

Dynamics of Extensive Drug Resistance Evolution of *Mycobacterium tuberculosis* in a Single Patient During 9 Years of Disease and Treatment

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Mycobacterium tuberculosis is one of the hardest to treat bacterial pathogens with a high capacity to develop antibiotic resistance by mutations. Here we have performed whole-genome sequencing of consecutive *M. tuberculosis* isolates obtained during 9 years from a patient with pulmonary tuberculosis. The infecting strain was isoniazid resistant and during treatment it stepwise accumulated resistance mutations to 8 additional antibiotics. Heteroresistance was common and subpopulations with up to 3 different resistance mutations to the same drug coexisted. Sweeps of different resistant clones dominated the population at different time points, always coupled to resistance mutations coinciding with changes in the treatment regimens. Resistance mutations were predominant and no hitch-hiking, compensatory, or virulence-increasing mutations were detected, showing that the dominant selection pressure was antibiotic treatment. The results highlight the dynamic nature of *M. tuberculosis* infection, population structure, and resistance evolution and the importance of rapid antibiotic susceptibility tests to battle this pathogen.

Keywords. antibiotic resistance; *Mycobacterium tuberculosis*; evolution.

Mycobacterium tuberculosis represents one of the most devastating human pathogens in history. Effective combination treatment regimens against tuberculosis extend for months or years and the drugs have severe side effects [1]. This reduces patient compliance leading to suboptimal treatment, transmission of resistant strains, and compromises complete eradication of the infection. The development of resistance among *M. tuberculosis* strains has resulted in multidrug resistant and extensively drug resistant bacteria [2]. There is no known horizontal transfer of resistance genes in *M. tuberculosis*; instead antibiotic resistance depends solely on selection of de novo mutations during treatment [3]. The subsequent evolutionary success of resistant mutants depends largely on the resistance level and fitness of the mutant, epistatic effects between genetic alleles, and selective sweeps due to antibiotic treatment [4, 5].

Because antibiotics target vital processes in the cell, resistance mutations that occur in genes responsible for these

processes often confer a fitness cost to the cell, reducing the ability of the resistant mutant to be maintained in the population [4, 6]. However, following a costly mutation, compensating mutations may occur that mitigate the fitness cost of the original resistance mutation [7–10]. In the presence of an antibiotic, the mutation frequency of occurrence, together with level of resistance and fitness cost for each mutation, will therefore be the main determinants of mutation prevalence in the population [4, 6, 11, 12]. In the recommended *M. tuberculosis* treatment plan the antibiotic selection pressure comes from multiple antibiotics at the same time, creating a complex selection landscape in each individual patient [1]. To add to the complexity, epistatic effects between resistance mutations and the genetic background may also play a part in the evolutionary fate of mutations [7, 9, 13, 14].

Previous whole-genome sequencing (WGS) studies on the evolution of antibiotic resistance of *M. tuberculosis* during treatment in patients show somewhat contradictory results [5, 15–19]. Some conclude that a majority of fixed mutations in the population were associated with drug resistance [16, 18, 19] and that antibiotic selection was the dominating factor driving evolution under treatment [16, 18]. Other studies report a high degree of hitchhiking mutations and a large diversity of mutations unrelated to drug resistance between longitudinal samples [5, 15]. Most studies observed a stepwise evolution of antibiotic resistance [15, 16, 18]. Some reported coexistence of mutationally different clonal populations of the same resistance phenotype at the same time point during infection [15, 17, 19].

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Here we studied the evolution of extensive drug resistance in a *M. tuberculosis* strain belonging to the Beijing lineage in a single patient over 9 years (1991–1999). The infecting strain was initially resistant to isoniazid and during the course of infection further resistance developed to 8 additional compounds used for treatment, eventually leading to the death of the patient. To address the genetic composition of the infecting *M. tuberculosis* population over the course of infection and determine the evolutionary trajectories of resistant subpopulations, we performed WGS of both population samples and several single isolates from the populations at each sampling point. Genetic changes were compared with treatment regimens and changes in phenotypic resistance patterns to determine how resistance evolved in response to antibiotic treatment.

