Supplementary Data

accompanying the manuscript

A novel mechanism for the scission of double-stranded DNA: BfiI cuts both 3′-5′ and 5′-3′ strands by rotating a single active site

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Supplementary Methods

Mass spectrometry of oligonucleotides containing 3′-phosphorothiolate modifications

Oligonucleotides containing 3′-phosphorothiolate modifications were characterised on a MicroMass LCT mass spectrometer using negative mode electrospray ionization and direct infusion syringe pump sampling. Samples were diluted using methanol, ammonium acetate (*ca*. 20-50 μM) and diethylamine (*ca*. 0-0.1%). Series of multicharged peaks were observed fitting the formula $(M\text{-}nH)^{n-}$, where M indicates the free acid where all phosphate groups are protonated, and n indicates the number of protons removed to leave a charge. Where possible, spectra were transformed using the system software to give the average molecular mass (M_{Ave}).

Bottom strand of 14/15s: n = 4-9. $C_{144}H_{184}N_{54}O_{88}P_{14}S$ requires 4545.0548; observed M_{Ave} = 4544.9512 (deviation from the required: -0.1036 Da; -22.7 ppm).

Bottom strand of 25/25s: n = 8-15. $C_{241}H_{306}N_{92}O_{147}P_{24}S$ requires 7619.0488; observed M_{Ave} = 7619.0664 (deviation: +0.0176 Da; +2.3 ppm).

Top strand of 25s/NICK: n = 6-13. $C_{244}H_{307}N_{95}O_{149}P_{24}S$ requires 7730.11; observed M_{Ave} = 7729.49 (deviation: -0.62 Da; -80.2 ppm).

Data analysis

The reactions of WT BfiI and the truncated heterodimer H105A/WT-N on DNA substrates (Equation (1) in the main text) were fitted to the following equations:

$$S(t) = 100 \times e^{(-k_1 t)},$$

$$Ci(t) = 100 \times \frac{k_1}{k_1 - k_2} \times \left[e^{(-k_2 t)} - e^{(-k_1 t)} \right],$$

$$P(t) = 100 - S(t) - Ci(t),$$

where $S(t)$, $Ci(t)$ and $P(t)$ are, respectively, the concentrations at time t of the DNA substrate, the covalent intermediate and the hydrolysis product; k_1 and k_2 are the rate constants for the formation and cleavage of the covalent intermediate. The data with WT BfiI on both the 14/15 and 14/15s substrates (Figures 3A and 3B in the main text) was analysed with a global fitting procedure that yielded a common value for k_2 for both the 14/15 and 14/15s data sets but with individual values for k_1 for each set.

The reaction of the BfiI heterodimer WT/H105A on the 14/15s substrate (Figure 3C in the main text) was fitted to the rate equations for the scheme in Figure 3D, as follows:

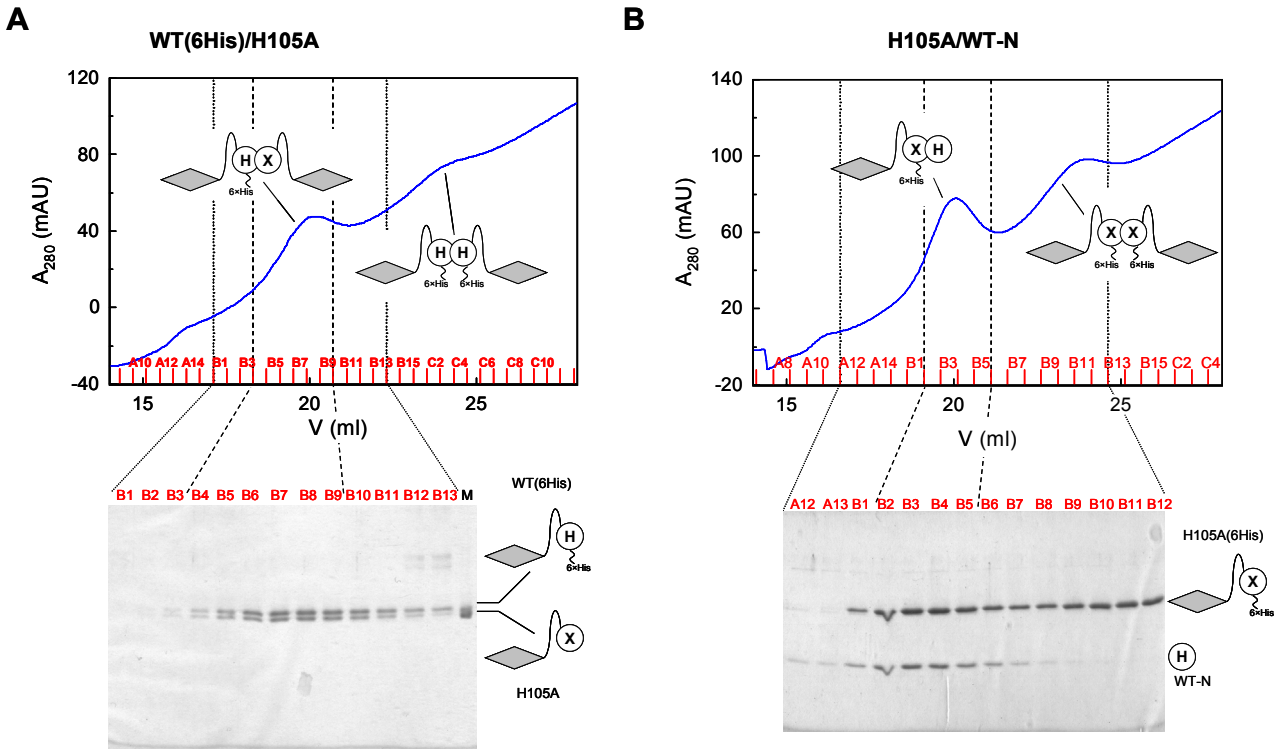
$$S(t) = A \times e^{(-k_1 t)} + (100 - A) \times e^{(-k_3 t)} + (100 - A) \times \frac{k_3}{k_3 - k_1} \times \left[e^{(-k_1 t)} - e^{(-k_3 t)} \right],$$

