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Changing the DNA Recognition Specificity of the EcoDam DNA-(Adenine-N6)-Methyltransferase by Directed Evolution

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EcoDam is an adenine-N6 DNA methyltransferase that methylates the GATC sites in the *Escherichia coli* genome. We have changed the target specificity of EcoDam from GATC to GATT by directed evolution, combining different random mutagenesis methods with restriction protection at GATT sites for selection and screening. By co-evolution of an enzyme library and a substrate library, we identified GATT as the best non-GATC site and discover a double mutation, R124S/P134S, as the first step to increase enzyme activity at GATT sites. After four generations of mutagenesis and selection, we obtained enzyme variants with new specificity for GATT. While the wild-type EcoDam shows no detectable activity at GATT sites in *E. coli* cells, some variants prefer methylation at GATT over GATC sites by about 10-fold in cells. *In vitro* DNA methylation kinetics carried out under single-turnover conditions using a hemimethylated GATC and a GATT oligonucleotide substrate confirmed that the evolved proteins prefer methylation of GATT sites to a similar degree. They show up to 1600-fold change in specificity *in vitro* and methylate the new GATT target site with 20% of the rate of GATC methylation by the wild-type enzyme, indicating good activity. We conclude that the new methyltransferases are fully functional *in vivo* and *in vitro* but show a new target-site specificity.

Keywords: enzyme design; directed evolution; DNA methylation; DNA recognition

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Introduction

Synthetic biology aims to design new organisms to produce desired compounds including fuel, food, or bioactive molecules.[1–3] This aim requires the redesign of genetic elements, metabolic pathways, and individual enzymes with respect to their substrate specificity. When combined with rational and computational design, directed evolution is a very promising approach for enzyme design.[4,5] To develop this method, we applied directed evolution to modify the DNA recognition specificity of the EcoDam DNA methyltransferase, which specifically recognizes GATC sequences and methylates the adenine residue within.[6,7] DNA methylation has a number of important epigenetic roles in bacteria including the control of gene expression, coordination of DNA replication and the cell cycle, and post-replicative mismatch repair.[8–11] In addition, it is involved in restriction–modification systems to protect bacteria from foreign DNA, and DNA methyltransferases are pathogenicity factors in several pathogens.[9,12,13] DNA MTases are particularly prone to directed evolution because they modify DNA at specific sites. Thus, the blueprint of the activity and specificity of an enzyme variant can be detected on the DNA coding for that particular protein, giving a unique coupling of genotype (DNA sequence) and phenotype (enzymatic properties) on individual DNA molecules.[14] Given this connection, DNA MTases compete with ribozymes[15,16] for being the enzyme model system best suited for *in vitro* evolution. The special properties of DNA MTases so far have been exploited in projects aiming to redirect the specificity of DNA MTases[17–19] and to broaden our knowledge on natural evolution pathways.[20,21]

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Abbreviation used: epPCR, error-prone PCR.

Results and Discussion

The principle of the selection of EcoDam variants for new specificities is illustrated in Fig. 1a. After random mutagenesis, the pool of MTase variants is transformed into the HMS174(DE3) *Escherichia coli* cells for the expression of the enzyme. In the cell, each enzyme variant modifies its own expression plasmid. The modified plasmids are isolated and digested with an appropriate restriction enzyme that is inhibited by adenine-N6 methylation. Uncleaved (i.e., methylated) plasmids are enriched by retransformation (Fig. 1b). Practical experiences showed us that it was necessary to treat the cleaved mixture with λ-exonuclease prior to the retransformation. This enzyme degrades the restriction enzyme cleavage products and, thereby, prevents their re-ligation in the cell after transformation. Since we always observed false positives in the form of plasmids that lost the BspEI restriction site or gained a GATC site overlapping with the BspEI site, we performed only one round of selection after mutagenesis and screened several hundred colonies for most highly protected candidates, which were then used as starting material in the next round of mutagenesis. For screening, the EcoDam variants were expressed in *E. coli* cells, the plasmids were isolated, and the methylation was analyzed by cleavage with BspEI (see below). For changing the specificity of EcoDam, we employed a combination of site-saturation mutagenesis using randomized oligonucleotides for site-directed mutagenesis,[22] error-prone PCR (epPCR),[23] and DNA shuffling.[24] In addition, we developed a new system for mutual co-evolution of enzyme variants and new target sites (Fig. 1c and d). We used a BspEI site (TCCGGA) followed by two randomized bases cloned into the

