Corrections

MICROBIOLOGY. For the article “Identification of eIF2Bγ and eIF2γ as cofactors of hepatitis C virus internal ribosome entry site-mediated translation using a functional genomics approach” by Martin Krüger, Carmela Beger, Qiang-Xin Li, Peter J. Welch, Richard Tritz, Mark Leavitt, Jack R. Barber, and Flossie Wong-Staal, which appeared in number 15, July 18, 2000, of Proc. Natl. Acad. Sci. USA (97, 8566–8571), the authors note the following correction. Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF257077, human eIF2Bγ sequence).

MICROBIOLOGY. For the article “Attenuation of virulence in Mycobacterium tuberculosis expressing a constitutively active iron repressor” by Yukari C. Manabe, Beatrice J. Saviola, Li Sun, John R. Murphy, and William R. Bishai, which appeared in number 22, October 26, 1999, of Proc. Natl. Acad. Sci. USA (96, 12844–12848), the authors note the following correction. In Table 2, the IB-1 accession number should read Rv0757. The IB-2 accession number should read Rv0761c. The IB-4 gene name is rrs, the accession number should read MTB00019, and the description should read: “A portion of the 16S ribosomal RNA.”
Attenuation of virulence in Mycobacterium tuberculosis expressing a constitutively active iron repressor

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Iron is an essential nutrient for the survival of most organisms and has played a central role in the virulence of many infectious disease pathogens. Mycobacterial IdeR is an iron-dependent repressor that shows 80% identity in the functional domains with its corynebacterial homologue, DtxR (diphtheria toxin repressor). We have transformed Mycobacterium tuberculosis with a vector expressing an iron-independent, positive dominant, corynebacterial dtxR hyperrepressor, DtxR(E175K). Western blots of whole-cell lysates of M. tuberculosis expressing the dtxR(E175K) gene revealed the stable expression of the mutant protein in mycobacteria. BALB/c mice were infected by tail vein injection with 2 × 10^6 organisms of wild type or M. tuberculosis transformed with the dtxR mutant. At 16 weeks, there was a 1.2 log reduction in bacterial survivors in both spleen (P = 0.0002) and lungs (P = 0.006) with M. tuberculosis DtxR(E175K). A phenotypic difference in colonial morphology between the two strains also was noted. A computerized search of the M. tuberculosis genome for the palindromic consensus sequence to which DtxR and IdeR bind revealed six putative “iron boxes” within 200 bp of an ORF. Using a gel-shift assay we showed that purified DtxR binds to the operator region of five of these boxes. Attenuation of M. tuberculosis can be achieved by the insertion of a plasmid containing a constitutively active, iron-insensitive repressor, DtxR(E175K), which is a homologue of IdeR. Our results strongly suggest that IdeR controls genes essential for virulence in M. tuberculosis.

With more than one-third of the world’s population latently infected with Mycobacterium tuberculosis, the global burden of tuberculosis is staggering. The emergence of multidrug-resistant strains and the increased susceptibility of the HIV-infected further highlights the need for elucidation of the molecular pathogenesis of M. tuberculosis and its virulence genes. Iron plays a critical role in the regulation of virulence of many bacterial pathogens. (1) In tuberculosis, there is indirect clinical and in vitro evidence that iron regulation is important to the virulence of this microbial pathogen (2–5).

In a phylogenetically related organism, Corynebacterium diphtheriae, iron depletion results in the derepression of virulence genes such as the diphtheria toxin (tox) gene by DtxR (diphtheria toxin repressor). The corynebacterial DtxR has a homologue in M. tuberculosis, IdeR (iron-dependent repressor). In the amino terminal 140 aa that contain the Fe2+ and DNA-binding domains of DtxR, IdeR shares 80% identity with DtxR (6). In 1995, IdeR was first described by Doukhan et al. (7) in conjunction with the sigA sigB cluster of genes. Subsequently, the ability of corynebacterial IdeR to bind to the corynebacterial tox operator region in a metal ion-dependent manner was demonstrated by gel-shift assay (8). Mutation of ideR in M. smegmatis resulted in derepressed siderophore production in high iron conditions (9). These findings parallel those described in corynebacterial dtxR and suggest that the homology between these two genes may allow for cross-genus functional complementation.

