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A Promoter Mutation Causes Differential Nitrate Reductase Activity of *Mycobacterium tuberculosis* and *Mycobacterium bovis*

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The recent publication of the genome sequence of *Mycobacterium bovis* showed >99.95% identity to *M. tuberculosis*. No genes unique to *M. bovis* were found. Instead numerous single-nucleotide polymorphisms (SNPs) were identified. This has led to the hypothesis that differential gene expression due to SNPs might explain the differences between the human and bovine tubercle bacilli. One phenotypic distinction between *M. tuberculosis* and *M. bovis* is nitrate reduction, which not only is an essential diagnostic tool but also contributes to mycobacterial pathogenesis. We previously showed that *narGHJI* encodes a nitrate reductase in both *M. tuberculosis* and *M. bovis* and that NarGHJI-mediated nitrate reductase activity was substantially higher in the human tubercle bacillus. In the present study we used a genetic approach to demonstrate that an SNP within the promoter of the nitrate reductase gene cluster *narGHJI* is responsible for the different nitrate reductase activity of *M. tuberculosis* and *M. bovis*. This is the first example of an SNP that leads to differential gene expression between the human and bovine tubercle bacilli.

Mycobacterium tuberculosis infects one-third of the world's population, with a fatality rate higher than that for any other bacterial organism. *Mycobacterium bovis*, which causes tuberculosis in a range of animal species, generates worldwide annual losses to agriculture of $3 billion. Both fall within the *M. tuberculosis* complex (TBC), a group of closely related mycobacteria including *M. bovis* BCG, a live attenuated vaccine strain against tuberculosis, *M. africanum*, and *M. microti* (14). Comparative genome analyses has shown that *M. bovis* does not have any unique genes compared to *M. tuberculosis*. Instead, there are numerous single-nucleotide polymorphisms SNPs that distinguish *M. bovis* from *M. tuberculosis* (11). Therefore, it has been suggested that SNPs are responsible for the differences between the human and the bovine tubercle bacilli. One such difference is nitrate reduction leading to accumulation of nitrite. *M. tuberculosis* rapidly reduces nitrate (23). We provided evidence that *M. bovis* also accumulates nitrite but at a much lower rate. We also found that nitrate reduction leading to accumulation of nitrite in *M. tuberculosis* and *M. bovis* is mediated by the same *narGHJI*-encoded nitrate reductase and asked why nitrite accumulation in *M. tuberculosis* was so much stronger than in the bovine mycobacteria (21).

Therefore, we compared the *narGHJI* promoter of *M. tuberculosis* and *M. bovis* in both laboratory and clinical strains and found that *M. tuberculosis* carries a thymine residue at nucleotide −215 prior to the start codon of *narG* whereas *M. bovis* carries a cytosine residue at this position. Interestingly, *M. bovis* BCG and other members of the TBC such as *M. africanum* and *M. microti* also contained a cytosine residue at this position (21). Comparing the available genome sequences of *M. tuberculosis* and *M. bovis*, we found additional SNPs within the coding sequences of *narGHJI*, which could account for the variable activity of NarGHJI. Consequently, in this study we explored the functional role of the SNP within the promoter of *narGHJI*. The thymine residue at position −215 prior to the start of *narG* was replaced with a cytosine residue on a plasmid carrying the *narGHJI* gene of *M. tuberculosis*. Either the wild-type or the mutated *narGHJI* gene cluster was introduced into *M. smegmatis*, a nonpathogenic mycobacterium that is not able to accumulate nitrite from nitrate, and the nitrate reductase activities of recombinant strains were compared. We also replaced the thymine residue with the cytosine residue on the chromosome of *M. tuberculosis* itself to test whether the SNP controls the difference in nitrate reductase activity between *M. tuberculosis* and *M. bovis*.

