LC64 (CGT GAT CGC TAC GGG CAT; antisense, where the underlined nucleotide is the polymorphic nucleotide within the promoter of *narGHJI*) as a sensor probe. The anchor probe LC63 was labeled with fluorescein as a donor for fluorescence resonance energy transfer (FRET), and the sensor probe LC64 was labeled with LightCycler Red 640 as an acceptor for FRET.

Detection of A/G transition of *oxyR*. For DNA amplification, LC90 (CGG GTG CCG CTG ACC GCG) was used as a forward primer, and LC91 (CCA GCC GGC TTC GCG TGG) was used as a reverse primer. The polymorphic nucleotide was targeted by using LC92 (GCC GGT CAC GCA CTG CAC GAC G; antisense) as an anchor probe and LC94 (GGC CAG CCA CAC CGC; antisense) as a sensor probe. The anchor probe LC92 was labeled with fluorescein as a donor for FRET, and the sensor probe LC94 was labeled with LightCycler Red 705 as an acceptor for FRET.

Detection of RD 1 region. The 3' end of RD1 was detected by using LC73 (CTA GAC GAG GCC GCT CAA G) and LC74 (AAG ACG TGG CCT TTC TGC TG) as forward primers and LC75 (ACG GGT TAC TGC GAA TAC CG) as a reverse primer. The amplification product was targeted by using LC78 (GCT ATG CCA GAC AGA TGC TGG ATC; antisense) as an anchor probe and LC79 (GGC GGC TGG GTG GAA TGC C; antisense) as a sensor probe. The anchor probe LC78 was labeled with fluorescein as a donor for FRET, and the sensor probe LC79 was labeled with LightCycler Red 640 as an acceptor for FRET.

RESULTS

Anaerobic nitrate reductase activity of wild-type and *narG* mutant strains of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG.

In other bacteria *narGHJI* encodes a nitrate reductase that is typically upregulated in the absence of oxygen. We tested nitrite accumulation in anaerobic cultures containing *M. tuberculosis* and 10 mM nitrate. Peaking at ca. 1 mmol/liter after 2 days, the concentration of nitrite slowly decreased over the next 5 days (Fig. 1). In contrast to *M. tuberculosis*, the amount of accumulated nitrite in cultures inoculated with *M. bovis* was considerably lower, and *M. bovis* BCG produced even less nitrite, showing minimal accumulation over 7 days (Fig. 1). Next, *narG* mutants of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG were generated. Since a mutant strain without an antibiotic resistance marker is clearly preferable, we used a suicide plasmid with a counterselectable marker system as described by Pavelka and Jacobs (24) to generate an unmarked deletion of *narG* in all three species. Disruption of *narG* deleted a *Sma*I restriction site within the *narG* gene. Therefore, Southern hybridization of *Sma*I-digested DNA showed a larger fragment for the mutant strains compared to the wild-type strains (Fig. 2). Nitrite accumulation activity of the three *narG* mutant strains was compared under anaerobic conditions. All strains were cultured in fully supplemented MB medium containing 10 mM nitrate, and aliquots were analyzed at various time points. The *narG* mutants of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG were unable to accumulate nitrite (Fig. 1). Thus, in all three species nitrite accumulation under anaerobic conditions was exclusively mediated by *narGHJI*.

Diagnostic nitrate reductase activity of *M. tuberculosis* and the *narG* mutant of *M. tuberculosis*.

Nitrate reductase activity has been used for many years in mycobacterial diagnostics. In the experiments described above, cultures were exposed to nitrate for 7 days; during this time continuous nitrite accumulation was measured at various time points. In diagnostic microbiology, however, mycobacterial nitrite accumulation is typically tested after just 2 h of incubation of bacilli in buffer containing nitrate (15, 18). Thus, diagnostic nitrate reductase activity is really more accurately described as the rapid accumulation of nitrite by the bacilli within 2 h. To test for diagnostic nitrate reductase activity, *M. tuberculosis* and the *narG* mutant of *M. tuberculosis* were inoculated from solid medium into phosphate buffer, and production of nitrite was measured after incubation at 37°C for 2 h. Nitrite accumulation by *M. tuberculosis* became visible and correlated with the amount of inoculated bacteria, whereas the *narG* mutant of *M. tuberculosis* showed no discernible production of nitrite (Fig. 3). As expected, neither *M. bovis* nor *M. bovis* BCG accumulated detectable amounts of nitrite from nitrate under these conditions within 2 h (data not shown). These results clearly indicate