METHODS

Isolates

M. tuberculosis isolates were obtained from a previous collection of samples from 1 Swedish patient during 9 years (1991–1999) of pulmonary tuberculosis [20]. The high sequence similarity strongly suggests that the patient carried the same single clone during 9 years without reinfection. The samples (populations) were collected at 8 different time points before and during treatment. Each sample was grown on Löwenstein-Jensen slants at 37°C to subsequently isolate up to 10 single colonies. The isolates were analyzed for phenotypic drug susceptibility using the

BACTEC 460TB system (Becton and Dickinson). Both populations and individual isolates were kept frozen at -70°C until used in this study.

DNA Extraction

DNA was isolated from 8 population samples and from 7–10 previously isolated colonies from each of the 8 different population samples, in total 77 samples (Figure 1). The population/isolate samples were cultured for 3 to 4 weeks on Löwenstein-Jensen slants at 37°C and DNA was isolated by chloroform-isoamyl alcohol extraction according to a protocol described earlier [21].

Whole-Genome Sequencing and Bioinformatics

The genomic DNA was sequenced with Illumina HiSeq 2000 using 2×90 read length with an average of 50 \times coverage by the BGI sequencing facility (Hongkong, China). Reads were trimmed from adaptor sequences and by quality as listed in Supplementary Table 1. The isolate S91/222-1.4 was sequenced using a polymerase chain reaction (PCR)-free library preparation to obtain higher reference coverage of GC-rich regions. The S91/222-1.4 sequence data was used for de novo assembly (CLC Genomics Workbench v12) and the best matching complete *M. tuberculosis* genome was identified by BLASTn at the National Center for Biotechnology Information (NCBI) as CCDC5079 (CP002884). Reference assembly of S91/222-1.4 raw reads onto CCDC5079 was then used to identify variations