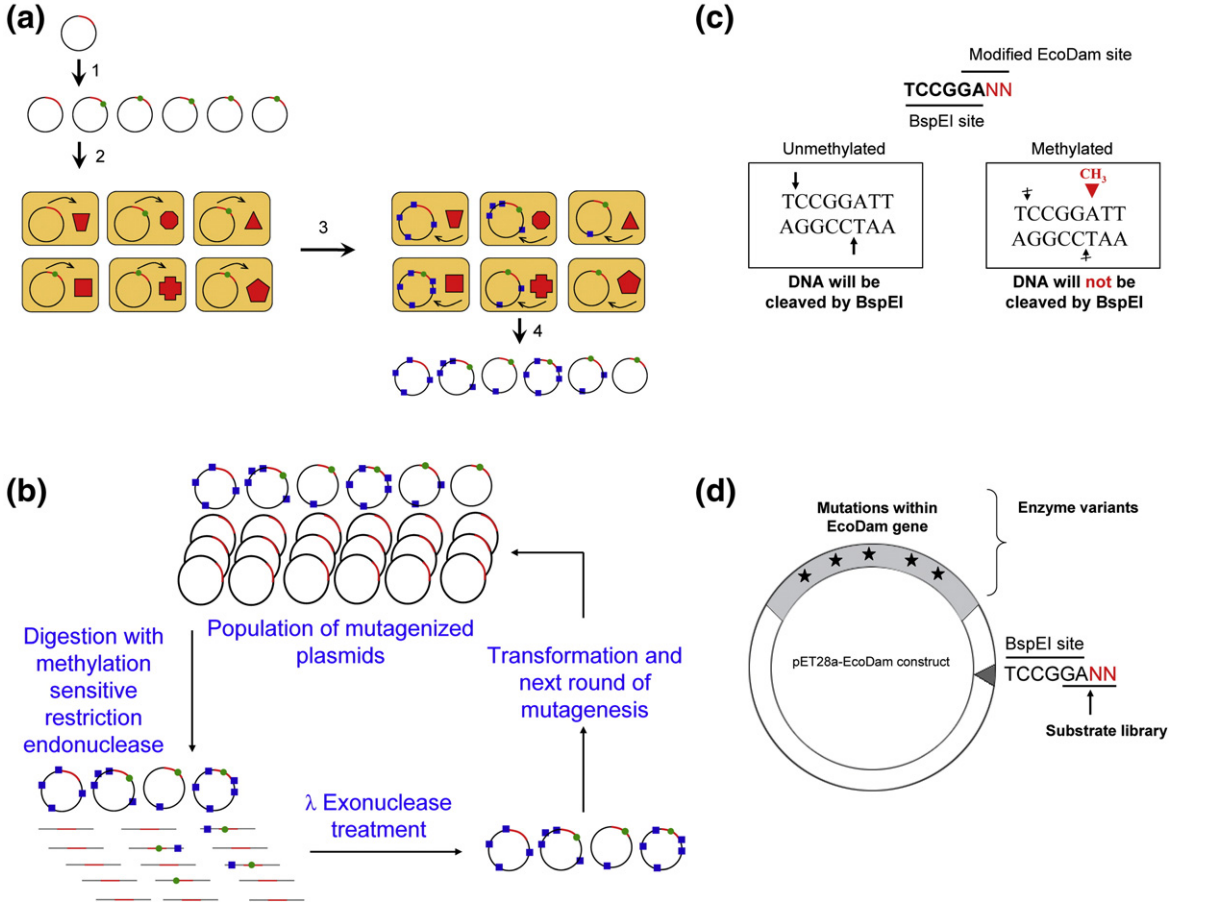

Fig. 1. Principles of *in vitro* evolution experiments with DNA MTases. (a) Coupling of genotype and phenotype with DNA methyltransferases. Step 1: a plasmid containing the gene for a DNA MTase (red color) is subjected to random mutagenesis (mutations indicated by green dots). Step 2: the library of MTase expression plasmids is transformed into bacteria, and the MTase variants get expressed (red symbols). Step 3: the MTases methylate the DNA in the cell (blue squares). Step 4: plasmids can be prepared, the methylation pattern of which reflects the activity and specificity of the MTase encoded by the corresponding plasmid. (b) The plasmid library can be screened using restriction digestion and retransformation. Finally, the mutant genes encoding variants with interesting properties can be retrieved. (c) Example of screening for methylation at modified GATC sites by using overlapping BspEI and GATT sites. Methylation of the terminal A residue in the upper strand of the BspEI site will inhibit DNA cleavage by this enzyme. (d) Scheme of the mutual co-evolution of enzyme activity and target site by cloning a library of enzyme variants and modified target site in the same plasmid and selection for DNA methylation at the modified target site.

EcoDam expression vector. Thereby, the BspEI site (the cleavage of which is inhibited by the methylation of the adenine residue) overlaps with modified EcoDam sites (GANN instead of GATC). All substrate libraries were prepared such that the original GATC site was excluded. By combination of different enzyme libraries with the substrate library, we were able to identify pairs of modified enzymes and matching new target sites in one selection step.

First-generation combined enzyme–substrate library prepared by site-saturation mutagenesis