Using a positive genetic selection system to clone dtxR alleles, Sun et al. (10) recently isolated and characterized a series of DtxR mutants created by PCR mutagenesis. One of the mutants that bound to the tox operator (toxO) and constitutively repressed reporter gene expression in an iron-independent manner was characterized and found to have a single amino acid substitution of lysine for glutamic acid at position 175 [DtxR(E175K)]. In merodiploid strains harboring both wild-type dtxR and mutant dtxR(E175K) genes, Sun et al. found the mutant to be dominant over the wild-type allele. We postulated that this dominant corynebacterial mutant may be able to constitutively repress IdeR-regulated genes in M. tuberculosis. To test this hypothesis, we constructed an M. tuberculosis strain expressing the dtxR(E175K) positive dominant allele and tested its virulence in a mouse model of tuberculosis.

**Methods**

**Strains, Plasmids, and Cultures.** The bacterial strains and plasmids used in this study are listed in Table 1 (10–14). Escherichia coli cultures were grown in LB or Luria agar supplemented with ampicillin (100 μg/ml) or hygromycin (200 μg/ml). M. tuberculosis CDC1551 and M. smegmatis cultures were grown in standard Middlebrook 7H9 broth (Difco), supplemented with albumin dextrose complex, 0.1% glycerol, and 0.05% Tween 80 at 37°C in roller bottles (15).

**Construction of dtxR(E175K) Shuttle Plasmid.** A 1.5-kb BamHI–HindIII fragment of DNA from pSMD2 was cloned into pNBV1. The resulting recombinant plasmid, pNBV1/SAD (self-activating DtxR), was cloned in E. coli DH5α and purified by using the QiaGen system (Qiagen, Chatsworth, CA) (16). Purified plasmids then were electroporated into M. tuberculosis CDC1551 by standard protocols (15).

**Western Blot Analysis.** Recombinant E. coli and mycobacteria were lysed in 3 M urea, 0.5% Triton X-100, 3.25 μM DTT, 2% Pharmalyte (Amersham Pharmacia Biotech), 100 μg/ml PMSF, and 2 μg/ml leupeptin. Using 0.1-mm glass beads, the samples were homogenized twice in a Mini-bead-beater (Biospec Products, Bartlesville, OK) at maximum speed for 1 min. Samples were centrifuged to remove cellular debris and unlysed cells. After separation by 12% SDS/PAGE, proteins were transferred to nitrocellulose membranes (Hybond, Amersham Pharmacia) by semidyne technique (Transblot SD, Hercules, CA) and blocked...
with 5% nonfat milk in PBS with 0.1% Tween 20 (PBS-T) for 1 hr. Membranes then were incubated overnight in PBS-T with rabbit anti-DtxR polyclonal antibodies at the appropriate concentration at 4°C (17). After washing, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody diluted in PBS-T for 2 hr. The Supersignal Chemiluminescent Substrate (Pierce) was used for autoradiograph development.

**Murine Tuberculosis Infection Model.** BALB/c mice (6–8 weeks old) were infected by tail vein injection with 2 × 10⁶ organisms of wild-type or *M. tuberculosis* DtxR(E175K). Bacterial infection was monitored over a 119-day period. Colony-forming units (cfu) in spleen and lungs were assessed at 4-week intervals by serial dilutions of organ homogenates plated on 7H10 Middlebrook agar containing 50 µg/ml cycloheximide, 50 µg/ml carbenicillin, 20 µg/ml trimethoprim, and 200 units/ml polymyxin (18).

**DNA Gel-Shift Binding Assay.** The DNA migration retardation assay was performed as described (19). Purified DtxR protein was isolated by methods as described (20). Radiolabeled DNA iron box fragments were generated by PCR using 100 ng of ³²P-end-labeled primer and template DNA from gel-purified 100-bp cold fragments containing the iron box of interest. Binding reactions were carried out in 10 mM Tris-OAc (pH 7.4), 1 mM EDTA, 50 mM KCl, 1 mM DTT, 5% glycerol, and 50 µg/ml calf thymus DNA. Binding reactions were equilibrated for 30 min and then loaded onto a nondenaturing 6% acrylamide gel (21).