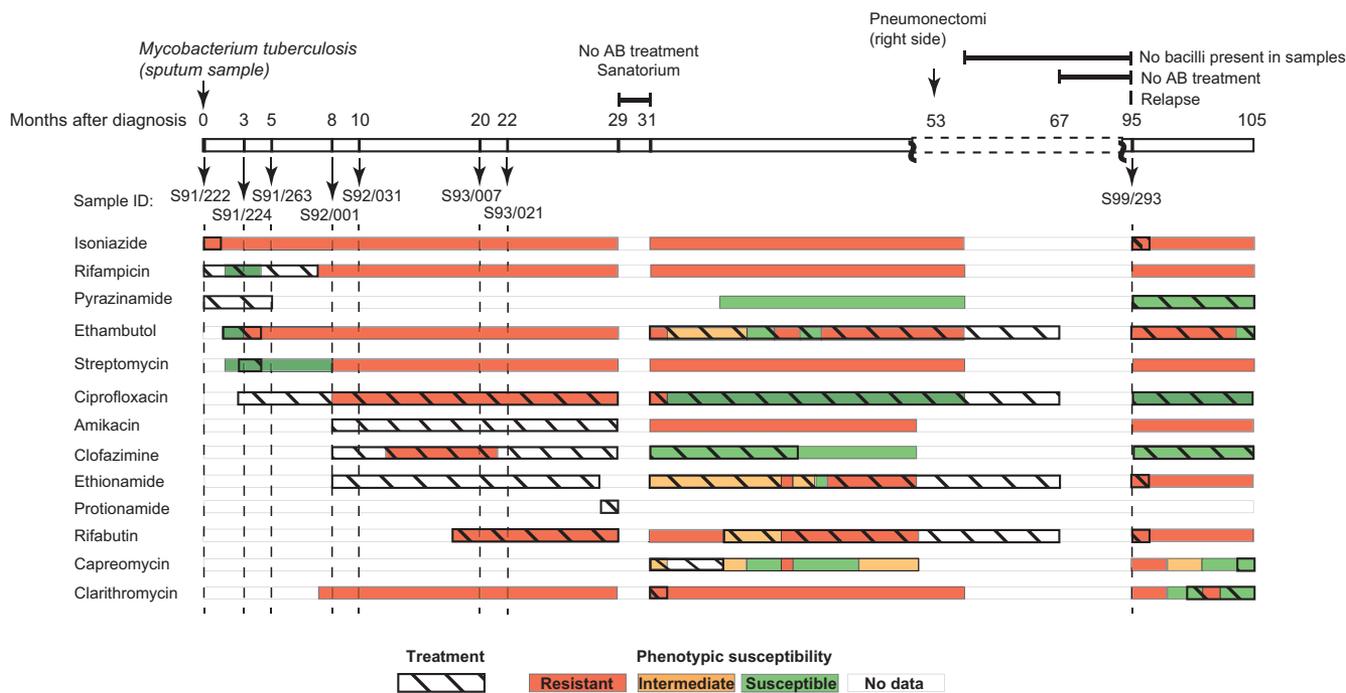


Figure 1. Illustration of time points for sampling, antibiotic treatment, and phenotypic resistance. The sampling consisted of 8 time points (S91/222, S91/224, S91/263, S92/001, S92/031, S93/007, S93/021, and S99/293) from 0 to 95 months, as indicated with arrows and vertical dashed lines in the upper part of the figure. The patient was diagnosed with lung tuberculosis at time 0. The time line is drawn to scale only up to month 25. Each antibiotic has a time line where antibiotic (AB) treatment (striped), phenotypic resistance (red), intermediate resistance (orange), and susceptibility (green) from the patient records are indicated.

and generate a reference genome to which all other isolates were assembled and sequence variation analyzed [22]. Single nucleotide polymorphisms (SNPs) and structural variations were identified using the respective tools in CLC Genomic Workbench v12 and the Microbial Genomics Plugin using the following criteria: (1) for the population samples a SNP frequency of at least 10% and 5 reads with the same mutation was used as cutoff to validate each mutation in the population; and (2) for the isolated clones a SNP frequency >70% was used as cutoff to validate each mutation. In addition, each validated mutation in a single clone or population sample was visually reconfirmed from the assemblies of WGS data. The Pro-Glu/Pro-Pro-Glu (PE/PPE) gene families were excluded from all analyses due to their repetitive abundance and high frequency of sequence errors reported [23]. All sequences including metadata were deposited at NCBI under BioProject number PRJNA641253.

RESULTS

To elucidate the dynamics of genetic variation in a *M. tuberculosis* strain during disease and treatment of an infected patient, from diagnosis of pulmonary tuberculosis until her death 9 years later, we performed WGS of 8 population samples from different time points during infection and 7–10 clones isolated from each of the populations [20]. From the patient records it was clear that the infecting strain developed resistance to different drugs used in the treatment in a stepwise manner (Figure 1). The infecting strain belonged to the Beijing lineage and was genetically very similar to the previously sequenced isolate CCDC5079 (CP002884) [22] differing by only 242 SNPs and small indels and 21 structural variations mainly including the presence/absence of IS6110 elements (Supplementary Table 2). The resulting reference assembly comprised 4.4 mega base pairs and the fraction of reference coverage of each of the subsequent samples was >99%.

To identify preexisting resistance mutations in the infecting strain, all SNPs and indels (in total 242) that differed between the isolates from the initial sample and the susceptible CCDC5079 strain was subjected to a literature search to identify SNPs in genes previously determined to lead to antibiotic resistance [22]. Two mutations were found in genes previously linked to isoniazid resistance, *katG* S315T [24] and low-level ethambutol resistance, *embB* T1082A [25]. These findings are in accordance with the initial susceptibility test showing resistance to isoniazid and an early but varying presence of ethambutol resistance in an otherwise susceptible strain (Figure 1).

Temporal Genetic Changes in the Infecting Population Show Strong Selection for Resistance Mutations in Response to Treatment

The index isolate was taken when the patient was first diagnosed with tuberculosis in March 1991. Treatment was then initiated with isoniazid, rifampicin, and pyrazinamide. After phenotypic antibiotic susceptibility testing the infecting strain

was found to be resistant to isoniazid as could be explained by the *katG* S315T mutation [24] (Figure 1 and Figure 2). Isoniazid was therefore replaced with ethambutol.