$$Ci(t) = A \times \frac{k_1}{k_1 - k_2} \times \left[e^{(-k_2 t)} - e^{(-k_1 t)} \right] +$$
$$+ (100 - A) \times \frac{k_1 k_3}{(k_3 - k_1)(k_3 - k_2)(k_1 - k_2)} \times \left[(k_1 - k_2) \cdot e^{(-k_3 t)} - (k_3 - k_2) \cdot e^{(-k_1 t)} + (k_3 - k_1) \cdot e^{(-k_2 t)} \right],$$

$$P(t) = 100 - S(t) - Ci(t),$$

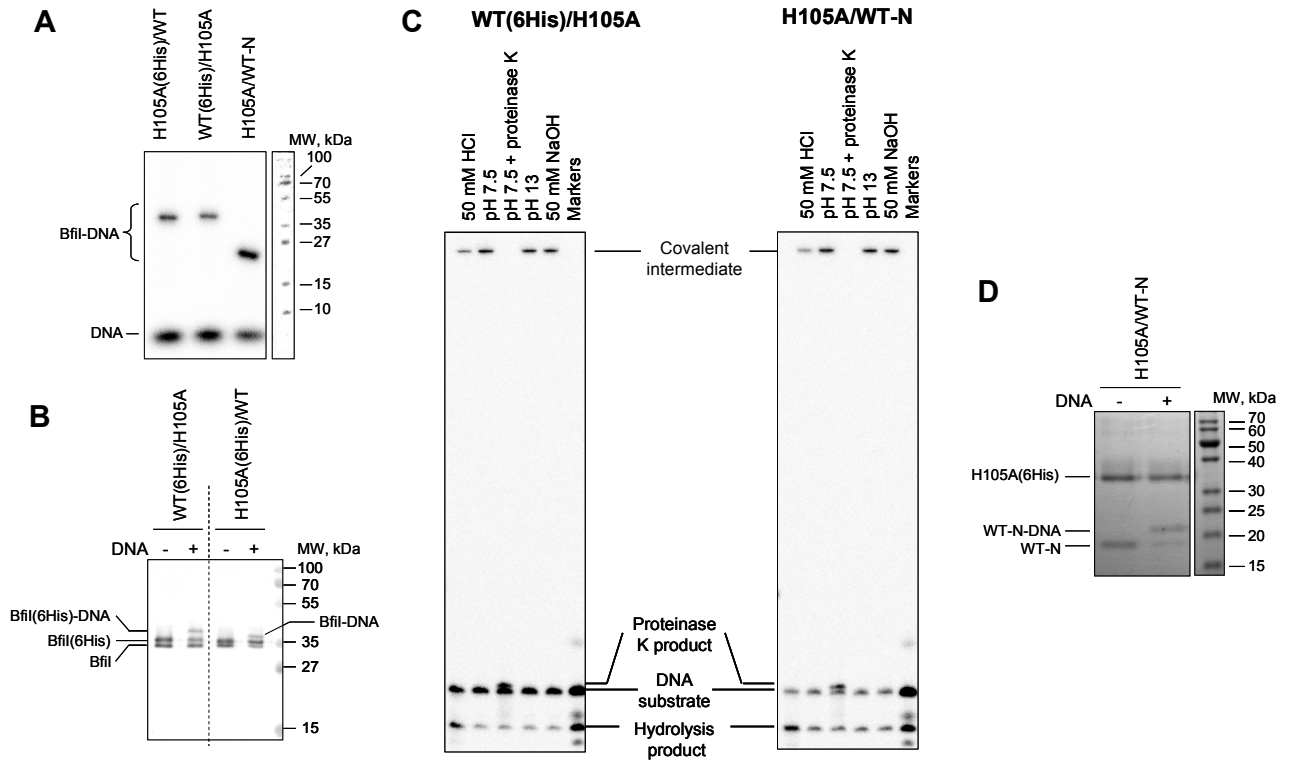
where $S(t)$, $Ci(t)$, $P(t)$ and both k_1 and k_2 are all defined as above: A denotes the initial fraction (%) of the DNA substrate carrying the heterodimer in the productive orientation and k_3 the rate constant for the dissociation of the heterodimer in the non-productive orientation and its re-binding in the productive orientation.

