Experimental versus In Silico Fluorescent Amplified Fragment Length Polymorphism Analysis of *Mycobacterium tuberculosis*: Improved Typing with an Extended Fragment Range

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Whole-genome fingerprinting fluorescent amplified fragment length polymorphism (FAFLP) data were compared with in silico data for the sequenced strains of *Mycobacterium tuberculosis* (H37Rv and CDC1551). For this G+C-rich genome, many predicted fragments were not detected experimentally. For H37Rv, only 108 (66%) of the 163 predicted *Eco*RI-*Mse*I fragments between 100 and 500 bp were visualized in vitro. FAFLP was also used to identify polymorphism in 10 clinical isolates of *M. tuberculosis* characterized previously by IS*6110* typing, examining fragments of up to 1,000 bp in size rather than up to 500 bp as was done previously. Five isolates had unique IS*6110* profiles and were not known to be epidemiologically related, two isolates were the same single-band IS*6110* type but were not known to be epidemiologically related, and the remaining three isolates were epidemiologically related with identical IS*6110* profiles. Analysis of fragments in the 500- to 1,000-bp range using nonselective primers differentiated better between strains than analysis of fragments in the 50- to 500-bp range using a set of four selective primers. Seventeen polymorphic fragments were identified between 500 and 1,000 bp in size compared with nine polymorphic fragments between 50 and 500 bp. Using the 500- to 1,000-bp analysis, a level of discrimination similar to that of IS*6110* typing was achieved which, unlike the IS*6110* typing, was able to differentiate the two *M. tuberculosis* strains, each of which had only a single copy of IS*6110*.

Mycobacterium tuberculosis is a pathogenic acid-fast bacillus with a genome rich in guanine and cytosine (G+C) bases and an average G+C content of 65.6% (7). It is the causative agent of tuberculosis, a disease responsible for a total of 2 to 3 million human deaths annually. Currently, one-third of the world's population is thought to be infected, and the World Health Organization has estimated that if present trends continue, 200 million people are at risk of developing the disease in the next 20 years (22).

M. tuberculosis isolates cannot be resolved by phenotypic strain typing, and their genomes lack heterogeneity. IS*6110* restriction fragment length polymorphism typing has been used to distinguish isolates of the *M. tuberculosis* complex (22). The method is based on the presence of different numbers of copies (varying from 0 to 25) of an insertion sequence, IS*6110*, in strains of *M. tuberculosis* and the variability of their positions in the genome. The technique is of limited value, as it cannot easily differentiate between *M. tuberculosis* isolates that contain only a few or no copies of the insertion sequence, as has been found in countries with a high incidence of tuberculosis, such as India (40% of isolates), and to a lesser degree (8% of isolates) in the United Kingdom (9, 15).

Amplified fragment length polymorphism (AFLP) analysis is a PCR-based technique involving the restriction of bacterial

genomic DNA with two restriction enzymes, the ligation of adapters to the restriction fragments, and selective amplification of sets of the restriction fragments with adapter-specific primers. The amplified fragments are then sized by gel electrophoresis (24). The addition of a fluorescent label to one of the PCR primers allows the amplified fragments to be detected with an automated DNA sequencer.

AFLP analysis has been used in the typing of strains of different pathogens (10, 13, 19, 20) and also for plant and animal genetic mapping, medical diagnostics, and phylogenetic studies (18). The whole-genome fingerprinting technique fluorescent AFLP (FAFLP) analysis has also been used to identify polymorphisms in different strains of *M. tuberculosis* (12). FAFLP offers improved resolution compared with restriction fragment length polymorphism typing of isolates with a single copy of IS*6110*.

Using FAFLP, Goulding et al. (12) differentiated among 65 clinical *M. tuberculosis* isolates of known IS*6110* type by analyzing DNA restriction fragments from 50 to 500 bp in size. Thirty-eight discriminatory fragments were identified, allowing differentiation of the isolates. Most of the epidemiologically related groups clustered in the same way as with IS*6110* profiling, but a group of seven epidemiologically unlinked isolates with identical single-copy IS*6110* profiles was split into four clusters by FAFLP analysis (12).