Three months after initiation of treatment, the strain was phenotypically resistant against both isoniazid and ethambutol. The ethambutol resistance could be explained by the *embB* T1082A mutation present already in the initial sample [25]. Although the susceptibility testing showed no phenotypic resistance towards rifampicin, WGS revealed the presence of 3 different *rpoB* (β -subunit of RNA polymerase) mutations in the population, H526L (standard *Escherichia coli* nomenclature, *M. tuberculosis* nomenclature H445L, frequency 10%), D516V (*M. tuberculosis* nomenclature D435V, frequency 10%), and H526Y (*M. tuberculosis* nomenclature H445Y, frequency 83%) (Figure 3A and Supplementary Table 3). These mutations are commonly found among clinical rifampicin-resistant *M. tuberculosis* isolates [26–28]. In contrast to the population sequence, the dominating *rpoB* mutation in the clones was D516V (9 of 10 clones) and only 1 clone had the H526L mutation (Figure 3A).

After an additional 2 months (5 months after diagnosis) of treatment with rifampicin, ethambutol, pyrazinamide, streptomycin, and ciprofloxacin, the strain was phenotypically resistant also to rifampicin and the *rpoB* D516V mutation dominated the population sample and was present in all clones (Figure 2 and Figure 3A). From now on the *rpoB* D516V mutation was present in all samples and none of the other *rpoB* mutations were detected again. At this time point the strain was still considered phenotypically susceptible to streptomycin but in the WGS data known streptomycin resistance mutations in *rpsL* K88R (S12 ribosomal protein) and *rrs* C517T (16S rRNA) had emerged in the population sample (Figure 2, Figure 3B, and Supplementary Table 3) [29, 30].

Eight months after the diagnosis, treatment consisted of ciprofloxacin, amikacin, clofazimine, and ethionamide and the strain was phenotypically resistant to isoniazid, ethambutol, streptomycin, rifampicin, ciprofloxacin, and clarithromycin. Mutations associated with resistance were *katG* S315T (INH), *embB* T1082A (ETB), *rpoB* D516V (RIF), and there were 3 different mutations in genes associated with streptomycin resistance at frequencies of 26%–48% (*rpsL* K43R and K88R, and *rrs* C517T) in the population sample (Figure 2, Figure 3B, and Supplementary Table 2) [29, 30]. The variability in streptomycin resistance mutations was maintained at the fifth sampling point (10 months), with *rpsL* K88R and *rrs* (C517T) still present in the population sample (Figure 2). Two single clones were found to carry either the *rpsL* K43R or the *rrs* C517T mutations while the rest had the *rpsL* K88R mutation. The strain was now also phenotypically resistant to clofazimine, likely by mutations in the transcriptional repressor (*rv0678*) of the efflux pump MmpL5 (Figure 1 and Supplementary Table 3) [31].

After an additional 10 months (20 months) of treatment with ciprofloxacin, amikacin, clofazimine, and ethionamide, and