MATERIALS AND METHODS

Strains and cultures. *M. smegmatis* mc^2 155 (19), *M. tuberculosis* H37Rv ATCC 25618, *M. tuberculosis* Erdmann ATCC 35801, and *M. bovis* ATCC 19210 were used in this study. Strains were cultured in 7H9 liquid (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.

Accumulation of nitrite under anaerobic conditions. The presence of nitrite can be demonstrated by using naphthylamide and sulfanilic acid reagents, which form a red diazonium dye when reacting with nitrite (14). 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 experiments in Fig. 1 and 3, 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 ADS and nitrate. Addition of Tween 80 is necessary to prevent mycobacteria from clumping in liquid culture. The exact formula of 1 liter of 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; 1 liter of trace elements contained 40 mg of $ZnCl_2$, 200 mg of $FeCl_3 \cdot 6H_2O$, 10 mg of $CuCl_2 \cdot 4H_2O$, 10 mg of $MnCl_2 \cdot 4H_2O$, 10 mg of $Na_2B_4O_7 \cdot 10H_2O$, and 10 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. After the culture was washed three times, the pellet was resuspended in fully supplemented MB medium, reaching an optical density at 600 nm of about 0.2, corresponding to approximately 10^7 bacilli/ml. An anaerobic environment was achieved with the Anaero *Gen* anaerobic system (Oxoid Ltd., Basingstoke, England) in a standard

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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 API system, bioMérieux, Marcy-l'Etoile, France) were added to aliquots of culture and the mixture was centrifuged at 15,000 × *g* for 15 min at room temperature. The absorbance of the 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 duplicate.

Diagnostic nitrate reductase activity. According to recommendations by the American Society for Microbiology, the diagnostic nitrate reductase activity test must be performed with actively growing cultures which are inoculated directly into phosphate buffer supplemented with nitrate and incubated for 2 h at 37°C. Culture prior to testing should be done on solid medium (13, 14). Thus, to test for diagnostic nitrate reductase activity, mycobacteria were cultured on 7H10 agar (Difco Laboratories, Inc.) supplemented with 0.2% glycerol, and 10% ADS. For the experiment in Fig. 4, bacilli were inoculated into phosphate buffer supplemented with 10 mM nitrate. Following 2 h of incubation at 37°C, naphthylamide and sulfanilic acid reagents were added and a photograph was taken.

Site-directed mutagenesis to replace the thymine residue with a cytosine residue at position −215 prior to the *narG* start codon of *M. tuberculosis*. Isolation of a cosmid carrying *narGHJI* from *M. tuberculosis* has been described previously (26). An 8.4-kbp EcoRV fragment with the entire *narGHJI* operon from *M. tuberculosis* including 0.9 kbp of 5′-untranslated DNA was subcloned in the mycobacterial shuttle vector pMV306 (22). An EcoRV-BspHI 0.9-kb fragment (see Fig. 2) containing the promoter region of *narGHJI* of *M. tuberculosis* was subcloned into pMV306, from which a 1,955-bp XbaI-NheI fragment had been deleted previously to facilitate subsequent long-range PCR for site-directed mutagenesis. To replace the thymine residue with a cytosine, the following mismatch olignucleotide primers, each complementary to opposite strands of the vector, were constructed: forward primer CCA GAA AAC CAT CGT GAT CGC TAC GGG CAT GC and reverse primer GCA TGC CCG TAG CGA TCACGA TGG TTT TCT GG. Using *Pfu* Turbo Hotstart DNA polymerase (Stratagene, La Jolla, Calif.), PCR amplifications were performed for 18 cycles using 50°C for 30 s for annealing, 68°C for 10 min for elongation, and 95°C for 30 s for denaturation. The PCR product was gel purified, and point mutations were confirmed by sequencing. The entire 0.9-kb promoter region was sequenced to exclude further mutations, which might have occurred during PCR. The promoter region was reintroduced into the *narGHJI* operon of *M. tuberculosis*. The resulting construct was named pAP28 and carried the entire *narGHJI* operon of *M. tuberculosis*, including the mutagenized promoter region in the mycobacterial shuttle plasmid pMV306.