**Results**

**Expression of the Corynebacterial dtxR Gene in Mycobacteria.** The 1.5-kb corynebacterial DNA fragment cloned in pNBV1/SAD contained 500 bp of 5′ noncoding sequences as well as the entire dtxR(E175K) ORF. To determine whether the corynebacterial mutant dtxR(E175K) gene was expressed in mycobacteria, we transformed *M. smegmatis*, a fast-growing strain of mycobacteria, with pNBV1/SAD. Whole-cell lysates prepared from *M. smegmatis* cultures were separated by 12% SDS-PAGE. Fig. 1 shows a Western blot developed with polyclonal anti-DtxR antibodies. As may be seen, these antibodies recognize both DtxR and IdeR because of their significant antigenic similarity. Although the deduced molecular mass of IdeR (25.2 kDa) differs by only 0.1 kDa from DtxR (25.3 kDa) we have repeatedly observed anomalous accelerated migration of IdeR in our SDS/PAGE gels in which it runs at 23 kDa in spite of its mass of 25 kDa. This phenomenon also has been noted by Schmitt et al. (8). In preparations from *M. smegmatis* harboring pNBV1/SAD (Fig. 1, lane 4), two distinct bands appear. Because dtxR(E175K) is expressed from a multicopy plasmid, significantly more DtxR(E175K) protein is made than the chromosomally expressed IdeR. Similar results in *M. tuberculosis* transformed with pNBV1/SAD also were found (results not shown). The in vivo growth rate of wild-type *M. tuberculosis* was indistinguishable from that of *M. tuberculosis* DtxR(E175K) by the Bactec radiometric growth monitoring system.

**Attenuation of Virulence in M. tuberculosis Expressing the Constitutively Active DtxR Hyperrepressor.** After confirming that the corynebacterial mutant dtxR was expressed in transformed mycobacteria, we turned to an in vivo animal model to test the effect of the hyperrepressor on virulence. Forty-eight BALB/c mice were inoculated with 2 × 10⁶ cfu of *M. tuberculosis* CDC1551 or *M. tuberculosis* DtxR(E175K) by tail-vein injection. Both animal weights and the tissue burden of surviving bacteria were monitored over time. Mice infected with wild-type *M. tuberculosis* began to lose weight beginning at 13 weeks whereas the *M. tuberculosis* DtxR(E175K)-infected animals initially gained weight, then maintained stable weights for the duration of the experiment. At 17 weeks, there was a statistically significant difference of 1.7 g (*P* = 0.006 by two-tailed *t* test) between the wild-type and DtxR(E175K) groups.

Fig. 2 shows the survival of the two *M. tuberculosis* strains in lungs and spleens of mice over time. At 17 weeks, there was a 1.2 log attenuation in virulence of the DtxR(E175K)-expressing strain compared with wild type, which was statistically significant in both spleen (*P* = 0.0002) and lungs (*P* = 0.006). Analysis of the colonies from the mouse tissues at 12 weeks showed that 99% of the colonies were hygromycin resistant, indicating maintenance of the pNBV1/SAD plasmid. Histopathologic inspection of spleen and lungs of wild-type and DtxR(E175K)-expressing...
strains corroborated our cfu data with fewer visible acid fast bacilli at 17 weeks in histologic sections of mouse organs from animals infected with the \textit{M. tuberculosis} DtxR(E175K) than with the wild type.

**Differences in Colonial Morphology Between Strains.** Colonies of \textit{M. tuberculosis} DtxR(E175K) grown from frozen stocks on 7H10 Middlebrook agar showed no difference in growth rate in \textit{vitro} as compared with wild-type CDC1551, but were noted to have a distinct colonial morphology (see Fig. 3). The recombinant strain colonies were rougher, appeared to be drier, and were more raised and wrinkled than wild-type colonies. In addition, yellow pigment was noted in the DtxR(E175K) expressor. Both strains exhibited a spreading phenotype and were crenated at the periphery.