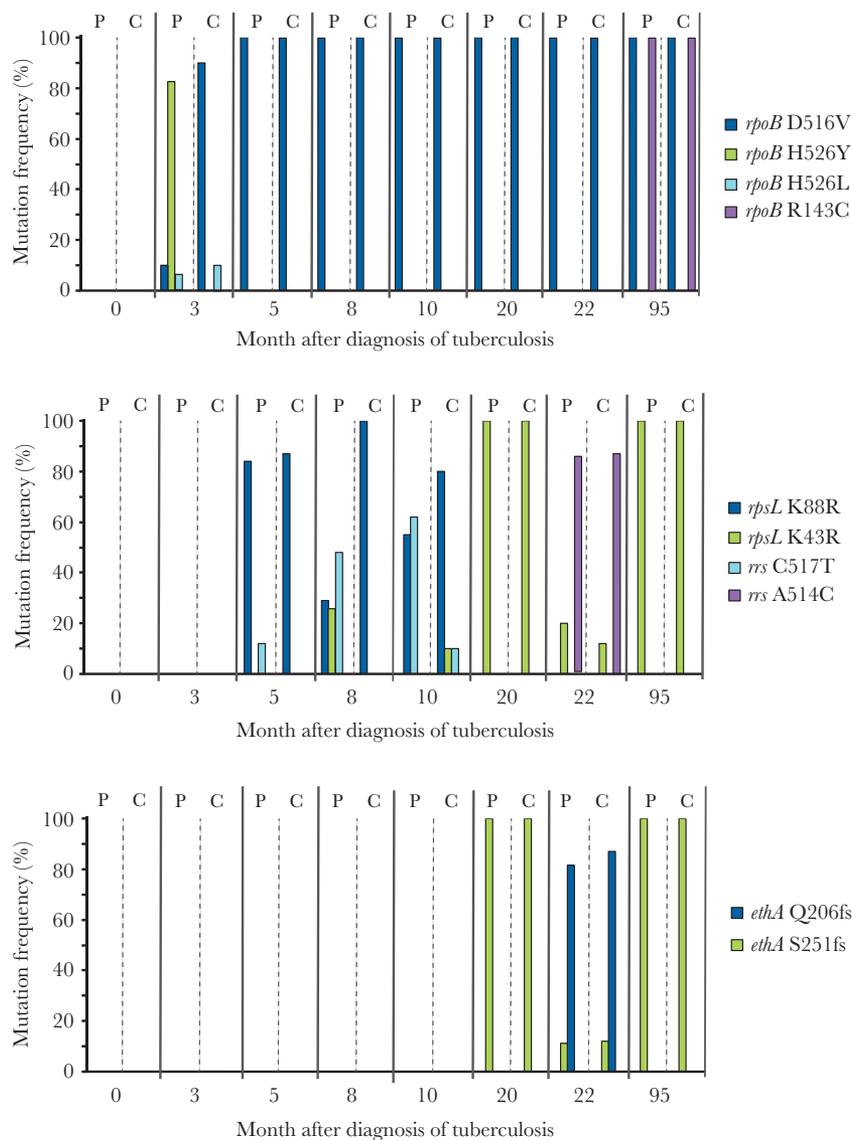


Figure 3. Clonal subpopulations of mutations connected to (A) rifampicin, (B) streptomycin, and (C) ethionamide resistance. The frequency (%) of each mutation is shown for both the population sample (P) and among the clonal samples (C). A, rifampicin resistance with mutations within the β -subunit of the bacterial RNA polymerase, *rpoB*. B, streptomycin resistance with mutations within 2 different genes, the ribosomal S12 protein *rpsL* and the 16S rRNA (*rrs*) gene. C, ethionamide resistance with mutations within the monooxygenase gene, *ethA*.

29 months of treatments all antibiotics were discontinued and the patient underwent sanatorium treatment for 2 months, after which antibiotic treatment was reinitiated (Figure 1). At 53 months after diagnosis the patient underwent a pneumonectomy of the right lung, which lead to a significant improvement. Antibiotic treatment was continued and no growth of bacilli in sputum samples was detected for a year and therefore all antibiotic treatment was discontinued.

In April 1999, 8 years after the tuberculosis diagnosis, the patient suffered a relapse with extensive bacterial growth in the left lung and the strain now tested resistant against 9 antibiotics: isoniazid, rifampicin, ethambutol, streptomycin, amikacin, ethionamide, rifabutin, capreomycin, and clarithromycin. All

sequenced clones at this time point were related to the strain isolated after 20 months of treatment but now the clones contained between 13 and 14 nonsynonymous mutations (Figure 2 and Supplementary Tables 3 and 4). The *katG* S315T (INH), *rpoB* D516V (RIF), *rpsL* K43R (STR), and *ethA* Q206fs (ETH) were all present 6 years earlier. A second mutation in *embB*, G406D, previously associated with ethambutol resistance, was present in all clones [25]. Also, a new *rrs* A1401G mutation was present in all clones (Figure 2 and Supplementary Table 3), previously detected in clinical isolates resistant to amikacin, kanamycin, and capreomycin [33, 34].

In total, 50 mutations in 38 different genes were observed among all 77 samples during the infection period

(Supplementary Tables 3 and 4). The average number of mutations (SNP, small deletions, and insertions) after 8 years of sampling was 13.5 per clone. The mutational spectrum was heavily dominated by nonsynonymous mutations with an average SNPs ratio of 8.5:1 of nonsynonymous to synonymous SNPs (1.54 mutations per genome and year) and more than a third were resistance mutations (0.63 mutations per genome and year). Mutations previously shown to be involved in antibiotic resistance were also the only mutations dominating the populations or clones at more than 1 time point (Figure 2 and Supplementary Table 3). Predicted resistance mutations also largely correlated with susceptibility test results and appeared in connection with treatment with the corresponding drug [20]. The level of similarity between clones from the same population was high and at the last time point 11 mutations were shared by all 10 clones with an additional 1 to 2 mutations/clone.