Supplementary Figure S1:



Supplementary Figure S1. Purification of BfiI heterodimeric variants.
(A) The WT(6His)/H105A heterodimer. A mixture of two homodimers of BfiI (WT enzyme with the 6×His tag and H105A mutant without the tag) was denatured in 6 M GdmCl and refolded. The resultant mixture of homo- and heterodimers was separated by Ni^{2+}-chelating chromatography. The peaks corresponding to the WT(6His)/H105A heterodimer (with a single His tag) and the homodimer of WT(6His) (with two tags) are indicated. The H105A homodimer lacking His tags does not bind to the Ni^{2+}-NTA resin. The continuous increase of absorption at 280 nm is due to the increase in imidazole concentration. Eluted fractions were analyzed by SDS-PAGE (bottom of the image). The lane numbers correspond to the fraction numbers in the chromatogram. Lane "M" contains a mixture of BfiI proteins with and without the His tag.
(B) The H105A/WT-N heterodimer. The H105A homodimeric mutant of BfiI (with a 6×His tag) and the N-terminal domain of WT enzyme (without a tag) were denatured in GdmCl. After renaturation, the mixture of homo- and heterodimers was separated as in (A) and analysed by SDS-PAGE. The bands corresponding to the WT N-terminal domain and the H105A subunit with the His tag are indicated. The fractions between the dashed lines (B4-B9 in (A), B2-B5 in (B)) were pooled, concentrated and dialysed against Storage Buffer.

Supplementary Figure S2:



Supplementary Figure S2. Characterisation of the covalent BfiI-DNA intermediate.

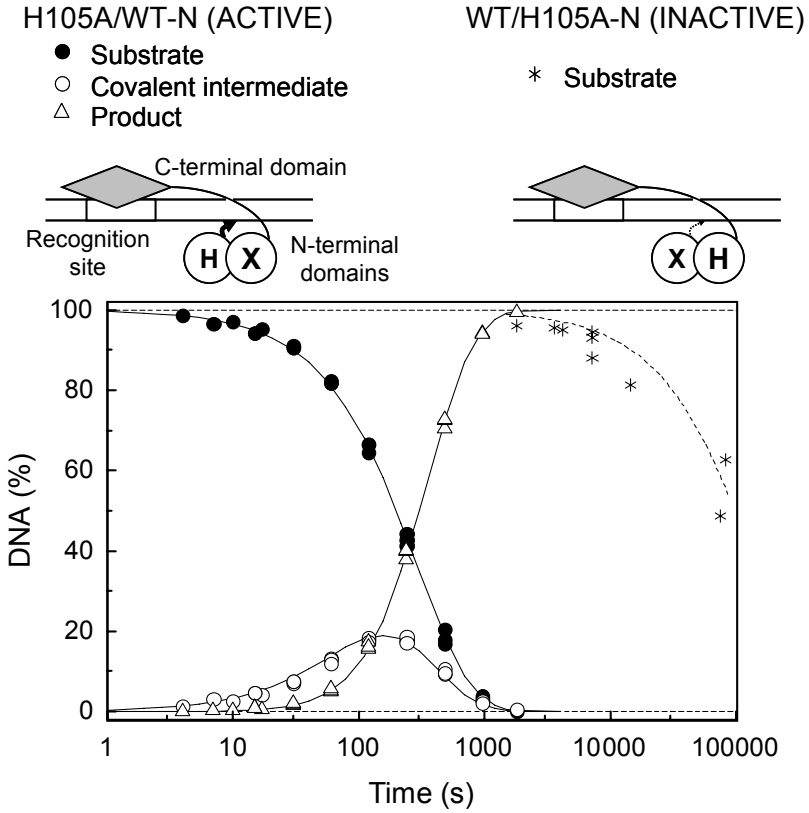
(A) Samples from the reactions of the BfiI heterodimers – H105A(6His)/WT, WT(6His)/H105A and H105A/WT-N – on radiolabelled 14/15s were subjected to SDS-PAGE and the gel analysed by phosphorimager. The labelled bands corresponding to the free DNA and protein-DNA intermediates are indicated. The WT(6His)/H105A and the H105A(6His)/WT heterodimers form protein-DNA adducts whose electrophoretic mobilities correspond to the full length BfiI subunit, with and without the His tag (40 and 42.5 kDa respectively), attached to the labelled DNA (4.2 kDa). H105A/WT-N forms a protein-DNA adduct whose mobility corresponds to the N-terminal domain of BfiI (26 kDa) attached to the 4.2 kDa DNA.