Structural and biochemical data showed that most of the specific interactions of EcoDam with DNA are formed by residues in a short β-hairpin structure with R124 interacting with the fourth base pair, L122, and P134 interacting with the third one.[25,26] The first guanine is recognized by K9. Based on the crystallographic data, we started the design of EcoDam variants towards new specificity by site-saturation mutagenesis at the two residues (R124 and P134) that are directly involved in DNA recognition of the TC part of the GATC site (RP library) combined with a GANN substrate library (Fig. 2). Successful library generation was confirmed by sequencing of some clones. Already after one selection cycle, several clones that provided partial protection against BspEI cleavage were obtained (Fig. 3). We screened 200 clones for BspEI protection and found 18 of them protected to at least 40%. Sequencing revealed that, in 9 of them, a GATC site overlapping with the BspEI site appeared and 2 of them had mutations within the BspEI site. These alterations were not present in the randomized oligonucleotide mixture that was used to prepare the library; thus, they occurred spontaneously in the *E. coli* cells. Our ability to select spontaneous mutations that are known to occur at very low frequency underscores the very strong selection pressure established in the selection procedure. Seven of the remaining clones contained a GATT site (Table 1). Among the clones methylating GATT sites, we observed a strong enrichment for the R124S and P134S exchanges. The R124S exchange disrupts the contact to the cytosine, explaining the change in specificity at the fourth base pair. The P134S mutation had been identified 20 years back in a natural isolate of the phage T4 Dam enzyme and shown to increase its activity,[27] which might explain why it was enriched here.