The present study examines whether including DNA fragments of up to 1,000 bp in the analysis increases the resolving power of FAFLP and, by comparing the results with the predicted in silico results obtained from the two sequenced strains

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of *M. tuberculosis* (8; http://www.tigr.org/CMR2), investigates whether a G+C-rich genome can be sized and identified accurately.

MATERIALS AND METHODS

The extracted DNA was the same as that used in the study by Goulding et al. (12). Ten of the 65 original strains with known epidemiological relationships were sampled (104, 129, 157, 202, 203, 218, 245, 251, 252, and 253). The genomes of isolates 104, 129, 203, 218, and 245 had unique IS*6110* profiles and were not known to be epidemiologically related. Isolates 157 and 202 were the same single-band IS*6110* type but were not known to be epidemiologically related. The epidemiologically related isolates 251, 252, and 253 had identical IS*6110* profiles. In addition, the sequenced *M. tuberculosis* strains, H37Rv and CDC1551, were used for reference (8, 21). The DNAs from the reference strains were extracted using the cetyltrimethylammonium bromide method as described previously (23).

Experimental FAFLP. We used the method described previously by Goulding et al. (12). Briefly, 500 ng of genomic DNA was digested in a total volume of 20 μl consisting of 5 U of *Mse*I (New England Biolabs, Hitchin, Hertfordshire, United Kingdom), 2 μl of 10× *Mse*I buffer, 0.2 μl of 10× bovine serum albumin, and 1.0 μl of DNase-free RNase A (10 μg/μl) for 1 h at 37°C. To this digest was added 5 U (1.0 μl) of *Eco*RI (Invitrogen, Paisley, United Kingdom), 1.68 μl of 0.5 M Tris HCl (pH 7.6), and 2.1 μl of 0.5 M NaCl (total volume, 25 μl), and the reaction mixtures were incubated for a further hour at 37°C. Slight variations of this method were carried out to optimize the reaction, including increasing the digest times from 1 to 2 and 3 h and adding known PCR-enhancing agents (5% [vol/vol] dimethyl sulfoxide [DMSO], 10% [vol/vol] glycerol, 60 mM tetramethylammonium chloride [TMAC], or 1% [vol/vol] deionized formamide) to the digest or to the PCR mixture. Endonucleases were inactivated at 65°C for 10 min prior to ligation. To the double-digested DNA was added 25 μl of a solution containing 40 U of T4 DNA ligase (New England Biolabs), 10 pmol of *Eco*RI adapter, 100 pmol of *Mse*I adapter, and 5 μl of 10× T4 ligase buffer. This reaction mixture was incubated at 12°C for 17 h, heated at 65°C for 10 min to inactivate the ligase, and stored at −20°C.

PCRs were performed in 20-μl volumes containing 2 μl of ligated DNA, 0.1 μM 5-carboxy-fluorescein-labeled nonselective primer specific to the *Eco*RI adapter, 0.25 μM *Mse*I nonselective primer specific to the *Mse*I adapter, 2 μl of 10× *Taq* polymerase buffer, 200 μM each deoxynucleoside triphosphate, 1.5 mM MgCl$_2$, and 0.5 U of *Taq* polymerase. Touchdown PCR cycling conditions were used for amplifying the fragments: a 2-min denaturation step at 94°C (one cycle) followed by 30 cycles of denaturation at 94°C for 20 s, a 30-s annealing step (see below), and a 2-min extension step at 72°C. The annealing temperature for the first cycle was 66°C; for the next nine cycles, the temperature was decreased by 1°C at each cycle. The annealing temperature for the remaining 20 cycles was 56°C. This was followed by a final extension at 60°C for 30 min. PCR was performed in a PE-9600 thermocycler (Perkin-Elmer Corp., Norwalk, Conn.).

The amplification products were separated on a 5% denaturing polyacrylamide gel (FMC SingGel) on an ABI 377 DNA sequencer (Perkin-Elmer Corp.). The sample (1 μl) was added to 2.5 μl of loading dye, which was a mixture containing 5 μl of formamide, 1 μl of dextran blue–50 mM EDTA loading solution, and 0.5 μl of the internal lane standard GeneScan 2500 labeled with the fluorophore ROX (PE Biosystems). The sample mixture was heated at 95°C for 2 min, cooled on ice, and immediately loaded onto the gel. The electrophoresis conditions were 2 kV at 51°C for 16 h, using 1× Tris-borate-EDTA as the buffer. GeneScan and GenoTyper software (PE Biosystems) were used to ascertain the presence or absence of precisely sized fragments.