**Identification of Iron Boxes.** An imperfect palindromic consensus sequence of the iron box for DtxR/Ider has been established by \textit{in vivo} and \textit{in vitro} methods (8, 22, 23). This consensus sequence is listed at the top of Fig. 4. To identify genes that may be regulated by Ider, we searched the \textit{M. tuberculosis} genome for iron boxes that were in untranslated regions within 200 bp of an ORF. We chose two half-site sequences with allowance for a variable number of intervening base pairs for our search. In the 4.41 MB of the \textit{M. tuberculosis} genome (24), 58 sequences with acceptable homology to the consensus sequence were identified. Six of these were in untranslated regions and had corresponding downstream ORFs.

A DNA gel-binding assay was used to assess the ability of DtxR to bind to these putative iron-regulated operator regions drawn from the \textit{M. tuberculosis} genome. Fig. 5 shows the results of gel-binding assays using 32P-end-labeled 100-bp DNA fragments containing five of the putative iron boxes (IB1–5). Binding of DtxR to the tox operator could be abolished with the addition of unlabeled tox DNA, but not with nonspecific DNA. All five of these putative iron boxes were bound by DtxR to a similar degree as that observed with the tox operator. The iron box upstream of the narG homologue, IB6, did not bind to DtxR (data not shown).

Table 2 identifies the ORFs downstream of these six iron boxes. BLAST searches reveal that these genes encode a PhoP homologue (a transmembrane sensor of a two-component sensor-regulator pair), a homologue of the Htra serine protease, 16S ribosomal RNA, an alcohol dehydrogenase AdhB, and a homologue of the \textit{M. tuberculosis} 19-kDa antigen (a protein shown to be involved in the human immune response to tuberculosis) (25). IB6, which was not shifted by DtxR \textit{in vitro}, appears upstream of a nitrate reductase subunit gene, narG.

**Discussion**

The concentration of free ferrous iron (Fe^{2+}) is extremely limited \textit{in vivo}. For this reason, many pathogenic prokaryotes such as \textit{Vibrio cholerae}, \textit{E. coli}, \textit{Neisseria gonorhoeae}, and \textit{C. diphtheriae} coregulate virulence gene expression with iron sensing and scavenging systems (26–28). In \textit{C. diphtheriae}, one such mechanism of iron regulation relies on a repressor, DtxR, which binds to a specific palindromic sequence in the operator regions of the genes that it controls (29). In low iron states, the metal ion triggered conformational change that allows it to bind to the DNA is disrupted, the repressor loses affinity for the operator site, and gene expression occurs. Recently, a positive dominant

![Fig. 2. Virulence comparison of wild-type \textit{M. tuberculosis} and \textit{M. tuberculosis} DtxR(E175K) mutant. (A) The log cfu of the homogenized spleens of mice sacrificed at 4-week intervals. (B) The log cfu of homogenized lungs at 4-week intervals. Each point represents the mean log cfu of 5–6 mice ± 1 SD (error bars). * denote statistically significant differences between groups at a given time point.](image)

![Fig. 3. (A) A 10-week-old representative colony of \textit{M. tuberculosis} DtxR(E175K) on 7H10 agar. (B) A 10-week-old representative colony of wild-type \textit{M. tuberculosis} (strain CDC1551) on 7H10 agar.](image)

![Fig. 5. Autoradiographs of gel-binding assay between DtxR and putative \textit{M. tuberculosis} DtxR/Ider binding sites. Shown are 100-bp 32P-end-labeled DNA fragments containing toxO (lanes 1 and 2), Ib1-1 (lanes 3 and 4), Ib2-1 (lanes 5 and 6), Ib3-1 (lanes 7 and 8), Ib3-2 (lanes 9 and 10), and Ib5-1 (lanes 11 and 12) separated in a nondenaturing 6% polyacrylamide gel. Odd-numbered lanes contain DNA only (unbound), and even-numbered lanes contain DNA preincubated with purified DtxR (bound).](image)
DtxR(E175K) mutant unresponsive to iron was generated by random PCR mutagenesis using a genetic selection system (10). Significant amino acid identity between corynebacterial DtxR and mycobacterial IdeR has been described. In the amino terminal 140 aa there is a DNA binding helix-turn-helix motif, a primary metal ion binding site, and a protein–protein interaction domain. Corynebacterial DtxR and mycobacterial IdeR share 80% amino acid identity in this portion of both proteins. Evidence of functional homology between IdeR and DtxR has been shown previously by Schmitt et al. (8). Because these two organisms are phylogenetically related, we postulated that the iron-independent corynebacterial DtxR(E175K) may be able to repress the expression of genes under the control of IdeR in mycobacteria in a constitutive fashion.

