ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2004, p. 4447–4449 Vol. 48, No. 11
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.11.4447–4449.2004

Novel Mutations within the *embB* Gene in Ethambutol-Susceptible Clinical Isolates of *Mycobacterium tuberculosis*

Ann S. G. Lee,[1]* Siti Noor Khadijah Othman,[1] Yu Min Ho,[1] and Sin Yew Wong[2]

Division of Medical Sciences, National Cancer Centre,[1] and Department of Infectious Diseases, Tan Tock Seng Hospital,[2] Singapore, Singapore

Received 30 March 2004/Returned for modification 10 May 2004/Accepted 15 July 2004

Genetic analysis of the *embB* gene revealed mutations in 17 (68%) of 25 ethambutol (EMB) resistant isolates (M306I, M306V, M306L, Q497R) but also in 4 (20%) of 20 EMB-susceptible isolates of *Mycobacterium tuberculosis*, namely, an ATG→ATM substitution resulting in M306I, G406N, and the novel alterations M423I and A659T.

Ethambutol (EMB) [(*S*,*S*′)-2,2′-(ethylenediimino)di-1-butanol] is a first-line drug used for antituberculosis therapy. It is often used in combination with isoniazid, rifampin, pyrazinamide, and streptomycin. Membrane-associated arabinosyl transferases have been implicated as the targets for EMB (2, 3, 14, 15). The *Mycobacterium tuberculosis emb* operon is a gene cluster of three contiguous genes, namely, *embC*, *embA*, and *embB*, which encode mycobacterial arabinosyl transferases (26). These enzymes are involved in the polymerization of the cell wall arabinan (4, 6, 9, 24, 25, 32). Inhibition of arabinan synthesis by EMB results in the accumulation of mycolic acids, leading to cell death.

Alterations at codon 306 of *embB* have been identified as being the most common alteration in EMB-resistant *M. tuberculosis* clinical isolates (8, 12, 17–20, 23, 29). Initial work on 51 EMB-resistant isolates had shown that 89% of these isolates had alterations at residue 306 of *embB*, but these alterations were not detected in 30 EMB-susceptible isolates (23). A subsequent study confirmed this high frequency of *embB306* alterations, with 67% of 75 EMB-resistant isolates having mutations not found in EMB-susceptible strains (19). This led to several groups developing targeted strategies for the detection of *embB306* alterations (7, 16, 21, 30). Amino acids within the EMB resistance-determining region of EmbB proteins are well conserved among mycobacterial species, including those from *M. tuberculosis*, *M. leprae*, and *M. smegmatis* (2), and mutations within this region have been detected in EMB-resistant isolates of *M. tuberculosis*.

The aim of this present work was to screen all regions of the *embB* gene with previously reported mutations in order to assess the contribution of mutations within this gene to EMB resistance in *M. tuberculosis* clinical isolates from Singapore.

Drug susceptibility testing was done using the BACTEC 460 radiometric method (Becton Dickinson, Towson, Md.) (2.5 μg/ml). Twenty-five consecutive *M. tuberculosis* isolates resistant to EMB and 20 EMB-susceptible isolates from Singapore were collected as previously described (5, 10).

* Corresponding author. Mailing address: Division of Medical Sciences, National Cancer Centre, Singapore 169610, Republic of Singapore. Phone: 65 6436 8313. Fax: 65 6372 0161. E-mail: dmslsg@nccs.com.sg.

DNA extracted from the isolates was analyzed by amplifying four fragments, using the PCR primers shown in Table 1. The PCR products were purified (QIAquick PCR purification kit or QIAquick gel extraction kit; QIAGEN) and directly sequenced using the BigDye Terminator sequencing kit and the ABI PRISM 377 automated sequencer (PE Biosystems, Branchburg, N.J.). Confirmation of mutations was done by reamplification and resequencing.