Possible Compensatory Mutations and Virulence Mutations Did Not Persist in the Population

In addition to antibiotic selection, mutations that increase bacterial fitness by compensating for negative effects of other mutations or that increase bacterial virulence may increase in an infecting population over time. Mutations in *gyrA* R264L, *rpoB* R143C (*M. tuberculosis* nomenclature R167C), *ribF* (*rv2786c*), *pitA* (*rv0545*), *ppsC* (*rv2933*), *ppsA* (*rv2931*), *rv3404c*, *recN* (*rv1696*), and *espK* (*rv3879*) mutations that possibly could lead to compensation of fitness costs or changes in virulence were observed in clones (Supplementary Tables 3 and 4) [35–37]. However, none of these mutations were present at more than 1 time point. In contrast, a set of tandem duplications between IS6110 elements increased in frequency over the course of infection (Table 1 and Figure 4). The size of the

duplications ranged from 132 579 bp to 360 499 bp with a minimum common overlapping region of 51 128 bp, including putative virulence-associated genes such as *sapM* and *sats*, the RNA polymerase sigma factor *sigF*, and the toxin-antitoxin system *vapC44/vapB44*. The duplications were observed sporadically in 1 clone at the first, second, and third time points but increased drastically in frequency at time points 7 and 8 (duplication frequencies of 43% and 90%, respectively). In the last sampled population amplifications with different end points were present, indicating that this is not just an expansion of a single clone but due to individual duplication events. Duplications in this region has been observed previously and implicated in decreased virulence in clinical isolates from the Beijing lineage [38].

DISCUSSION

A hallmark of *M. tuberculosis* is the ability to form persistent long-term infections that are difficult to treat with antibiotics. The population structure of *M. tuberculosis* in the infected lung is highly compartmentalized, generating differential exposure of antibiotics that may promote coexistence of different resistant subpopulations in different parts of the lung [11, 12]. The rapid population sweeps observed here for resistance mutations, the small number of other mutations found, and the fact that only mutations associated with antibiotic resistance were found at more than 1 time point in the population indicate antibiotic treatments to be the dominant force of selection. Other WGS studies of longitudinal inpatient samples of *M. tuberculosis* isolates have also suggested antibiotic treatment to be the dominating force of selection [16, 18]. In contrast to some other studies, we found very few additional mutations and did not observe any hitchhiking mutations in addition to the

Table 1. Analysis of Genomic Duplications in Clones

Sampling, mo	Clones With Duplication/Total Clones	Isolate Name	Duplicated Region	Length, Base Pairs	IS6110 End Points
0	1/7	1.2	3 484 819–3 845 317	360 499	Yes
3	1/10	2.9	3 484 819–3 708 206	223 388	Yes
5	1/7	3.4	3 484 819–3 845 317	360 499	Yes
8	0/7
10	0/10
20	0/9
22	3/9	7.2	3 484 819–3 845 317	360 499	Yes
		7.5	3 484 819–3 845 317	360 499	Yes
		7.8	3 484 819–3 845 317	360 499	Yes
95	9/10	8.1	3 657 079–3 789 657	132 579	No
		8.2	3 544 002–3 708 206	164 205	Yes
		8.3	3 484 819–3 708 206	223 388	Yes
		8.4	3 544 002–3 845 317	301 316	Yes
		8.5	3 484 819–3 845 317	360 499	Yes
		8.6	3 544 002–3 845 317	301 316	Yes
		8.7	3 484 819–3 845 317	360 499	Yes
		8.8	3 544 002–3 708 206	164 205	Yes
		8.10	3 484 819–3 845 317	360 499	Yes