FIG. 2. Genomic locus of *M. tuberculosis* encompassing *narGHJI* and Southern blot analysis of *narG* mutants of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG. An *EcoRV* fragment of *M. tuberculosis* contains the entire *narGHJI* gene cluster, including 0.9-kb prior to the translation start of *narG*. *MscI/BsmI* restriction sites for the construction of the *narG* deletion in *M. tuberculosis*, and *Clal* restriction sites for construction of *narG* deletions in *M. bovis* and *M. bovis* BCG are depicted. The positions of the deleted fragments are shown ($\Delta M. tuberculosis$ and $\Delta M. bovis$, BCG). The positions of the forward primer and the reverse primer that were used for screening *narG* mutants are depicted as arrows. A Southern blot analysis showing wild-type (lanes 1 to 3) and mutant (lanes 4 to 6) strains is shown below. Genomic DNAs from *M. tuberculosis* (lane 1), *M. bovis* (lane 2), *M. bovis* BCG (lane 3), $\Delta narG$ *M. tuberculosis* (lane 4), $\Delta narG$ *M. bovis* (lane 5), and $\Delta narG$ *M. bovis* BCG (lane 6) were digested with *SmaI*. The position of the DNA probe for Southern blot analysis is shown. The loss of a *SmaI* site due to deletion within the *narG* gene results in an "upshift" of the specific band in all three mutant strains.

that the molecular mechanism of the nitrate reductase activity test, which is used in diagnostic microbiology for identification of *M. tuberculosis*, is also based on *narGHJI*.

Identification of *M. tuberculosis* by analyzing T/C polymorphism within the promoter of *narGHJI* by means of LightCycler technology. *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG all possess the *narGHJI* operon. However, NarGHJI-dependent accumulation of nitrite appeared to be strikingly different. One explanation for this could be a variation within the promoter of *narGHJI*. Therefore, we sequenced a 500-bp region preceding the translation start of *narG* from *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG. At position -215 prior to the GTG-start codon, a thymine residue was detected in *M. tuberculosis*, and a cytosine residue was found in *M. bovis* and *M. bovis* BCG. Next, we analyzed the nucleotide polymorphism of the *narG* promoter at position -215 in 35 strains of *M. tuberculosis*, 13 strains of *M. bovis*, and 9 strains of *M. bovis* BCG, and we also included

2 strains of *M. microti* and 3 strains of *M. africanum*. LightCycler technology provides rapid DNA amplification and sequence-specific detection of DNA using oligonucleotides labeled with two different fluorescence dyes. Typically, so-called sensor probes, which cover a polymorphic region, allow detection of as little as one nucleotide difference by melting off at a lower temperature. We constructed an oligonucleotide targeting position -215 with a perfect match to *M. bovis* and *M. bovis* BCG. Amplification of a 155-bp fragment of the *narGHJI* promoter region encompassing position -215 was followed by melting peak analysis. The probe dissociated from the target at 63°C when genomic DNA from *M. bovis*, *M. bovis* BCG, *M. africanum*, or *M. microti* was used as a template, indicating a perfect match between the target DNA and the hybridization probe. When *M. tuberculosis* was used as a template, the probe dissociated at 57°C, indicating a mismatch between the target DNA and the hybridization probe (Fig. 4 and Table 1). These

FIG. 3. Diagnostic nitrate reductase activity of *M. tuberculosis* and the *narG* mutant of *M. tuberculosis*. Three-week-old cultures from *M. tuberculosis* wild-type and the *narG* mutant of *M. tuberculosis* on 7H10 agar plates were used to inoculate three loops (tube a), one loop (tube b), and one-third loop (tube c) of bacilli into phosphate buffer containing 10 mM nitrate and tested for the accumulation of nitrite after 2 h at 37°C.

results suggested that the thymine residue at position –215 prior to the GTG start codon of *narG* is specific for *M. tuberculosis*. In contrast, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti* appear to carry a cytosine at this position of the *narGHJI* promoter.

Identification of *M. bovis* and *M. bovis* BCG by analysis of *oxyR* and RD1 polymorphisms using LightCycler technology.