IS*6110* profiling was done according to standard procedures to determine if the isolates were epidemiologically independent (28). All isolates with the same nucleotide substitutions in this study were deemed to be epidemiologically unassociated as they had distinct IS*6110* fingerprints.

Overall, mutations in the *embB* gene were detected in 17 (68%) of the 25 EMB-resistant isolates (Table 2). Mutations at *embB306* were detected in 12 of these 25 (48%) EMB-resistant isolates. Notably, all of the 12 EMB-resistant isolates with *embB306* mutations were also resistant to isoniazid. All five EMB-resistant isolates with mutations at codon 497 were resistant to at least three antituberculosis drugs. Three isolates monoresistant to EMB had no detectable mutations in *embB*.

This is the first report of a double substitution (ATG→ATM, where M represents the nucleotides A and C), resulting in a Met→Ile alteration at the frequently altered codon 306 of *embB* in an EMB-susceptible isolate (Table 2). This isolate was resistant to both isoniazid and rifampin.

In addition, three other alterations were also detected in EMB-susceptible isolates, G406D and two novel mutations, M423I and A659T. The isolate with the G406N substitution was also resistant to isoniazid and rifampin, while the isolate with the M423I alteration was monoresistant to isoniazid and the isolate with the A659T alteration was monoresistant to streptomycin. In total, alterations in the *embB* gene were detected in 4 (20%) of the 20 EMB-susceptible isolates (Table 2).

There is a possibility that these mutations may have occurred in susceptible isolates due to cross-contamination of the PCR product, heteroresistance involving mixed cultures, or errors in the susceptibility testing, though every effort was undertaken to avoid this.

Alterations in *embB* in EMB-susceptible isolates at codons other than codon 306 have been documented in only two isolates with the G406D alteration (20) and one isolate with the

TABLE 1. Oligonucleotide primer sequences for amplification of the *embB* gene[a]

Primer	Description	Sequence	Nucleotides	Annealing temp (°C)	PCR product size (bp)
embB1(F)	First fragment, sense	5′ CTG AAA CTG CTG GCG ATC AT	7601–7620	58.0	415
embB1(R)	First fragment, antisense	5′ GGT CTG GCA GGC GCA TCC	8015–7998		
embB2(F)	Second fragment, sense	5′ TGG AGG CCA GCA AAC CCG	8082–8099	58.0	451
embB2(R)	Second fragment, antisense	5′ TAG TAG TAA CGC AGG TTC TC	8532–8513		
embB3(F)	Third fragment, sense	5′ GCT GTT CGC CGC CGT AGG	8743–8760	62.0	528
embB3(R)	Third fragment, antisense	5′ GAA CCC GAA TCG CCG TCC AG	9270–9251		
embB4(F)	Fourth fragment, sense	5′ TTC GCC CGA GCA AAG ATG	9752–9769	61.0	368
embB4(R)	Fourth fragment, antisense	5′ TCG CGG GAC AGG TAG GTG	10119–10102		

[a] The *M. tuberculosis* sequence used to design the primers was obtained from GenBank, accession number U68480.

S347T alteration (8). This paucity of information is due in part to some studies targeting only *embB306* (17, 29), several reporting no mutations (1, 12, 19, 23), and others not including EMB-susceptible isolates (22, 31).

Interestingly, all three EMB monoresistant isolates in this present study did not have any detectable alterations in *embB*. In contrast, all 58 EMB-susceptible isolates in this and other studies with *embB* alterations were resistant to other antituberculosis drugs as well (8, 17, 20, 29). These observations support the hypothesis that a target other than EmbB may exist for EMB which may be activated during combination treatment with other first-line antituberculosis drugs, resulting in susceptibility to EMB (17).

Importantly, if all alterations of *embB306* are considered polymorphisms, then only a minority of EMB-resistant isolates would be mutated. A similar scenario was observed in studies defining the role of the *katG* gene in isoniazid resistance in *M. tuberculosis*. Members of our group and others have shown that the predominant alteration in *katG* is R463L, which is detected in both isoniazid-resistant and -susceptible isolates and hence is considered a polymorphism and an unreliable indicator of isoniazid resistance (11, 13, 27). Thus, it is imperative for all studies elucidating the molecular mechanisms of drug resistance in *M. tuberculosis* to include drug-susceptible isolates as controls.