Reproducibility. To check the reproducibility of the FAFLP analysis, at least two different sets of digestions and ligations were carried out on the DNAs of the clinical isolates. For each digestion-ligation, at least three different PCRs were carried out separately, and each was run on a different gel.

In silico FAFLP. The complete genome sequences of *M. tuberculosis* strains H37Rv and CDC1551 were analyzed with the Restriction Digest Tool of The Institute for Genomic Research (http://www.tigr.org/tigr-scripts/CMR2/restrict_display.pl) using *Eco*RI and *Mse*I. Data concerning the sizes and predicted number of *Eco*RI-*Mse*I fragments were imported into a spreadsheet, and fragment sizes were adjusted to allow for the addition of primer sequences during PCR, enabling direct comparison between the predicted in silico and in vitro results.

TABLE 1. Comparison of in silico-predicted number of *M. tuberculosis Mse*I/*Eco*RI fragments with actual number of fragments detected by FAFLP analysis[a]

Strain	Restriction fragment size range (bp)	[MgCl$_2$] (mM)	Predicted no. of fragments	No. of fragments detected	% Predicted fragments detected
H37Rv	100–500	1.5	163	108	66
H37Rv	100–500	2.0	163	76	47
H37Rv	501–1,000	1.5	160	12	8
H37Rv	501–1,000	2.0	160	24	15
CDC1551	100–500	1.5	160	88	55
CDC1551	100–500	2.0	160	109	68
CDC1551	501–1,000	1.5	164	24	15
CDC1551	501–1,000	2.0	164	42	26

[a] 1.5 and 2 mM [MgCl$_2$] in the PCR.

RESULTS

Experimental versus in silico analysis. For H37Rv, 66% of the 163 predicted *Eco*RI-*Mse*I fragments between 100 and 500 bp, and only 8% of the 160 predicted *Eco*RI-*Mse*I fragments between 501 and 1,000 bp, were detected (Table 1). For CDC1551, we found that 55% of the 160 predicted *Eco*RI-*Mse*I fragments between 100 and 500 bp and 15% of the 164 predicted *Eco*RI-*Mse*I fragments between 501 and 1,000 bp were detected (Table 1). When the experiments were repeated with increased magnesium in the PCR mixture (2 mM MgCl$_2$ concentration instead of 1.5 mM) (Table 1), the number of fragments visualized increased but did not match the in silico results.

To assess how accurate FAFLP analysis could be for *M. tuberculosis*, the experimental analysis of H37Rv was repeated using a nonselective *Eco*RI-labeled primer and a selective *Mse*I+TA primer. This primer combination was expected to result in the amplification of fewer fragments, making the comparison of fragment size data between the predicted (in silico) and the observed (i.e., experimental) data easier.

Only 5 of the 11 predicted fragments (sizes, 195, 250, 252, 434, and 821 bp) were present in the experimental results of the *Mse*I+TA-*Eco*RI FAFLP analysis of H37Rv (Table 2). These five fragments were within ±2 bp of their predicted sizes. Four fragments of other sizes were detected that were not predicted in silico (Table 2).

FAFLP analysis of 10 clinical isolates. The 10 *M. tuberculosis* isolates showed 17 discriminatory fragments between 800 and 1,000 bp in size when nonselective *Eco*RI and *Mse*I primers were used (Table 3). This discriminated between two epidemiologically unlinked strains, 157 and 202, with single copies of IS*6110*. Isolate 202 possessed two fragments, 898 and 996 bp in size, which were absent from isolate 157. Figure 1 shows the

TABLE 2. Fragments predicted in silico and generated by FAFLP analysis of *M. tuberculosis* strain H37Rv using *Mse*I + TA selective primer and *Eco*RI nonselective primer

Method	Presence of fragment (size in bp)[a]													
	50	63	186	195	250	252	361	434	646	649	663	821	827	966	977
In silico	+		+	+	+		+	+	+	+	+			+	+
Experimental		+	+	+	+	+						+	+		

[a] +, present.