Next, we sought to include LightCycler-based identification and differentiation of *M. bovis* and *M. bovis* BCG. We generated FRET probes targeting either A/G polymorphism of *oxyR*, which is specific for bovine mycobacteria (26), or RD1, a 9,650-bp deletion that is specific for *M. bovis* BCG (27). *M. bovis* and *M. bovis* BCG carry an adenine residue at nucleotide 285 of the *oxyR*, whereas *M. tuberculosis*, *M. africanum* and *M. microti* carry a guanine residue. The FRET probe targeting A/G polymorphism of *oxyR* had a perfect match to *M. tuberculosis*. Amplification of a 200-bp fragment was followed by melting peak analysis of the amplification product. As shown in Table 1, the probe dissociated from the target at 59°C for *M.*

bovis and *M. bovis* BCG and at 68°C for *M. tuberculosis*, *M. africanum*, and *M. microti*.

RD1, a DNA fragment comprising 9,650 bp, is absent in *M. bovis* BCG but present in *M. bovis*, *M. tuberculosis*, *M. africanum*, and *M. microti*. PCR identification of *M. bovis* BCG, as described by Talbot et al., is based on three primers (27). Two primers are complementary to regions flanking the RD1 sequence. In *M. bovis* BCG, which lack RD1, these primers bind and amplify a 200-bp fragment. In strains from other species of the MTBC, these primers bind but the 9,650-bp sequence is too large to efficiently amplify. However, a third primer, which is complementary to the 3' end within the RD1 sequence, results in amplification of a 150-bp product, which is thus specific for *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*. The 200- and 150-bp products, respectively, are then visualized on an agarose gel. We adapted this multiplex PCR method to the LightCycler system by generating a FRET probe complementary to 12 bp of genomic DNA outside of, but adjacent to, the 3' end of RD1 (Fig. 5). An additional 7 bp

FIG. 4. LightCycler analysis of the polymorphic nucleotide at position –215 within the promoter region of *narGHJI* of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti*. Strains were cultured in the MGIT automated culture system, and DNA was prepared from an aliquot of positive culture by mechanical disruption. Melting peak analysis of the amplification product was done after the last amplification cycle. The melting curve analysis is displayed as the first negative derivative of the fluorescence (–dF/dT) versus temperature. F2 refers to channel 2, which is used by the LightCycler’s optical unit to measure signals from LightCycler Red 640 at 640 nm.

extending toward RD1 varied in their homology to *M. bovis* BCG and the other members of the MTBC, respectively. Three primers were used for amplification, two of which were complementary to regions flanking the RD1 sequence, whereas the third primer targeted the 3' end within RD1. Primers LC73 and LC75 amplified a 320-bp fragment from *M. bovis* BCG, and primers LC74 and LC75 amplified a 290-bp fragment from *M. tuberculosis*, *M. bovis*, *M. africanum*, or *M. microti*. Differentiation between these two different amplification products was achieved by subsequent melting peak analysis. As shown in Fig. 6 and Table 1, a melting peak of 49°C was specific for *M.*

bovis BCG, and a melting peak of 62°C was specific for *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*.

DISCUSSION

In other bacteria, several nitrate reductases have been identified. NarGHJI is only one of at least three different nitrate reductases, and its presence has been demonstrated in both obligate aerobes such as *Bacillus* spp. and facultative anaerobes such as *Escherichia coli* (1, 10). Apart from NarGHJI, a second nitrate reductase, often referred to as Nas, mediates in

FIG. 5. Multiplex PCR design for LightCycler-based analysis of RD1. The RD1, which comprises 9,560 bp, is deleted in *M. bovis* BCG (II) but present in *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*. (I). The PCR primers LC73, LC74, and LC75 are shown as arrows and oriented in the direction of amplification. The sensor probe LC79 is aligned to the target specific for MTBC except for BCG (I) and to the target specific for BCG (II). Mismatches between the sensor probe and the targets are shown as boldface, lowercase letters.

FIG. 6. LightCycler analysis of RD1 of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti*. Strains were cultured in the MGIT automated culture system, and DNA was prepared from an aliquot of positive culture by mechanical disruption. Melting peak analysis of the amplification product was done following the last amplification cycle. The melting curve analysis is displayed as the first negative derivative of the fluorescence ($-dF/dT$) versus temperature. F2 refers to channel 2, which is used by the LightCycler's optical unit to measure signals from LightCycler Red 640 at 640 nm.

concert with an assimilatory nitrite reductase the utilization of nitrate as a nitrogen source via ammonia. A third nitrate reductase, called Nap, may play a role in the disposal of excess reducing equivalents (19). By generating *narG* deletion mutants in *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG, we ruled out that nitrate reductases other than NarGHJI contributed to nitrite accumulation under anaerobic conditions in the three species.