Construction of the *M. bovis* library and isolation of cosmids carrying *narGHJI* from *M. bovis*. A cosmid library from *M. bovis* was constructed in the mycobacterial shuttle vector pYUB412, which replicates as an episomal vector in *Escherichia coli*, and integrates into the *attP* sites of different mycobacterial species, by applying the double *cos* vector strategy as described previously (1). Briefly, arms of pYUB412 were prepared by digestion with XbaI and BclI. Chromosomal DNA fragments were prepared by partial digestion with Sau3A (average size, >35 kb), treatment with alkaline phosphatase to prevent multiple inserts, and ligation to the two pYUB412 arms at a molar ratio of 1:10 (insert to arms). The ligations were packaged in vitro with Giga Pack Gold (Stratagene) packaging mix, transduced to STABL-2 (GIBCO BRL, Gaithersburg, Md.), and selected on Luria-Bertani plates containing carbenicillin (100 μg/ml). More than 5×10^5 independent clones were pooled, and DNA for transformation was obtained by a large-scale DNA preparation using a standard alkaline lysis method. To ensure that the the entire genome of *M. bovis* was represented, a panel of auxotrophic mutants of *M. smegmatis* was tested for complementation to prototrophy by the library as described previously (2). *narGHJI* of *M. bovis* was cloned by colony blot hybridization using standard procedures. For colony blot hybridization, a DNA probe targeting a 527-bp region within the *narG* gene was constructed, using TCG GAC TTT GAC GCATTC GC as a forward primer and GTA TCG GCG TAG GTG ATG CG as a reverse primer.

Alleleic exchange to replace the thymine residue with a cytosine residue at position −215 prior to the *narG* start codon on the chromosome of *M. tuberculosis*. We used a homologous recombination technique with pYUB657, a suicide plasmid with a counterselectable marker system, to generate unmarked mutations as described previously by Pavelka and Jacobs (16). Two contructs based on pYUB657 and targeting the *narG* promoter region were built. First, a 2.4-kbp EcoRV-AgeI fragment from pAP28 containing the mutagenized nucleotide (thymine to cytosine) at position −215 was cloned into the PacI site of pYUB657. The EcoRV-AgeI fragment included 1.5 kbp of the *narG* gene and 0.9 kbp of the 5′ untranslated region prior to the *narG* start codon (see Fig. 2). Next, a 1-kbp EcoRV-NotI fragment from the *narGHJI* operon of *M. bovis* which includes 0.1 kbp of the *narG* gene and 0.9 kbp of the 5′ untranslated region prior to the *narG* start codon (Fig. 2), was cloned into the PacI site of pYUB657.

M. tuberculosis was transformed, and clones resistant to hygromycin had presumably integrated the plasmid (primary recombinants). Integration was tested by using LightCycler technology. A 155-bp DNA fragment of the 5′ untranslated region prior to *narG*, which contained the SNP at position −215, was amplified using LC66 (AAC CGA CGG TGT GGTTGA C) as a forward primer and LC67 (ATC TCG ATG GAT GGG CGT C) as a reverse primer. The SNP was targeted using LC63 (GTC GCC ACG CGT CCA GAA AAC C) (antisense) as an anchor probe and LC64 (CGT GAT CGC TAC GGG CAT) (antisense) as a sensor probe (the underlining shows a single-nucleotide polymorphism). 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. DNA was amplified as described previously (21). 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. Clones that had integrated the plasmid showed two different melting peaks, one at 58°C indicating a thymine and the other at 65°C indicating a cytosine. 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 optical unit to measure signals from LightCycler Red 640 at 640 nm. Integration of the plasmid was confirmed using Southern blot analysis. Genomic DNAs from potential clones were digested with AflII, and a DNA probe, targeting a 527-bp region within the *narG* gene, was constructed 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 7,757-bp fragment indicated the wild type of *M. tuberculosis*, whereas 20- and 19.6-kbp fragments indicated integration of the plasmid in *M. tuberculosis*.