DISCUSSION

TABLE 3. Sizes of discriminatory fragments generated by FAFLP analysis of 10 IS*6110*-typed *M. tuberculosis* strains[a]

Strain no.	Presence of fragment (size in bp)[b]																
	819	823	828	830	847	879	887	891	896	898	906	908	911	928	931	968	996
104				+					+				+		+		
129				+					+		+		+		+	+	
157	+	+	+	+		+	+	+			+	+		+	+		
202	+	+	+	+		+	+	+		+	+	+		+		+	+
203			+	+					+		+	+	+		+	+	
218				+					+		+		+		+		
245			+	+					+		+		+		+	+	+
251			+						+		+		+		+		
252									+		+		+		+		
253									+		+		+		+		

[a] Originally studied by Goulding et al. (12) using *Mse*I and *Eco*RI nonselective primers. Isolates 251, 252, and 253 are known to be epidemiologically linked.
[b] +, present.

presence of the 996-bp fragment in isolate 202. The epidemiologically related isolates 251, 252, and 253 showed identical profiles apart from one fragment, 828 bp in size, present only in isolate 251 (Table 3).

Reproducibility. The FAFLP results were reproducible (Fig. 2); however, some background was seen in a small number of replicates, and consequently, only fragments read as above 75 fluorescent units (Fig. 2, *y* axis) were included. The FAFLP data showed slight variation in the relative peak heights of fragments of corresponding size (Fig. 2).

Previous work with *Escherichia coli* K-12 has shown that sizing is accurate to within 1 bp and that 48 of 48 predicted fragments were visualized (2) in FAFLP analysis. This was not, however, true of *M. tuberculosis* H37Rv and CDC1551 (Table 1). For H37Rv, only 108 (66%) of the 163 predicted *Eco*RI-*Mse*I fragment sizes between 100 and 500 bp were visualized in vitro, and when H37Rv was investigated with *Mse*I+TA-*Eco*RI, only 45% of the predicted fragments were visualized. The four unexpected fragments generated by this study were thought to be caused by incomplete digestion of the *M. tuberculosis* genomic DNA. It is possible that, due to the rich G+C content of the genome, secondary structures form that block the restriction sites from the action of the restriction enzymes. Usually an incomplete digest would mean generating a larger number of fragments than expected because the restriction enzyme cuts the genome at that position sometimes but not always, generating two fragments instead of one, i.e., the authentic one and a larger one. This does not appear to be the case for *M. tuberculosis*, as fewer fragments than expected are seen, even in the larger size range, indicating that certain restriction sites are rarely (or never) cut or that some digested fragments are not amplified or are amplified poorly. Various strategies were tried (the use of DMSO, glycerol, formamide, and TMAC and increasing digestion times) to overcome these potential problems. DMSO has been shown to improve the amplification efficiency and specificity of PCR (3). It is thought to reduce secondary structure by disrupting the base pairing