Two clones (R124R/P134A and R124S/P134A) were found to recognize other altered sites (GAAC and GATG, respectively). The R124R/P134A clone keeps the R124 contact to the C4 but shows an altered target site at position 3. The R124S/P134A clone was found with both GATT and GATG targets, suggesting that it might be promiscuous with respect to the recognition of the fourth base of the target sequence, which would make it an interesting candidate for further evolutionary optimization.[20]

First-generation enzyme–GATT library prepared by site-saturation mutagenesis

We speculated that a smaller library combining the site saturation at R124 and P134 (RP library) with a fixed GATT target site might allow for the identification of clones that showed a higher level of protection and prepared such library (Fig. 2). Again, after one round of selection, protected clones were collected and sequenced, but we did not observe a better

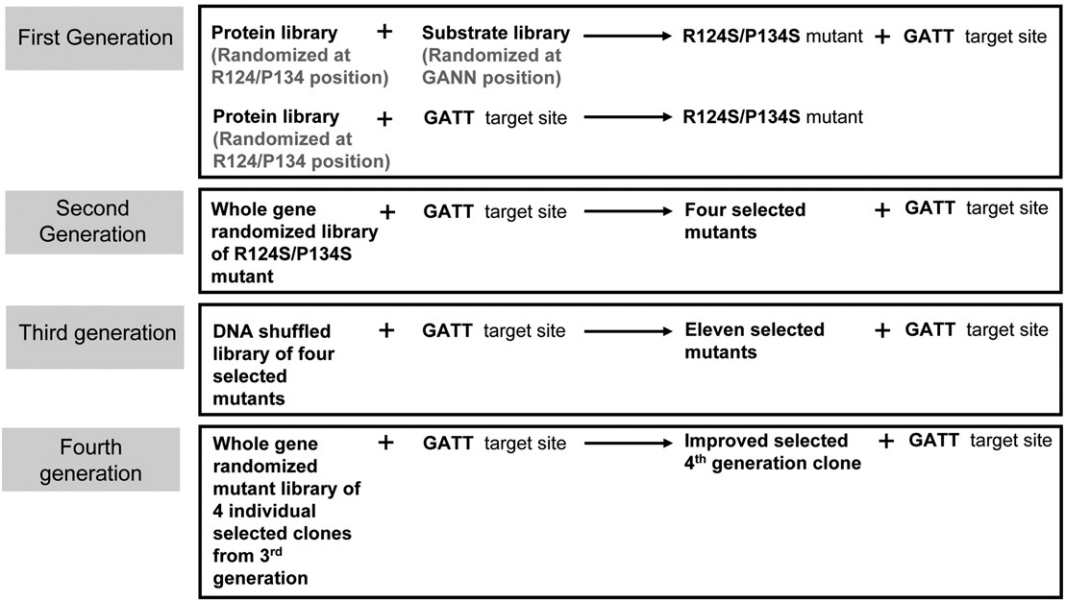

Fig. 2. Experimental setup of this study. We conducted four successive rounds of mutagenesis and selection. In the first round, site-saturation mutagenesis was performed at R124 and P134 and the enzyme variant library was combined with a substrate library, which led to the identification of GATT as the best non-GATC target. In the second round, whole-gene randomization was carried out, followed by DNA shuffling in the third and another step of whole-gene randomization in the fourth round.