Next, several clones resistant to hygromycin were grown in 10 ml of 7H9 medium without selection. From these cultures, clones were selected on 7H10 plates containing 2% sucrose. Clones that were resistant to sucrose had presumably lost the plasmid (secondary recombinants), as confirmed by testing the growth of clones on plates with hygromycin. Clones that were sensitive to hygromycin were again subjected to LightCycler analysis. Clones that exchanged thymine for a cytosine showed a single melting peak at 63°C.

RESULTS

Accumulation of nitrite in *M. smegmatis* expressing the *narGHJI* operon from *M. tuberculosis* with a T→C mutation at nucleotide −215. Initially we sought to test whether the SNP within the promoter of *narGHJI* had any effect on nitrate reductase activity. *narGHJI* from *M. tuberculosis* transformed in *M. smegmatis* mc^2155, a fast-growing nonpathogenic mycobacterium, allows rapid analysis of nitrate reduction mediated by recombinant *narGHJI*, since the strain itself does not accumulate measurable amounts of nitrite (26). Therefore, we used *M. smegmatis* as a host to compare the expression of wild-type *narGHJI* from *M. tuberculosis* and a mutated allel of *narGHJI*. To generate the T→C mutation at nucleotide −215 within the *narGHJI* promoter, an EcoRV-BspI fragment (see Fig. 2) containing 0.9 kbp of the 5′ untranslated region prior to the start codon of *narG* was subjected to site-directed mutagenesis. The mutagenized promoter region was reintroduced into the *narGHJI* operon and transformed into *M. smegmatis*. *M. smegmatis* expressing either *narGHJI* wild type or the *narGHJI* with the mutagenized promoter was cultured anaerobically in minimal medium containing 10 mM nitrate. Figure 1 shows that replacing the thymine residue with a cytosine residue at nucleotide −215 of the *narGHJI* promoter caused a substantial reduction in the accumulation of nitrite from nitrate.

Mutation of the promoter SNP on the chromosome of *M. tuberculosis* and comparison of nitrite accumulation of *M. bovis* and the mutated *M. tuberculosis*. To test whether the promoter

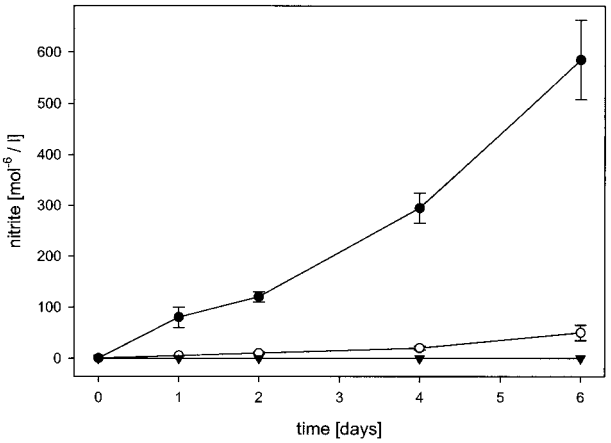

FIG. 1. Anaerobic nitrate reductase activity of *M. smegmatis* expressing *narGHJI* wild type and *narGHJI*(T215C) from *M. tuberculosis*. *M. smegmatis* (10^7 bacteria/ml) transformed with *narGHJI* wild type (closed circles), *narGHJI* (T215C) (open circles), and pMV306 vector control (triangles) was cultured in MB medium supplemented with 10 mM nitrate, and aliquots were tested for production of nitrite after 1, 2, 4, and 6 days.