Another interesting finding of this study was the presence of resistance to other antituberculosis drugs when alterations of *embB* were present. Further investigations are necessary in order to understand the involvement of these drugs in the molecular mechanism of EMB resistance.

In conclusion, alterations at *embB306* may not confer resistance to EMB but may be common polymorphisms in clinical isolates of *M. tuberculosis*. The clinical significance of this alteration is dubious, and further evaluation of EMB-susceptible isolates from other geographic regions is warranted.

TABLE 2. Mutations in the *embB* gene in clinical isolates of *M. tuberculosis*

Phenotype (n[a])	Codon	Amino acid change	Mutation	No. (%) of isolates
EMB resistant (25)		None	None	8 (32)
	306	Met→Ile	ATG→ATC	3 (12)
	306	Met→Ile	ATG→ATA	6 (24)
	306	Met→Val	ATG→GTG	2 (8)
	306	Met→Leu	ATG→CTG	1 (4)
	497	Gln→Arg	CAG→CGG	5 (20)
EMB susceptible (20)		None	None	16 (80)
	306	Met→Ile	ATG→ATM[c]	1 (5)
	406	Gly→Asp	GGC→GAC	1 (5)
	423[b]	Met→Ile	ATG→ATA	1 (5)
	659[b]	Ala→Thr	GCG→ACG	1 (5)

[a] *n*, no. of isolates.
[b] Novel mutation.
[c] M represents the nucleotides A and C.

We thank Irene H. K. Lim, Lynn L. H. Tang, and Tong-Seng Lim for excellent technical assistance. We gratefully acknowledge the Central Tuberculosis Laboratory, Department of Pathology, Singapore General Hospital, for providing isolates.

This work was supported by the National Medical Research Council of Singapore.

REFERENCES

1. **Abbadi, S., H. G. Rashed, G. P. Morlock, C. L. Woodley, O. El Shanawy, and R. C. Cooksey.** 2001. Characterization of IS6110 restriction fragment length polymorphism patterns and mechanisms of antimicrobial resistance for multidrug-resistant isolates of *Mycobacterium tuberculosis* from a major reference hospital in Assiut, Egypt. J. Clin. Microbiol. **39:**2330–2334.
2. **Alcaide, F., G. E. Pfyffer, and A. Telenti.** 1997. Role of embB in natural and acquired resistance to ethambutol in mycobacteria. Antimicrob. Agents Chemother. **41:**2270–2273.
3. **Belanger, A. E., G. S. Besra, M. E. Ford, K. Mikusova, J. T. Belisle, P. J. Brennan, and J. M. Inamine.** 1996. The embAB genes of Mycobacterium avium encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. Proc. Natl. Acad. Sci. USA **93:**11919–11924.
4. **Besra, G. S., K. H. Khoo, M. R. McNeil, A. Dell, H. R. Morris, and P. J. Brennan.** 1995. A new interpretation of the structure of the mycolyl-arabinogalactan complex of Mycobacterium tuberculosis as revealed through characterization of oligoglycosylalditol fragments by fast-atom bombardment mass spectrometry and 1H nuclear magnetic resonance spectroscopy. Biochemistry **34:**4257–4266.
5. **Boudville, I. C., S. Y. Wong, and I. Snodgrass.** 1997. Drug-resistant tuberculosis in Singapore, 1995 to 1996. Ann. Acad. Med. Singapore **26:**549–556.
6. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64:**29–63.
7. **Cooksey, R. C., G. P. Morlock, B. P. Holloway, J. Limor, and M. Hepburn.** 2002. Temperature-mediated heteroduplex analysis performed by using denaturing high-performance liquid chromatography to identify sequence polymorphisms in *Mycobacterium tuberculosis* complex organisms. J. Clin. Microbiol. **40:**1610–1616.
8. **Escalante, P., S. Ramaswamy, H. Sanabria, H. Soini, X. Pan, O. Valiente-Castillo, and J. M. Musser.** 1998. Genotypic characterization of drug-resistant Mycobacterium tuberculosis isolates from Peru. Tuber. Lung Dis. **79:**111–118.
9. **Khoo, K. H., E. Douglas, P. Azadi, J. M. Inamine, G. S. Besra, K. Mikusova, P. J. Brennan, and D. Chatterjee.** 1996. Truncated structural variants of lipoarabinomannan in ethambutol drug-resistant strains of Mycobacterium smegmatis. Inhibition of arabinan biosynthesis by ethambutol. J. Biol. Chem. **271:**28682–28690.
10. **Lee, A. S., I. H. Lim, L. L. Tang, A. Telenti, and S. Y. Wong.** 1999. Contribution of *kasA* analysis to detection of isoniazid-resistant *Mycobacterium tuberculosis* in Singapore. Antimicrob. Agents Chemother. **43:**2087–2089.
11. **Lee, A. S., L. L. Tang, I. H. Lim, M. L. Ling, L. Tay, and S. Y. Wong.** 1997. Lack of clinical significance for the common arginine-to-leucine substitution