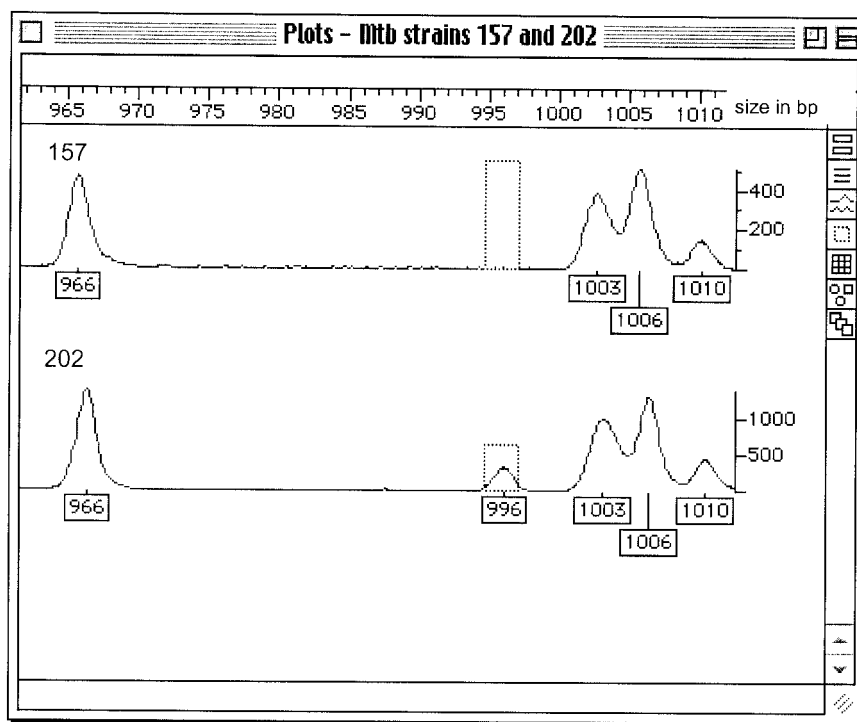FIG. 1. FAFLP profiles of *M. tuberculosis* single-copy IS*6110* isolates 157 and 202. The plot shows the fragment sizes in the range from 965 to 1,010 bp. The numbered boxes show the size in base pairs of each fragment. A fragment at 996 bp is present in isolate 202 and absent in isolate 157. The vertical scale measures the efficiency of PCR amplification of each fragment in fluorescent units.
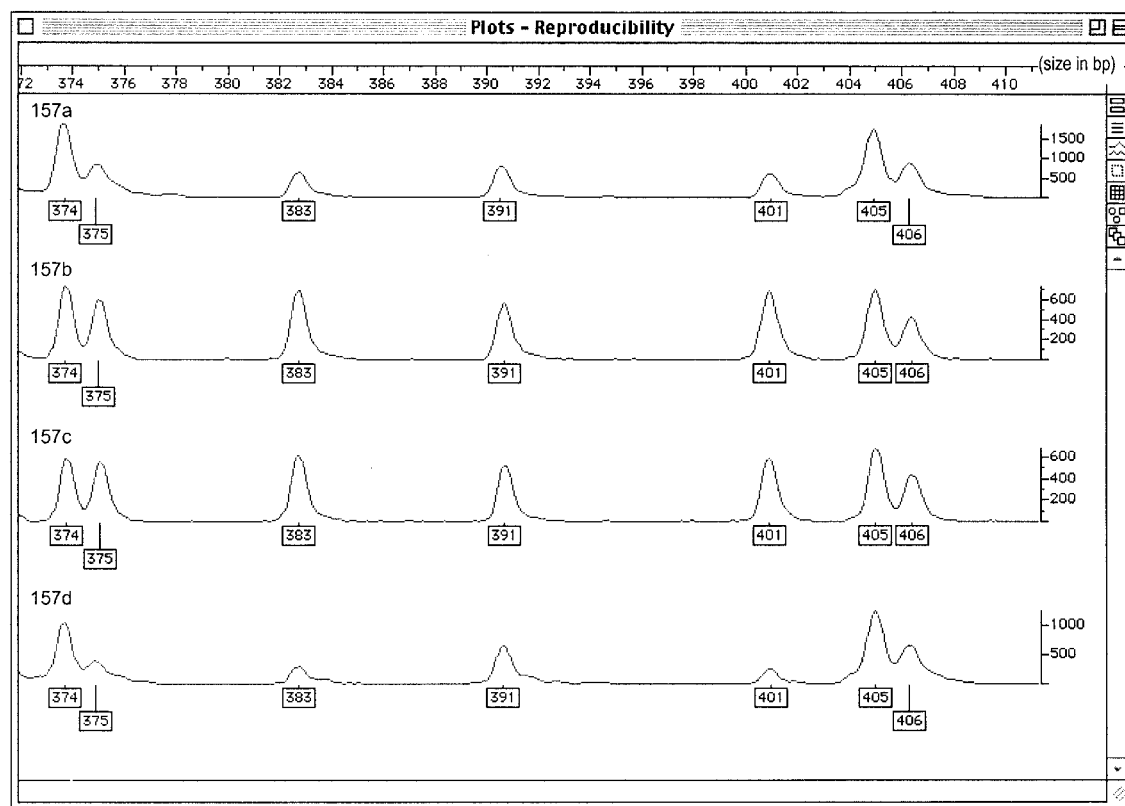
FIG. 2. FAFLP reproducibility study for *M. tuberculosis* clinical isolate 157. The four plots show the FAFLP profiles for the same isolate across a 40-bp window (372 to 412 bp). The four reactions were carried out separately and run on different gels. The vertical scale measures the efficiency of PCR amplification of each fragment in fluorescent units.