In comparison to *M. bovis* and *M. bovis* BCG, *M. tuberculosis* strongly accumulates nitrite from nitrate. At present the American Society for Microbiology recommends testing nitrite accumulation in mycobacteria by incubating bacilli at 37°C for 2 h in buffer containing nitrate (15, 18). We tested nitrite accumulation of the *narG* mutant of *M. tuberculosis* under these conditions and demonstrated that the molecular mechanism for this “diagnostic nitrate reductase activity” of *M. tuberculosis*, which could be more precisely described as rapid nitrite accumulation within 2 h by *M. tuberculosis*, is also mediated by *narGHJI*.

Single nucleotide polymorphism, as well as the absence or presence of entire DNA regions ranging in size from 2 to 12 kb, has been described as a variable between different tubercle bacilli. At present, we do not know whether the polymorphic nucleotide detected at position -215 prior to the start codon of *narG* is functional and plays a role in the different nitrite accumulation that is observed between *M. tuberculosis* and other members of the MTBC. By comparing the sequence data we obtained from sequencing 500 bp upstream of the *narG* gene with the genomic sequence data available for *M. tuberculosis* strains H37Rv/CDC1551 and *M. bovis*, the single nucleotide polymorphism at position -215 was confirmed. Within this 500-bp region, no additional nucleotide difference between *M. tuberculosis* and *M. bovis* was found. Previous studies showed that a single nucleotide polymorphism in *oxyR* separates the

bovine mycobacteria from other members of the MTBC, and the absence of an DNA region encompassing 9,650 bp, called RD1, is specific for *M. bovis* BCG (3, 26). Whereas *oxyR* is nonfunctional in mycobacteria, the absence of RD1 is not only specific for *M. bovis* BCG but also contributes to attenuation of the vaccine strain (16, 25, 27).

We recently used LightCycler technology for the diagnosis of mycobacteria based on amplification and detection of the 16S rRNA gene (14). Since all members of the MTBC share identical 16S rRNA genes, further procedures were required for unambiguous identification of *M. tuberculosis*. Two recent molecular approaches greatly advanced differentiation within the MTBC, one is based on *gyrB* polymorphism, and the other is based on genomic deletion analysis (3, 21, 23). However, both methods require further manipulations after DNA amplification such as gel electrophoresis and DNA hybridization procedures. LightCycler allows DNA amplification and subsequent DNA analysis by hybridization in a one-tube format. In the present study, LightCycler technology was used to evaluate the single nucleotide polymorphism at position -215 of *narGHJI* for its role in diagnostic mycobacteriology. At the same time we subjected an adaptation of the analysis of the single nucleotide polymorphism of *oxyR*, as well as RD1, to the LightCycler system. We generated FRET probes targeting the single nucleotide polymorphism of the *narGHJI* promoter, and the single nucleotide polymorphism within *oxyR*. Detection of the presence or absence of the 9.6-kbp RD1 was achieved by constructing a FRET probe that was complementary to neither of the two possible target sequences, which resulted in a reduction of the melting peak specific for *M. bovis* BCG by 13°C in comparison to the melting peak specific for *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*. Thus, the combination of the three FRET probes allowed rapid identification and differentiation of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG. This approach is currently being applied in our laboratory

to DNA extracted from positive MGIT automated culture system specimens.

Diagnostic laboratories that receive predominantly specimens from human sources may use *M. tuberculosis*-specific FRET probe for initial diagnostic procedures, since *M. tuberculosis* will comprise almost all clinical isolates. In our previous study, we used a FRET probe specific for mycobacteria and a FRET probe specific for MTBC for identification of all mycobacteria and separation of MTBC from nontuberculous mycobacteria (14). Meanwhile, we submitted all specimens revealing MTBC to a second round of LightCycler analysis by using the *M. tuberculosis*-specific FRET probe, since in our laboratory the vast majority of these specimens contain *M. tuberculosis*. A thymine residue at position -215 of the *narGHJI* promoter establishes the unambiguous diagnosis, and no further processing, such as phenotypic testing for nitrate reductase activity or additional molecular analysis, is required. The LightCycler's optical unit is capable of measuring fluorescence from FRET probes in two separate channels simultaneously. Channel 2 (F2; 640 nm) is used to measure signals from LightCycler Red 640. Channel 3 (F3; 705 nm) is designed for using LightCycler Red 705. To utilize this technique, the FRET probe targeting RD1 was labeled with LightCycler Red 640, and the FRET probe targeting *oxyR* was labeled with LightCycler Red 705. Thus, the FRET probe specific for bovine mycobacteria combined with the FRET probe specific for *M. bovis* BCG allows simultaneous identification and differentiation of *M. bovis* and *M. bovis* BCG. Only definitive diagnosis of *M. africanum* and *M. microti* will require further testing.