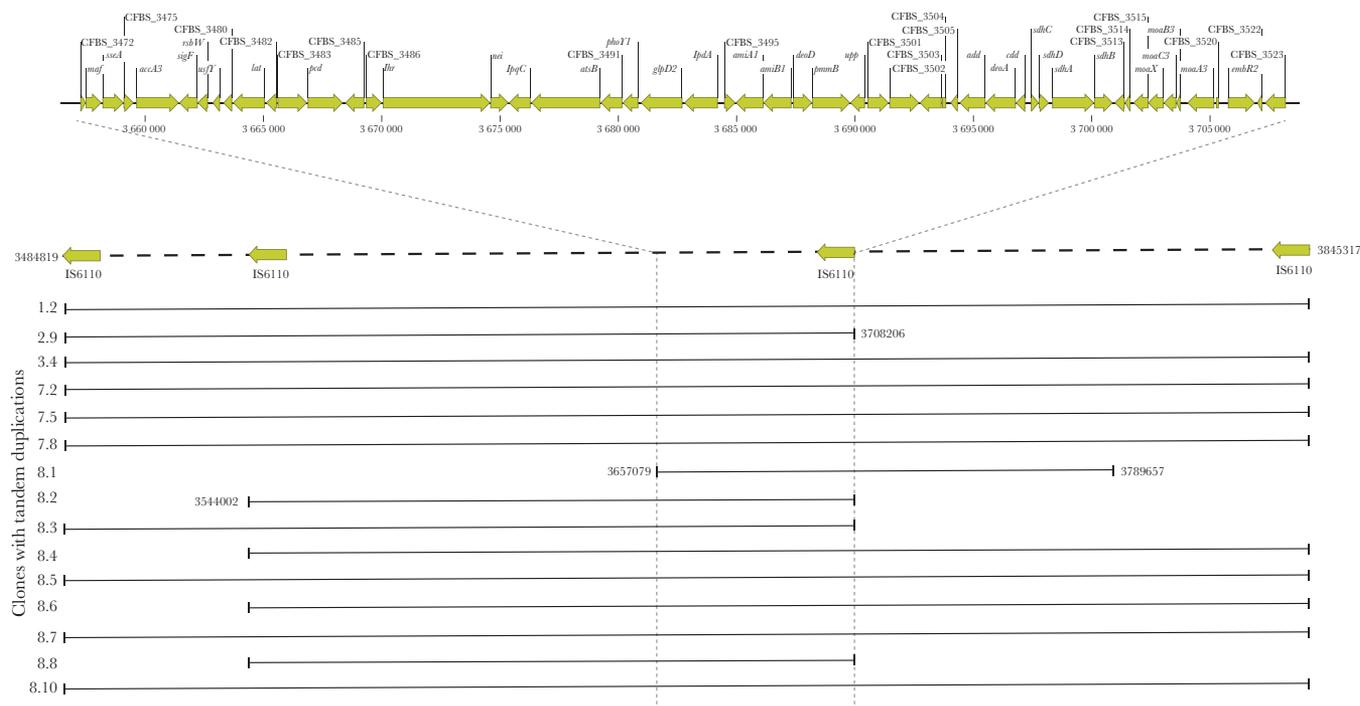


Figure 4. Tandem duplications. Clones with tandem duplications are shown including the length and position of the duplicated regions. The smallest common duplicated region is highlighted at the top including the genes within the region. IS6110 elements that serve as duplication end points are indicated with arrows on the dashed line.

resistance mutations [5, 15, 17]. A factor that can explain part of the observed differences between studies is differences in the selective factors, such as treatment regimens, between different patients as well as the compartmentalization of tuberculosis in the lungs, and the difficulty of retrieving representative samples that reflect the genetic variation in populations [11, 12]. In addition, whether genome sequence approaches are based on population samples or single-colony samples can also affect the results. Here, the level of sequence identity between clones from the same population was high and differences between population samples and clones from the same population was mainly observed in SNP frequencies for different genotypic resistant mutations for the same phenotypic susceptibility.

We never observed a mixture of resistant and susceptible genotypes for the same antibiotic among the clones and only once for the population samples (time point 7, rv0678 possible clofazimine resistance) indicating a strong selection by the treatments. However, multiple resistance mutations to the same drug existed simultaneously and varied over time for rifampicin, ethionamide, and streptomycin resistance. This could be due to longitudinal variability in the sampling of subpopulations of *M. tuberculosis* recovered from separate cavities of the lung [11, 12], complex genetics of resistance, variable selection by different treatments, or a combination of these factors. The 5 population switches of dominant subclones always matched the treatment regimens with additional resistance mutations mostly originating from the previous dominating clone.