In this paper, we have shown that the positive dominant DtxR(E175K) iron-independent repressor is expressed in mycobacteria and furthermore, that it is able to attenuate *M. tuberculosis* in a murine model of infection. Rational attenuation of *M. tuberculosis* offers the possibility of defining specific virulence factors of the organism and of developing live vaccines superior to bacillus Calmette–Guérin. However, gene replacement has proven difficult in *M. tuberculosis* because of high rates of illegitimate recombination. Addition of a dominant mutant gene is technically simpler than gene replacement in *M. tuberculosis* and permits comparison of a defined merodiploid strain from extracellular rather than intracellular sources (35).

The *ideR* gene has been found in *M. tuberculosis*, *M. bovis*, and *M. smegmatis*. In *M. smegmatis*, an *ideR* mutant showed defective regulation of siderophore biosynthesis (9). Potential IdeR-binding sites upstream of exochelin biosynthesis genes, such as *fxbA*, recently have been confirmed (36, 37). In addition, several IdeR recognition sequences have been identified by using computer searches of the *M. tuberculosis* genome (38). We have similarly identified six potential IdeR-binding sites in *M. tuberculosis*, five of which demonstrated significant binding with DtxR in a gel-shift assay. We postulate that the sixth sequence was unable to bind in our *in vitro* assay because of incorrect spacing between the two relatively well-conserved half-sites. We used DtxR rather than IdeR in this gel-shift assay because we specifically sought to identify genes responsible for the attenuated phenotype of *M. tuberculosis* DtxR(E175K). The predicted ORF downstream of IB-1 encodes a homologue of *phoP*, a phosphotransfer response regulator. A number of two-component pairs have been shown to regulate virulence pathways in bacterial pathogens. These include BvgA/BvgS in Bordatella pertussis, VanR/VanS in *Enterococcus faecium*, PhoP-PhoQ in Salmonella typhimurium, and OmpT/EnvZ in Shigella flexneri (39, 40). In *M. tuberculosis*, a two-component pair, *mitA-mtrB*, has been described previously and appears to play an intracellular role as expression of *mitA* increases upon entry into macrophages (41). Furthermore, *phoP* mutants in *Salmonella* are unable to synthesize many of the proteins expressed on interaction with macrophages (42). Downstream of IB-2 is *adhB*, an alcohol dehydrogenase. In *S. typhimurium*, it has been postulated that alcohol dehydrogenase genes such as *eutG* may confer a protective role from reactive aldehyde intermediates associated with inflammatory cell activation (43). IB-3 lies upstream of an ORF homologous to a HtrA-like serine protease, which in *E. coli* is thought to be required for growth of the organism at high temperature, and may play a role in degrading abnormal proteins within the periplasm (44, 45). It is a known virulence factor in several organisms including *S. typhimurium*, *Yersinia enterocolitica*, *Brucella abortus*, and *Brucella melitensis* (46–49). In an animal model, a *S. typhimurium htrA* mutant is attenuated and a safe and immunogenic live vaccine strain in mice (50). Both *M. avium* subspp. *paratuberculosis* and *M. tuberculosis* have putative serine proteases with significant homology to HtrA (24, 51).

The *rnlA* gene is a 16S rRNA gene that has been shown to be part of a group of rDNA operons in both slow and fast-growing mycobacteria with hypervariable multiple promoter regions. The *M. tuberculosis* *rnlA* operon has two promoters, one of which is conditionally induced, suggesting complex regulation of this essential gene (52).

Our results indicate that a dominant positive corynebacterial *dtxR* allele can attenuate the virulence of *M. tuberculosis* in a murine model. These data implicate the *M. tuberculosis* IdeR repressor as a regulator of genes essential for full virulence. Expanded study of IdeR and the genes under its control offers a promising avenue toward understanding the pathogenicity of *M. tuberculosis*.

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