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REFERENCES

- Blasco, F., C. Iobbi, G. Giordano, M. Chippaux, and V. Bonnefoy. 1989. Nitrate reductase of *Escherichia coli*: completion of the nucleotide sequence of the *nar* operon and reassessment of the role of the alpha and beta subunits in iron binding and electron transfer. *Mol. Gen. Genet.* **218**:249–256.
- Bonické, R., S. E. Juhasz, and U. Diemer. 1970. Studies on the nitrate reductase activity of mycobacteria in the presence of fatty acids and related compounds. *Am. Rev. Respir. Dis.* **102**:507–515.
- Brosch, R., S. V. Gordon, M. Marmiesse, P. Brodin, C. Buchrieser, K. Eiglmeier, T. Garnier, C. Gutierrez, G. Hewinson, K. Kremer, L. M. Parsons, A. S. Pym, S. Samper, D. van Soolingen, and S. T. Cole. 2002. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proc. Natl. Acad. Sci. USA* **99**:3684–3689.
- Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry, F. Tekai, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, et al. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**:537–544.
- Dye, C., S. Scheele, P. Dolin, V. Pathania, M. C. Ravigliione, et al. 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. *JAMA* **282**:677–686.
- Escoto, M. L., and I. N. de Kantor. 1978. Nitrate reductase activity of *Mycobacterium tuberculosis* and *Mycobacterium bovis* in the presence of electron donors. *J. Clin. Microbiol.* **7**:601–602.
- Fritz, C., S. Maass, A. Kreft, and F. C. Bange. 2002. Dependence of *Mycobacterium bovis* BCG on anaerobic nitrate reductase for persistence is tissue specific. *Infect. Immun.* **70**:286–291.
- Goke, M. N., A. Leppert, P. Flemming, B. Soudah, F. C. Bange, J. S. Bleck, A. Widjaja, B. Hogemann, J. Mellmann, J. Ockenga, I. Schedel, M. Gebel, and M. P. Manns. 2001. Intestinal tuberculosis: easier overlooked than diagnosed. *Z. Gastroenterol.* **39**:1015–1022. (In German.)
- Grosse, V., F. Bange, J. Tischendorf, R. Schmidt, and M. Manns. 2002. A mass in the eye. *Lancet* **360**:922.
- Hoffmann, T., B. Troup, A. Szabo, C. Hungerer, and D. Jahn. 1995. The anaerobic life of *Bacillus subtilis*: cloning of the genes encoding the respiratory nitrate reductase system. *FEMS Microbiol. Lett.* **131**:219–225.
- Horstkotte, M. A., I. Sobotka, C. K. Schewe, P. Schafer, R. Laufs, S. Rusch-Gerdes, and S. Niemann. 2001. *Mycobacterium microti* llama-type infection presenting as pulmonary tuberculosis in a human immunodeficiency virus-positive patient. *J. Clin. Microbiol.* **39**:406–407.
- Hutter, B., and T. Dick. 1999. Upregulation of *narX*, encoding a putative "fused nitrate reductase" in anaerobic dormant *Mycobacterium bovis* BCG. *FEMS Microbiol. Lett.* **178**:63–69.
- Kirschner, P., B. Springer, U. Vogel, A. Meier, A. Wrede, M. Kickenbeck, F. C. Bange, and E. C. Bottger. 1993. Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. *J. Clin. Microbiol.* **31**:2882–2889.
- Lachnik, J., B. Ackermann, A. Bohrsen, S. Maass, C. Diephaus, A. Puncken, M. Stermann, and F. C. Bange. 2002. Rapid-cycle PCR and fluorimetry for detection of mycobacteria. *J. Clin. Microbiol.* **40**:3364–3373.
- Master, R. N. 1992. Mycobacteriology, p. 3.0.1–3.16.4. In H. D. Isenberg (ed.), *Clinical microbiology procedures handbook*. ASM Press, Washington, D.C.