(11) and is therefore very useful for G+C-rich DNA templates (6). Both glycerol (16) and formamide (4, 17) improve the specificity and efficiency of PCR by changing the melting temperature of the primer-template hybridization reaction (Alkami quick guide for PCR: a laboratory reference for the polymerase chain reaction, vol. 1, Alkami Biosystems Inc., Berkeley, Calif., 1999). TMAC has been shown to improve the stringency of hybridization reactions, reduce potential DNA-RNA mismatch, and help eliminate nonspecific priming in PCR (5, 14).

Although DMSO, glycerol, and TMAC did not improve the method, preliminary results suggested that for FAFLP analysis of G+C-rich genomes, the use of deionized formamide in the digest step reduced the problem of unexpected fragments being produced and/or visualized. The identification and characterization of the four unpredicted restriction fragments generated by FAFLP analysis with the *Mse*I+TA and *Eco*RI primers, and their locations in the genome, may help to elucidate the differences that occur between the in silico and experimental data.

If the failure to detect expected fragments is not due to incomplete digestion but to cut fragments not being amplified because of secondary structure, the addition of dITP in place of dGTP during the PCR step may improve the FAFLP profile. The effect of the dITP may be to increase the number of fragments seen, but it may also have an effect on their relative peak heights, as there appears to be some variability in the

relative peak heights among different *M. tuberculosis* strains. However, this may be because amplification appears not to be uniform due to other factors, for example, if peaks with higher signal are comprised of multiple fragments of the same or similar sizes. Other potential causes for this lack of parity between in vitro and in silico data might be that there are errors in the sequences of H37Rv and CDC1551 or that passage of different stocks has led to differences between the isolates studied and the isolates sequenced. Mispriming of PCR may also have an effect on successful generation of expected fragments, but touchdown PCR is employed in FAFLP and should minimize this.

Another way of improving the FAFLP method for *M. tuberculosis* would be the use of different sets of enzymes, for example, *Xho*I and *Hha*I, which cut at IS*6110* as well as throughout the rest of the genome. This combination should give FAFLP profiles corresponding to the IS*6110* elements in a genome, as well as sampling throughout that genome.

The four-primer combination used in the study by Goulding et al. (12) of 65 IS*6110*-typed clinical isolates generated 38 discriminatory fragments under 500 bp in size. Of these fragments, only nine discriminated among the 10 (of 65) randomly selected isolates when studied as a subgroup. However, when nonselective primers were used, these 10 isolates gave a further 17 discriminatory fragments between 800 and 1,000 bp in size (Table 1). Two of these 17 polymorphic fragments (800 to 1,000 bp) differentiated between isolates 157 and 202, which

both contain only a single copy of IS*6110* (Fig. 1), making this a useful supplementary technique for *M. tuberculosis* isolates containing a single copy of IS*6110*. The profiles of the three isolates known to be epidemiologically linked (251, 252, and 253) were identical apart from one fragment observed in isolate 251 only. The reason for this is unclear, but it may be a reflection of real changes in the genome of isolate 251 compared with 252 and 253. The reason for the disproportionate number of polymorphisms in the higher size range is not understood. It is not due to the lack of reproducibility of amplification of larger-sized fragments.

In conclusion, experimental FAFLP does not exactly match in silico prediction, even when known PCR enhancers, such as TMAC (5) and formamide (17), are added to the digestion and/or PCR. The FAFLP technique is not as accurate for the G+C-rich genome of *M. tuberculosis* as for *E. coli* (1) and *Campylobacter* (10), as it does not strictly conform to the predicted model, but the fragments were precisely sized (± 2 bp). The results were reproducible (Fig. 2) but not as clean as for genomes with low G+C content, such as *E. coli*, which again may be due to the G+C-rich content of the *M. tuberculosis* genome. Fragments below a certain signal strength were therefore ignored. FAFLP is capable of resolving strains that cannot be differentiated using IS*6110* typing due to their low IS*6110* copy numbers, as demonstrated by the two polymorphic fragments (898 and 996 bp) that were present in isolate 202 but not in 157 (both of which contain single IS*6110* copies). This difference is significant, as up to 40% of *M. tuberculosis* isolates in certain countries have only one copy of IS*6110* (9). Our study also shows that FAFLP has a greater resolving power when fragments between 501 and 1,000 bp are included, as well as those between 100 and 500 bp.

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