at codon 463 of the katG gene in isoniazid-resistant Mycobacterium tuberculosis in Singapore. J Infect. Dis. **176:**1125–1127.
12. **Lee, H. Y., H. J. Myoung, H. E. Bang, G. H. Bai, S. J. Kim, J. D. Kim, and S. N. Cho.** 2002. Mutations in the embB locus among Korean clinical isolates of Mycobacterium tuberculosis resistant to ethambutol. Yonsei Med. J. **43:** 59–64.
13. **Leung, E. T., K. M. Kam, A. Chiu, P. L. Ho, W. H. Seto, K. Y. Yuen, and W. C. Yam.** 2003. Detection of katG Ser315Thr substitution in respiratory specimens from patients with isoniazid-resistant Mycobacterium tuberculosis using PCR-RFLP. J. Med. Microbiol. **52:**999–1003.
14. **Maddry, J. A., W. J. Suling, and R. C. Reynolds.** 1996. Glycosyltransferases as targets for inhibition of cell wall synthesis in M. tuberculosis and M. avium. Res. Microbiol. **147:**106–112.
15. **Mikusova, K., R. A. Slayden, G. S. Besra, and P. J. Brennan.** 1995. Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. Antimicrob. Agents Chemother. **39:**2484–2489.
16. **Mokrousov, I., O. Narvskaya, E. Limeschenko, T. Otten, and B. Vyshnevskiy.** 2002. Detection of ethambutol-resistant *Mycobacterium tuberculosis* strains by multiplex allele-specific PCR assay targeting embB306 mutations. J. Clin. Microbiol. **40:**1617–1620.
17. **Mokrousov, I., T. Otten, B. Vyshnevskiy, and O. Narvskaya.** 2002. Detection of *embB306* mutations in ethambutol-susceptible clinical isolates of *Mycobacterium tuberculosis* from Northwestern Russia: implications for genotypic resistance testing. J. Clin. Microbiol. **40:**3810–3813.
18. **Ramaswamy, S., and J. M. Musser.** 1998. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 update. Tuber. Lung Dis. **79:**3–29.
19. **Ramaswamy, S. V., A. G. Amin, S. Goksel, C. E. Stager, S. J. Dou, H. El Sahly, S. L. Moghazeh, B. N. Kreiswirth, and J. M. Musser.** 2000. Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **44:**326–336.
20. **Ramaswamy, S. V., S. J. Dou, A. Rendon, Z. Yang, M. D. Cave, and E. A. Graviss.** 2004. Genotypic analysis of multidrug-resistant Mycobacterium tuberculosis isolates from Monterrey, Mexico. J. Med. Microbiol. **53:**107–113.
21. **Rinder, H., K. T. Mieskes, E. Tortoli, E. Richter, M. Casal, M. Vaquero, E. Cambau, K. Feldmann, and T. Loscher.** 2001. Detection of embB codon 306 mutations in ethambutol resistant Mycobacterium tuberculosis directly from sputum samples: a low-cost, rapid approach. Mol. Cell Probes **15:**37–42.
22. **Sharaf-Eldin, G. S., N. S. Saeed, M. E. Hamid, A. M. Jordaan, G. D. Van der Spuy, R. M. Warren, P. D. Van Helden, and T. C. Victor.** 2002. Molecular analysis of clinical isolates of Mycobacterium tuberculosis collected from patients with persistent disease in the Khartoum region of Sudan. J. Infect. **44:**244–251.