A likely explanation is therefore that selection for mutants with low fitness cost and high resistance generated the clonal variance and selective sweeps, as has been seen before for rifampicin and streptomycin resistance mutations [17, 18, 39, 40]. For example, variation in rifampicin resistance levels and degree of fitness cost between the 3 mutations *rpoB* H526Y, D516V, and H526L has been reported [28, 41–43]. In addition, previous experiments in *E. coli* and *M. tuberculosis* showed epistasis of rifampicin resistance mutations in *rpoB* with streptomycin and isoniazid resistance mutations [13, 14, 43, 44]. Stochastic appearance of resistance mutations to other antibiotics among the existing subpopulations may also contribute to clonal sweeps. For example, the clonal variation of streptomycin resistance at the last 3 sampling points was probably due to evolution of ethionamide and ethambutol resistance in different *rpsL*/*rrs* clones and maybe also clofazimine resistance. When the first ethionamide resistance mutation (*ethA* Q206fs) evolved at 20 months in a clone with a preexisting *rpsL* K43R mutation, the previously dominating *rpsL* K88R and *rrs* (517T) mutations disappeared completely. The *rpsL* K43R mutation also has no or low fitness cost compared to a drug-susceptible strain and it is one of the most common clinical streptomycin-resistant mutations [30, 39, 45]. The combination of an *ethA* S251fs mutation in a clone with an *rrs* C514T mutation dominated at 22 months with a temporary dip in the frequency of *rpsL* K43R mutations. The last sample was again dominated by the *rpsL* K43R mutation but this time in combination with an *rrs* A1401G mutation

leading to high amikacin resistance and low-level capreomycin resistance [34].

A general explanation for antibiotic-resistant phenotypes without specific target mutations, which was the case for ciprofloxacin in our study, could be increased expression of efflux pumps [46]. Mutations in *pstB* (*rv0933*), *rv0678*, and *rv2326c* coding for efflux pumps were observed and upregulation of the PstB efflux pump has been shown to be connected to fluoroquinolone resistance [47]. Given the long period of infection, it is also plausible that differences in susceptibility testing laboratory procedures between different samples affect the reporting of resistance, especially when the minimum inhibitory concentration is close to the clinical breakpoint.

Several studies have shown that compensatory mutations can ameliorate fitness costs of resistance, often without loss of resistance [6, 7, 20, 48]. Previously described compensatory mutations in *M. tuberculosis* are mainly connected with mutations in the *rpoA* or *rpoC* genes compensating for rifampicin resistance, but no such mutations were found here [48]. At the later time points, possible compensatory mutations for rifampicin resistance were observed in *pitA*, *ppsA*, *ppsC*, and *recN* but they were only present at 1 sampling point [36, 37]. After the pneumonectomy, the patient improved in health and there were no detectable bacilli from sputum cultures. All antibiotic treatments were also discontinued but after 16 months without treatment the patient relapsed and the strain now contained additional resistance mutations. This is discouraging because it indicates that the combined resistance mutations did not confer a sufficiently high fitness cost for the patient's immune system to clear the infection or that the patient was exposed to risk factors that increased the risk of a relapse.

An alternative explanation for the success of the last clonal population could be that some of the new mutations found in the population at that time caused an increased virulence and associated population expansion. However, all identified mutations instead point towards lowered virulence. The frame shift mutation found in *espK*, a gene previously described to be involved in virulence, would likely lead to inactivation of EspK and be predicted to lead to decreased virulence [35]. Also, the large tandem duplications within the same region that increased in frequency at the 2 last time points have previously been observed to reduce virulence in a mouse model [38]. Overall, there is no indication that virulence-increasing mutations were selected in the patient over a period of 9 years.

In conclusion, this patient case illustrates the fast and stepwise evolution of resistance even with the simultaneous use of multiple antibiotics, illustrating a remarkable adaptability of *M. tuberculosis* in the human lung and the dynamics of different emerging subpopulations during infection. Most importantly, this unique patient material demonstrates the need for rapid diagnostics of both bacterial genotype and phenotype before treatment is initiated. Thus, had it been known that the strain

was isoniazid resistant and had reduced susceptibility to ethambutol, it is likely that the initial treatment regime could have been adjusted to provide a better outcome.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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