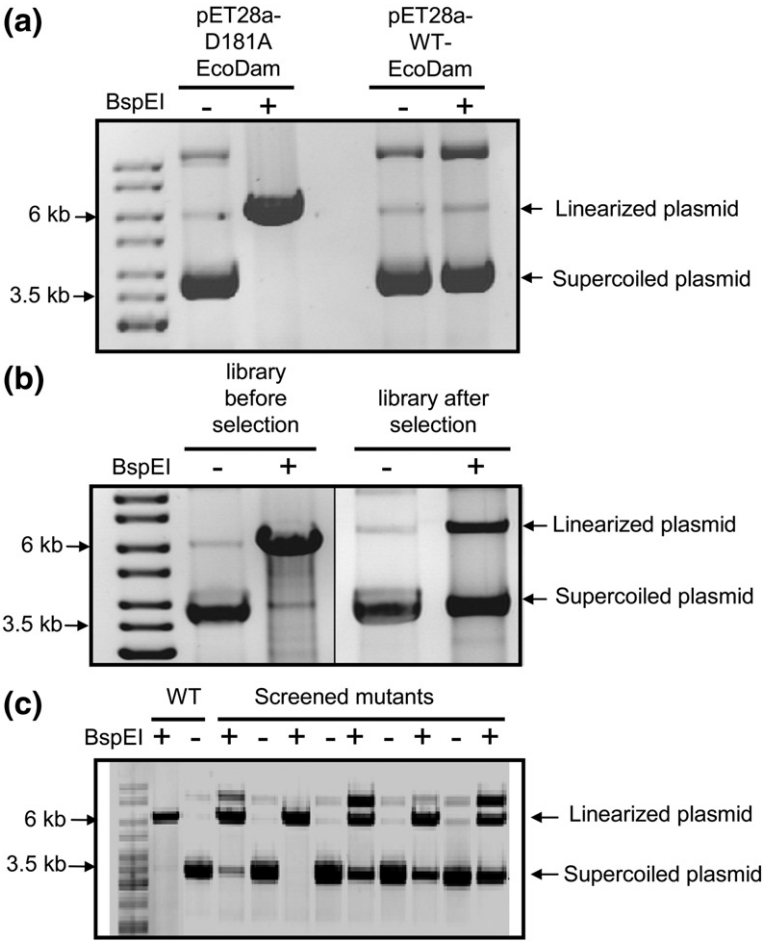

Fig. 3. Screening of the first-generation libraries. (a) Example of methylation sensitivity of BspEI restriction cleavage used for selection. Wild-type EcoDam and an inactive D181A variant were expressed in dam-negative *E. coli* cells; the plasmids containing a single overlapping BspEI/GATC site were isolated and cleaved by BspEI (for details, cf. Fig. 1c). (b) An example of the improvement of restriction protection at TCCGGANN sites after one round of selection of the RP-GANN library. (c) Example of the screening of clones obtained after the first round of selection. In this example, clone 1 shows a low level and clones 3–5 show a high level of protection against BspEI cleavage.

protection. Sequencing of several clones confirmed the enrichment of R124S and P134S exchanges as observed before. After a second and third selection cycle of the RP-GATT library, the level of protection did not increase (data not shown). Given the small size of the initial library, we concluded that selection is saturated already after the first cycle and better enzymes could be only obtained after randomization of additional parts of the enzyme.

Table 1. Summary of the results obtained from sequencing of independent EcoDam variants that show partial methylation at GATT sites

	Exchange at position R124	Exchange at position P134	Source
Clone 1	A	A	RP-GANN library
Clone 2	Y	S	RP-GANN library
Clone 3	Y	S	RP-GANN library
Clone 4	S	G	RP-GANN library
Clone 5	S	A	RP-GANN library
Clone 6	S	S	RP-GANN library
Clone 7	S	A	RP-GANN library
Clone 2-1	A	G	RP-GATT library
Clone 2-2	S	S	RP-GATT library
Clone 2-3	S	A	RP-GATT library
Clone 2-4	S	S	RP-GATT library
Clone 2-5	G	S	RP-GATT library
Clone 2-6	C	S	RP-GATT library
Clone 2-7	S	S	RP-GATT library
Clone 2-8	A	S	RP-GATT library
Clone 2-9	S	S	RP-GATT library
Clone 2-10	G	S	RP-GATT library
Clone 2-11	S	S	RP-GATT library

Clones 1–7 were obtained from the R124/P134-GANN substrate library. Clones 2-1 to 2-11 were obtained from the R124/P134-GATT library.

Second-generation library prepared by epPCR

We prepared a second-generation library by whole-gene randomization of the R124S/P134S variant (Fig. 2). After selection, around 400 clones were screened and 6 clones showed an improved level of methylation at GATT sites as judged from the comparison of the amount of superhelical and open circle *versus* linear DNA after BspEI cleavage (Fig. 4a). Sequencing showed that clones 3 and 4 were identified twice. Overall, the 4 clones contained 18 different mutations, in addition to the original R124S/P134S mutations (Table 2).