(B) Western-blot analysis of covalent protein-DNA intermediates formed by BfiI heterodimers. The 14/15s substrate (2.5 μM) was incubated with either the WT(6His)/H105A or the H105A(6His)/WT heterodimer (3 μM) for 20 s at 25°C in a total volume of 10 μl. The reactions were stopped with 13 μl of 1.0 M NaOH, neutralized with 4 μl of Neutralisation Solution (1 M H_3PO_4, 9% SDS), mixed with 5 μl of SDS-PAGE loading dye solution and heated for 3 min at 90°C. Samples (10-fold diluted) were analysed by 12% SDS-PAGE. After transfer to polyvinylidene fluoride membranes, proteins were detected using mouse polyclonal anti-BfiI antibodies (courtesy of dr. A. Žvirblienė, Institute of Biotechnology, Vilnius) and an alkaline phosphatase conjugate with BCIP/NBT substrates. In the absence of DNA, two bands were observed, corresponding the BfiI subunits with and without the 6×His tag (low and high mobilities respectively). Incubation of the heterodimers with the 14/15s substrate resulted in additional bands with reduced mobilities, presumably protein-DNA adducts. Formation of the presumed adducts concomitantly diminished the amounts of unreacted WT BfiI subunits (the low-mobility subunit from WT(6His)/H105A and the high-mobility subunit from H105A(6His)/WT), but did not affect the amounts of mutant H105A subunits (the high-mobility subunit from WT(6His)/H105A and the low-mobility subunit from H105A(His)/WT).

(C) Effect of pH and proteinase K on the covalent intermediate. Samples of the WT(6His)/H105A and the H105A/WT-N heterodimers of BfiI were incubated with radiolabelled 14/15s DNA. The reactions were stopped with 1 M NaOH. The DNA was precipitated with glycogen and ethanol and dissolved in one of the following solutions: 50 mM HCl; 50 mM NaOH; 50 mM sodium phosphate, pH 13; 50 mM potassium phosphate, pH 7.5, and 300 mM KCl (with or without proteinase K, 1 U/10 μl). After 20 min at 25°C, the samples were brought to pH 13 with 0.5 M sodium phosphate buffer. After heating at 90°C, the samples were analysed by 20% denaturing PAGE in the presence of 8 M urea. Positions of the covalent intermediate, free DNA and proteinase K product are indicated. The "Markers" lane contained the uncleaved substrate and a 5′-phosphorylated synthetic oligonucleotide corresponding to the hydrolysis product.

(D) Covalent intermediate with the H105A/WT-N heterodimer. Western blot experiments could not be performed with the truncated heterodimer H105A/WT-N, as the available anti-BfiI antibodies do not react with the N-terminal domain of BfiI, thereby preventing detection of the WT-N subunit either with or without attached DNA. Nonetheless, the WT-N subunit was detected during H105A/WT-N reactions, both as a free subunit and as a component of the reduced-mobility band formed upon DNA cleavage, by subjecting undiluted samples from H105A/WT-N reactions (performed as in panel B) to SDS-PAGE and staining the gel with "Page-Blue" dye (Fermentas). Positions of the free His-tagged H105 and the WT N-terminal domain are indicated. An additional band, with reduced mobility relative to the WT-N domain, is assigned to the covalent adduct of the WT-N domain with the DNA. Note that formation of the presumed covalent intermediate concurrently diminishes the amount of free WT N-terminal domain, but does not affect the amount of the full-length H105A subunit.

Supplementary Figure S3:



Supplementary Figure S3. Cleavage of the bottom DNA strand of the full length substrate 25/25s by BfiI heterodimers WT/H105A-N and H105A/WT-N. Single turnover reactions were performed as described in Materials and Methods. The heterodimer H105A/WT-N converts the intact substrate (filled circles) to the final product (open triangles) via the covalent enzyme-DNA intermediate (open circles). Solid lines are the best fit of Equation (1) to the experimental data, which yielded $k_1 = 0.0035 \pm 0.0001$ s^{-1} and $k_2 = 0.011 \pm 0.001$ s^{-1}. The preparation of WT/H105A-N displays only residual level of DNA cleavage (asterisks). Fitting a single exponential (dashed line) yielded a cleavage rate of 7×10^{-6} s^{-1}.