23. **Sreevatsan, S., K. E. Stockbauer, X. Pan, B. N. Kreiswirth, S. L. Moghazeh, W. R. Jacobs, Jr., A. Telenti, and J. M. Musser.** 1997. Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of *embB* mutations. Antimicrob. Agents Chemother. **41:**1677–1681.
24. **Takayama, K., E. L. Armstrong, K. A. Kunugi, and J. O. Kilburn.** 1979. Inhibition by ethambutol of mycolic acid transfer into the cell wall of *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **16:**240–242.
25. **Takayama, K., and J. O. Kilburn.** 1989. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **33:**1493–1499.
26. **Telenti, A., W. J. Philipp, S. Sreevatsan, C. Bernasconi, K. E. Stockbauer, B. Wieles, J. M. Musser, and W. R. Jacobs, Jr.** 1997. The emb operon, a gene cluster of Mycobacterium tuberculosis involved in resistance to ethambutol. Nat. Med. **3:**567–570.
27. **van Doorn, H. R., E. J. Kuijper, A. van der Ende, A. G. Welten, D. van Soolingen, P. E. de Haas, and J. Dankert.** 2001. The susceptibility of *Mycobacterium tuberculosis* to isoniazid and the Arg→Leu mutation at codon 463 of *katG* are not associated. J. Clin. Microbiol. **39:**1591–1594.
28. **van Embden, J. D. A., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, P. Hermans, C. Martin, R. McAdam, T. M. Shinnick, and P. M. Small.** 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J. Clin. Microbiol. **31:**406–409.
29. **Van Rie, A., R. Warren, I. Mshanga, A. M. Jordaan, G. D. van der Spuy, M. Richardson, J. Simpson, R. P. Gie, D. A. Enarson, N. Beyers, P. D. van Helden, and T. C. Victor.** 2001. Analysis for a limited number of gene codons can predict drug resistance of *Mycobacterium tuberculosis* in a high-incidence community. J. Clin. Microbiol. **39:**636–641.
30. **Victor, T. C., A. M. Jordaan, A. van Rie, G. D. van der Spuy, M. Richardson, P. D. van Helden, and R. Warren.** 1999. Detection of mutations in drug resistance genes of *Mycobacterium tuberculosis* by a dot-blot hybridization strategy. Tuber. Lung Dis. **79:**343–348.
31. **Wang, W., H. Li, X. Wu, A. Wang, Z. Wang, J. Liu, H. Chen, M. Lin, J. Wang, Y. Ye, and S. Li.** 2002. Clinical application and evaluation of the detection of five drug resistance genes in Mycobacterium tuberculosis. Zhonghua Jie He He Hu Xi Za Zhi. **25:**670–673. (In Chinese.)
32. **Wolucka, B. A., M. R. McNeil, E. de Hoffmann, T. Chojnacki, and P. J. Brennan.** 1994. Recognition of the lipid intermediate for arabinogalactan/arabinomannan biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. J. Biol. Chem. **269:**23328–23335.