- Master, S. S., B. Springer, P. Sander, E. C. Boettger, V. Deretic, and G. S. Timmins. 2002. Oxidative stress response genes in *Mycobacterium tuberculosis*: role of *ahpC* in resistance to peroxynitrite and stage-specific survival in macrophages. *Microbiology* **148**:3139–3144.
- McKinney, J. D., W. R. Jacobs, and B. R. Bloom. 1998. Persisting problems in tuberculosis, p. 51–139. In R. M. Krause (ed.), *Emerging Infections*. Academic Press, Ltd., London, England.
- Metchock, B. G., F. S. Nolte, and R. J. Wallace. 1999. *Mycobacterium*, p. 399–437. In P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Tenover (ed.), *Manual of clinical microbiology*, 7th ed. ASM Press, Washington, D.C.
- Moreno-Vivian, C., and S. J. Ferguson. 1998. Definition and distinction between assimilatory, dissimilatory, and respiratory pathways. *Mol. Microbiol.* **29**:664–666.
- Nakano, M. M., and P. Zuber. 1998. Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*). *Annu. Rev. Microbiol.* **52**:165–190.
- Niemann, S., D. Harmsen, S. Rusch-Gerdes, and E. Richter. 2000. Differentiation of clinical *Mycobacterium tuberculosis* complex isolates by *gyrB* DNA sequence polymorphism analysis. *J. Clin. Microbiol.* **38**:3231–3234.
- Niemann, S., E. Richter, H. Daluge-Tamm, H. Schlesinger, D. Graupner, B. Königstein, G. Gurath, U. Greinert, and S. Rusch-Gerdes. 2000. Two cases of *Mycobacterium microti*-derived tuberculosis in HIV-negative immunocompetent patients. *Emerg. Infect. Dis.* **6**:539–542.
- Parsons, L. M., R. Brosch, S. T. Cole, A. Somoskovi, A. Loder, G. Bretzel, D. van Soolingen, Y. M. Hale, and M. Salfinger. 2002. Rapid and simple approach for identification of *Mycobacterium tuberculosis* complex isolates by PCR-based genomic deletion analysis. *J. Clin. Microbiol.* **40**:2339–2345.
- Pavelka, M. S., and W. R. Jacobs. 1999. Comparison of the construction of unmarked deletion mutations in *Mycobacterium smegmatis*, *Mycobacterium bovis* bacillus Calmette-Guérin, and *Mycobacterium tuberculosis* H37Rv by allelic exchange. *J. Bacteriol.* **181**:4780–4789.
- Pym, A. S., P. Brodin, R. Brosch, M. Huerre, and S. T. Cole. 2002. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Mol. Microbiol.* **46**:709–717.
- Sreevatsan, S., P. Escalante, X. Pan, D. A. Gillies, S. Siddiqui, C. N. Khalaf, B. N. Kreiswirth, P. Bifani, L. G. Adams, T. Ficht, V. S. Perumaalla, M. D. Cave, J. D. van Embden, and J. M. Musser. 1996. Identification of a polymorphic nucleotide in *oxyR* specific for *Mycobacterium bovis*. *J. Clin. Microbiol.* **34**:2007–2010.
- Talbot, E. A., D. L. Williams, and R. Frothingham. 1997. PCR identification of *Mycobacterium bovis* BCG. *J. Clin. Microbiol.* **35**:566–569.
- Virtanen, S. 1960. A study of nitrate reduction by mycobacteria. *Acta Tuberc. Scand.* **47**:1–116.
- Warren, N. G., B. A. Body, and H. P. Dalton. 1983. An improved reagent for mycobacterial nitrate reductase tests. *J. Clin. Microbiol.* **18**:546–549.
- Wayne, L. G., and L. G. Hayes. 1998. Nitrate reduction as a marker for hypoxic shutdown of *Mycobacterium tuberculosis*. *Tuberc. Lung Dis.* **79**:127–132.
- Weber, I., C. Fritz, S. Rutkowski, A. Kreft, and F. C. Bange. 2000. Anaerobic nitrate reductase (*narGHJI*) activity of *Mycobacterium bovis* BCG in vitro and its contribution to virulence in immunodeficient mice. *Mol. Microbiol.* **35**:1017–1025.
- Wilkins, E. G., R. J. Griffiths, and C. Roberts. 1986. Pulmonary tuberculosis due to *Mycobacterium bovis*. *Thorax* **41**:685–687.