SNP accounts for the difference between *M. tuberculosis* and *M. bovis*, the thymine residue at nucleotide −215 of the *narGHJI* promoter was replaced with a cytosine residue on the chromosome of *M. tuberculosis* by using a two-step allelic exchange as described by Pavelka and Jacobs (16). Two well-characterized *M. tuberculosis* strains, H37Rv and Erdmann, were chosen. An EcoRV-AgeI fragment (Fig. 2) from the in vitro-mutagenized *narGHJI* gene of *M. tuberculosis* carrying the cytosine residue at nucleotide −215 served as a substrate for homologous recombination. We applied LightCycler technology to screen for primary recombinants by using a hybridization probe that carried a cytosine at nucleotide 4, corresponding to the SNP at nucleotide −215 of the promoter region. Primary recombinants, which by definition have integrated the plasmid with the mutated allele next to the wild-type allele on the chromosome, showed two different melting peaks at 58 and 64°C (Fig. 2). These clones were cultured in medium without hygromycin to initiate a second recombination event which could leave behind either the wild-type or the mutant allele. Screening with the LightCycler successfully distinguished between the former and the latter by showing a single melting peak at 58°C for the wild-type allele and 64°C for the mutant allele (Fig. 2).

Secondary recombinants were incubated anaerobically in minimal medium with nitrate. The ability to accumulate nitrite was reduced (Fig. 3). It is noteworthy that accumulation of nitrite by the mutant strains of *M. tuberculosis* was similar to that by wild-type *M. bovis*. To validate this result, we next replaced a 1-kbp EcoRV-NotI fragment (Fig. 2) of the *narGHJI* promoter on the chromosome of *M. tuberculosis* with the corresponding EcoRV-NotI fragment from the *narGHJI*