Third-generation library prepared by DNA shuffling and *in vivo* enzyme specificity analysis

We wanted to investigate the combinatorial effect of the mutations identified so far and prepared a third-generation library by DNA shuffling.[24] After DNA shuffling and one round of selection, 200 clones were screened for improved DNA methylation at GATT sites by the BspEI restriction protection

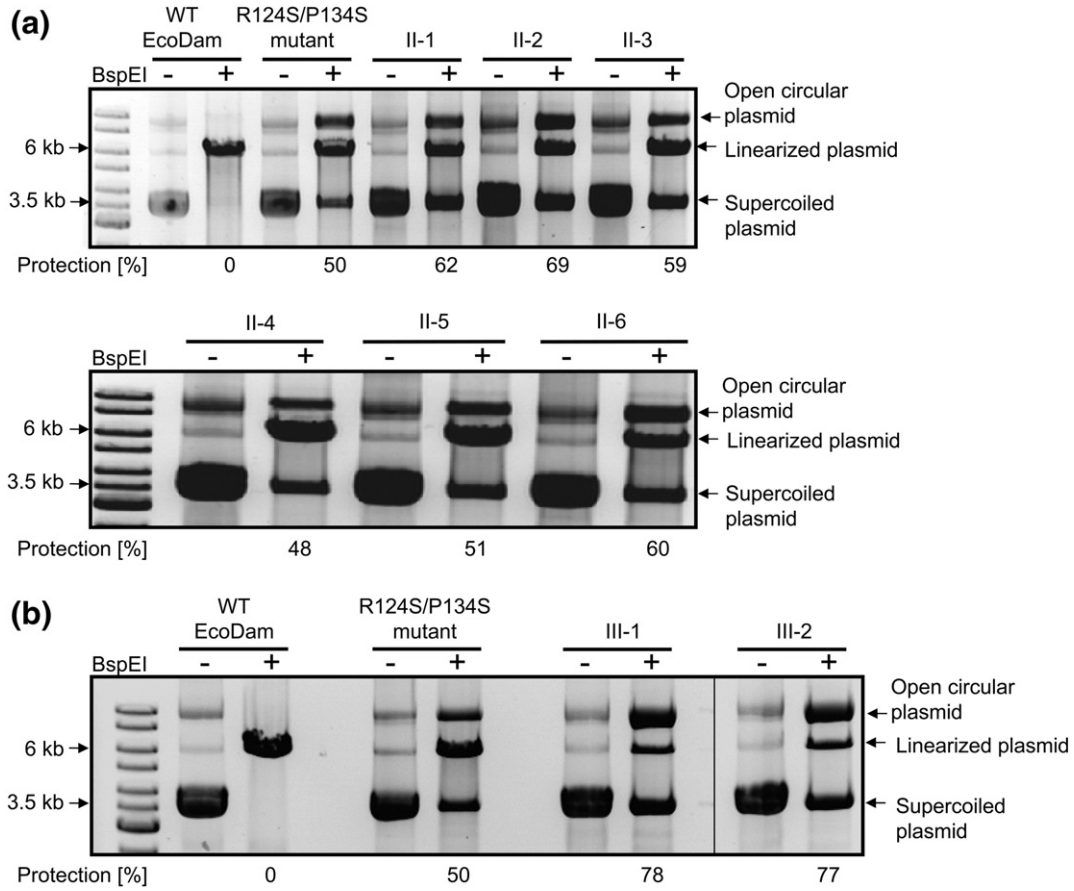

Fig. 4. Examples of the screening of clones from second-generation (a) and third-generation (b) library with BspEI after the first round of selection. The level of protection was estimated on the basis of the scanned intensities of the supercoiled and open circular plasmid fractions, when compared with the linearized band.

assay. Two clones were found to show increased protection against BspEI cleavage (clones III-1 and III-2 in Fig. 4b and Table 3); several others showed similar protection as observed in generation II (Table 3). Sequencing revealed the presence of 14 mutations, 10 of them shuffled from the previous rounds and 4 new ones (Table 3).