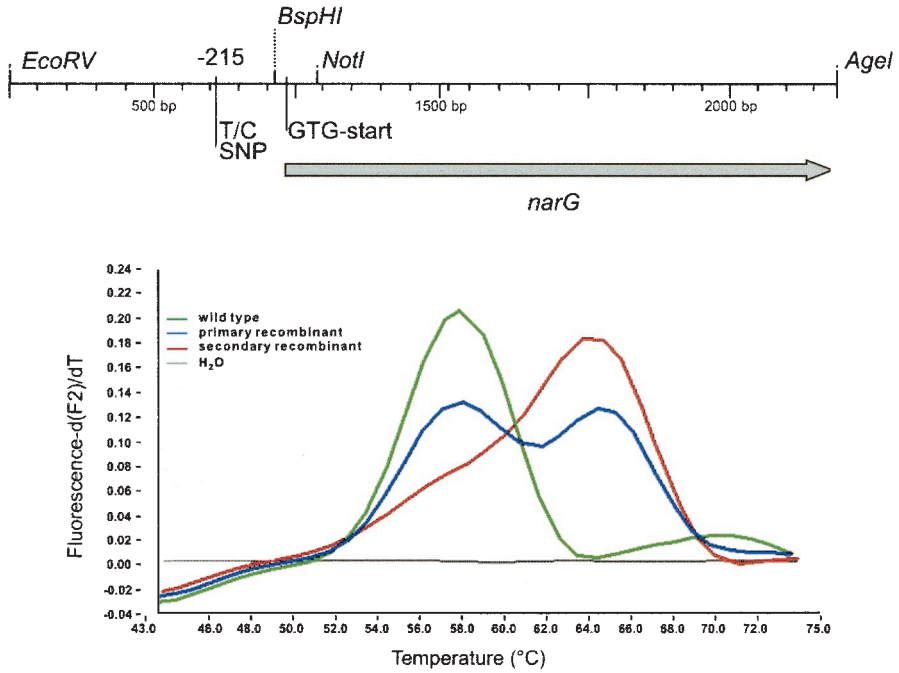

FIG. 2. Genomic locus encompassing *narG* and LightCycler analysis during allelic exchange. The EcoRV-AgeI fragment includes 1.5 kbp of the *narG* gene and 0.9 kbp of the 5′ untranslated region prior to the *narG* start codon. The T215C SNP within the promoter of *narG* is shown. An EcoRV-BspHI fragment from *narGHJI* of *M. tuberculosis* was subcloned and subjected to site-directed mutagenesis to replace the thymine residue at position −215 with a cytosine residue. The mutagenized fragment was reintroduced into *narGHJI* of *M. tuberculosis* (pAP28), and nitrate reductase activity was analyzed in *M. smegmatis*. An EcoRV-AgeI fragment from pAP28 was subsequently used as a substrate for allelic exchange in *M. tuberculosis* H37Rv and Erdmann. An EcoRV-NotI fragment from *narGHJI* of *M. bovis* was also used as a substrate for allelic exchange in *M. tuberculosis* Erdmann. Genomic DNA was prepared from wild-type and primary and secondary recombinants of *M. tuberculosis.* An example of each is shown here in the LightCycler, using FRET probes targeting the T215C SNP.

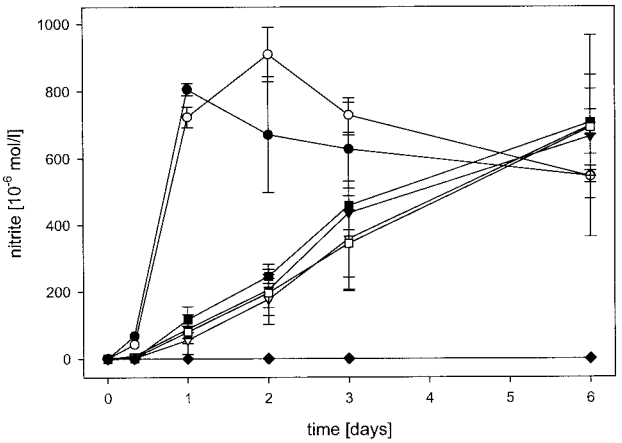

FIG. 3. Anaerobic nitrate reductase activity of *M. tuberculosis*, *M. bovis*, and various T215C mutants of *M. tuberculosis.* Mycobacteria (10^7 bacteria/ml) were cultured in MB medium supplemented with 10 mM nitrate, and aliquots were tested for production of nitrite after 1, 2, 4, and 6 days. Accumulation of nitrite from nitrate of the following strains was compared: *M. tuberculosis* Erdmann wild type (solid circles), *M. tuberculosis* H37Rv wild type (open circles), *M. tuberculosis* Erdmann (T215C) (solid triangles), *M. tuberculosis* H37Rv(T215C) (open triangles) (the two mutants were generated by using the EcoRV-AgeI fragment from *narGHJI* of *M. tuberculosis* that mutagenized in vitro to replace the thymine residue with the cytosine residue at position −215, *M. bovis* wild type (solid squares), and *M. tuberculosis* Erdmann (T215C) (open squares) (this mutant was generated by using the EcoRV-NotI fragment from *narG* of *M. bovis* as a substrate for allelic exchange). Bacilli incubated without nitrate were used as a negative control (solid diamonds).