To investigate the methylation preferences of the various EcoDam variants in *E. coli* cells, we expressed the mutant enzymes in dam-negative cells (JM110). Under these conditions, a second BspEI site on the plasmid that overlaps with a GATC site became available for cleavage. In dam-positive cells, this site is always protected by the endogenous EcoDam. In the dam-negative host, the EcoDam variants methylate the original GATC site or the new GATT site depending on their intrinsic preferences. The specificity of methylation was analyzed by a double digestion, indicating that the clones of the third generation showed an about 10-fold preference for DNA methylation at GATT sites *in vivo*, which is a remarkable result considering that wild-type EcoDam does not show detectable protection at GATT sites (Fig. 5), indicating an at least 50-fold preference for GATC methylation *in vivo*.

Table 2. Sequence analysis of six clones from the second-generation library (all clones contained the R124S and P134S exchanges)

II-1	II-2	II-3	II-4
M1T	N4S	Q75R	Y138H
K67R	S40P	E84V	
K139E	P104L	K241E	
F159L	L127Q		
G256S	L203H		
	M231L		
	K246N		
	S250N		
	K260Q		

Clones 3 and 4 were identified twice.

Fourth-generation library prepared by epPCR

So far, we evolved enzyme variants towards methylation at GATT sites. Our next step was to evolve the protein against GATC site methylation, while keeping the activity at GATT sites. To this end, a mixture of the genes of the four isolated third-generation mutants was used as template to generate a fourth-generation mutant library by whole-gene randomization. Proteins were expressed in dam-negative host and the plasmid pool was

Table 3. Sequence analysis of 11 individual clones after DNA shuffling (all clones contained the R124S and P134S exchanges)

III-1	III-2	III-3	III-4	III-5	III-6	III-7	III-8	III-9	III-10	III-11
	Q75R	Q75R E84V	E84V			E84V				Q75R
			P104L						C87R P104L	
K139E F159L	F159L	K139E S200G	K139E F159L	K139E F159L	K139E F159L	K139E F159L	K139E	K139E F159L	K139E	
						M231L		M231L		M231L
K241E	K241E G256S		K241E	K241E			K241E S274H A276R	K241E		G256S

isolated. After double digestion with BglII and BspEI, the 4.2-kb DNA fragment (originating from plasmids methylated at the GATT site but not methylated at the GATC site) was excised from the gel, the EcoDam gene was amplified by PCR, and the DNA was re-cloned. Two hundred clones were screened for improved specificity of methylation at GATT sites. One variant showed an increased specificity as evidenced by the loss of the 5- and 1.2-kb bands, indicative of methylation at the GATC sites, and the further improvement of the ratio of the 4.2- and 5-kb bands (Fig. 5c).