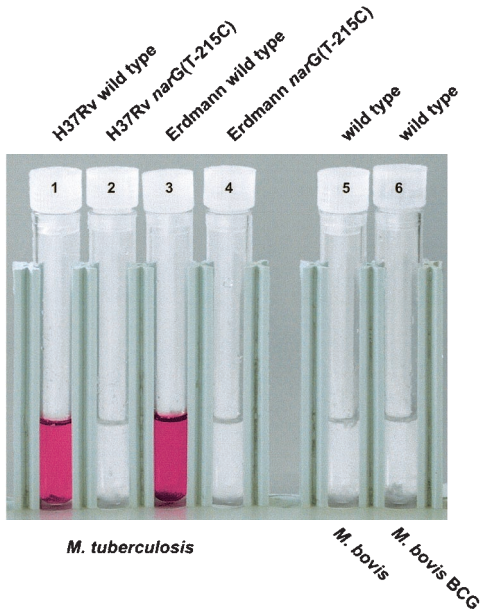

FIG. 4. Diagnostic nitrate reductase activity of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, and various T215C mutants of *M. tuberculosis.* All strains were subcultured on 7H10 agar plates for 3 weeks. One loop of bacilli was used for inoculation of phosphate buffer supplemented with nitrate. Following incubation for 2 h at 37°C, production of nitrite was tested. The presence of nitrite was visualized by adding naphthylamide and sulfanilic acid reagents, which form a red diazonium dye when reacting with nitrite.

promoter of *M. bovis*. This time, allelic exchange was done only in the Erdmann strain, following the same procedures as above. A clone of *M. tuberculosis* which acquired the T→C mutation in this way was incubated anaerobically in minimal medium with nitrate. Again, accumulation of nitrite was reduced and was similar to that by wild-type *M. bovis* (Fig. 3).

Influence of the SNP within the *narGHJI* promoter on diagnostic nitrate reductase activity. The ability to accumulate nitrite is one of the characteristic tests that differentiates human and bovine bacilli. Nitrite accumulation is typically tested after just 2 h of incubation of bacilli in buffer containing nitrate, leading to rapid accumulation of nitrite by *M. tuberculosis*, whereas *M. bovis* and *M. bovis* BCG seem to be nitrate reductase negative (14). We recently provided evidence that this "diagnostic nitrate reductase activity" in *M. tuberculosis* is also encoded by *narGHJI* (21). Thus, the *narGHJI* promoter mutation might also account for the difference in the diagnostic nitrate reductase activity between the human and the bovine bacilli. To test this idea, *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, and the mutants of *M. tuberculosis* carrying a cytosine at nucleotide −215 were incubated in the presence of nitrate for just 2 h as recommended by the American Society for Microbiology. Accumulation of nitrite became visible in wild-type *M. tuberculosis*, whereas the T→C mutants of *M. tuberculosis* showed no discernible production of nitrite (Fig. 4). As expected, neither *M. bovis* nor *M. bovis* BCG accumulated detectable amounts of nitrite from nitrate. Thus, within the time limit of 2 h, the negative test result for accumulation of nitrite in *M. bovis* is due to the SNP within the promoter of *narGHJI*. These results support our earlier study, in which we suggest that the phenotypic test for diagnostic nitrate reductase activity could be replaced by a genotypic analysis of the *narGHJI* promoter (21).

DISCUSSION

Although the human and bovine tubercle bacilli can be differentiated on the basis of host range, virulence, and physiological features, the genetic basis for these differences, including the different nitrate reductase activity, is unknown. Accumulation of nitrite in *M. tuberculosis* and *M. bovis* is mediated by NarGHJI. The membrane-bound nitrate reductase consists of NarG, NarH, and NarI, with NarG being the catalytic subunit, whereas NarJ is required for the assembly of the enzyme. When the genome sequences of *M. tuberculosis* and *M. bovis* became available (7, 9), we compared the *narGHJI* genomic locus in *M. tuberculosis* and *M. bovis*. No further SNPs within 0.9 kbp prior to the *narG* start codon between *M. tuberculosis* and *M. bovis* became apparent. However, several SNPs existed within the coding region of the *narGHJI* gene cluster. *M. tuberculosis* H37Rv and *M. bovis* differed by four SNPs, two in *narG*, one in *narH*, and one in *narI*. The two SNPs in *narG* were at nucleotides 1372 and 2462, changing valine to leucine and aspartate to glycine, respectively. In *narH*, the SNP, a transition from adenine to guanine at position 1038, was synonymous. In *narI*, the SNP was located at position 599, changing alanine to valine. No SNPs between *M. tuberculosis* and *M. bovis* were found in *narJ.* In principle, both the promoter SNP and/or the nonsynoymous SNPs within *narG* and/or

narI could be responsible for the different nitrate reductase activity of *M. tuberculosis* and *M. bovis.* In this study, we demonstrate that it is the SNP at nucleotide −215 prior to the start codon of *narG* that is important for the difference in nitrite accumulation between the human and the bovine tubercle bacilli, even though it does not exclude the possibility that other SNPs within the coding region of the *narGHJI* gene cluster might contribute to this phenotypic difference.

At present there are only two SNPs between *M. tuberculosis* and *M. bovis* that were analyzed in more detail, one in *pncA*, encoding pyrazinamidase, the other in *oxyR*, a pseudogene. The *pncA* gene contains a guanine residue at nucleotide 169 in *M. bovis* and *M. bovis* BCG, whereas other members of the TBC have a cytosine residue at that position. The C169G mutation confers resistance to pyrazinamide (5, 17, 18). The *oxyR* gene contains an adenine residue at nucleotide 285 in *M. bovis* and *M. bovis* BCG, whereas other members of the TBC have a guanine residue at that position (20). Neither *pncA* nor *oxyR* plays a role in mycobacterial virulence. We reported that *narGHJI*-encoded nitrate reduction is essential for the persistence of *M. bovis* BCG in mice (10, 26). Thus, the T215C mutation in the promoter of *narGHJI* is the first SNP to alter the expression of a gene associated with mycobacterial virulence.

Previous studies of mycobacterial nitrate reduction were limited to its role in the classification and identification of the genus *Mycobacterium* (4, 8, 23, 24). However, as well as addressing its role in mycobacterial persistence, other recent studies focussed on a possible role of enzymes involved in nitrate metabolism during mycobacterial infection. Wayne and Hayes showed that nitrate reduction increases during a hypoxic shiftdown of *M. tuberculosis* (25). Hutter and Dick demonstrated upregulation of NarX, a putative fused nitrate reductase in anaerobic dormant *M. bovis* BCG (12). The utilization of nitrate as an energy or nitrogen source by mycobacteria is reasonable, considering that nitrate is normally present in sufficiently high concentrations in the chronically infected host (3). Reduction of nitrate to nitrite mediated by NarGHJI might provide the pathogen with ATP through nitrate respiration and with nitrogen by further reduction of nitrite to ammonia (15). This could mean that upregulation of nitrate reductase activity gives *M. tuberculosis* a selective advantage in vivo, which in turn might be crucial for the virulence or tropism of human bacilli but not of bovine bacilli. In principle, deletions are more stable markers than point mutations, which may be subject to reversion. However, improving survival in vivo would also confer a higher stability to the thymine mutation in *M. tuberculosis*, which in turn would make this SNP a valuable marker in mycobacterial diagnostics. In fact, we have recently shown that analysis of the T215C SNP reliably allowed the identification of *M. tuberculosis* and its separation from other members of the TBC (21).

The promoter mutation might also serve as an evolutionary marker. Using deletion analysis, a new scenario for the evolution of the TBC has been proposed that places *M. tuberculosis* closer to a common progenitor of the complex than *M. bovis* (6). For example, the deletion TbD1 is specific for the modern *M. tuberculosis* strain and possibly occurred before the 18th century. TbD1 is absent in 87% of *M. tuberculosis* strains distributed worldwide, including representative strains from major epidemics such as the Haarlem, Bejing, and Africa clusters. The other major evolutionary event during the development of the TBC seems to be reflected by the loss of RD9. This deletion characterizes the *M. africanum*, *M. microti*, *M. bovis* lineage, corresponding to species with a host range as diverse as humans, cattle, voles, seals, goats, and badgers. It is therefore most likely that the RD9 deletion occurred many thousands of years ago (6). Association of the T215C mutation of *narGHJI* with TbD1 and RD9 could be examined in a larger population of TBC strains from all over the world, including ancient and modern *M. tuberculosis* strains as well as strains from other members of the TBC.

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