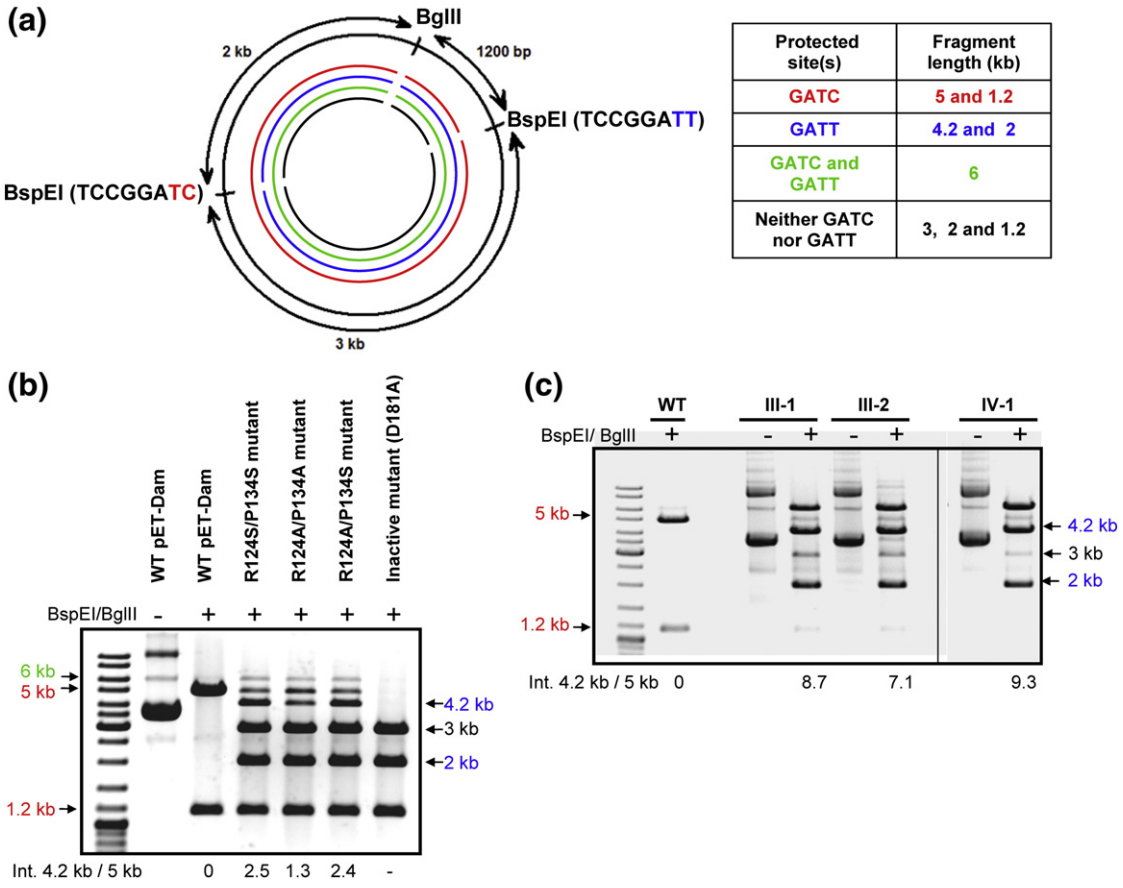

Fig. 5. *In vivo* specificity of EcoDam and its variants. The mutant dam expression plasmid containing two BspEI sites (one overlapping with GATC and the other with a GATT sequence) was grown in dam-negative *E. coli* cells. After expression of the EcoDam variants, plasmids were isolated and the methylation state at GATC and GATT sites was determined by digestion with BspEI. (a) Schematic drawing of the plasmid and the expected fragments after double digestion with BglII and BspEI. (b) Examples from the first-generation variants, which clearly show methylation at the GATT site. As an estimate of the specificity of the enzyme variants, the ratio of the intensities of the 4.2- and 5-kb bands (which are characteristic of GATT and GATC methylation, respectively) was determined. (c) Examples of the III-1 and III-2 clones from the shuffled library (third generation) and the IV-1 clone from the fourth generation, which clearly showed preferential methylation at the GATT site. Relative intensities of the 4.2- and 5-kb bands were indicated as described in (b).

In vitro investigation of enzyme specificity

Finally, we purified the initial R124S/P134S variant and the best variants obtained in the third and fourth generation and determined their DNA methylation specificity *in vitro* by DNA methylation kinetics using two oligonucleotide substrates containing one GATC or GATT site in an otherwise identical sequence context (Fig. 6). The GATC substrate was used in hemimethylated form to allow enzymatic methylation in only one strand and make the results comparable with the non-palindromic GATT substrate that has only one target adenine (in the upper DNA strand). Because of slow rates of GATT methylation by wild-type EcoDam and low activities of some of the selected variants, kinetics were performed under single-turnover conditions. The results indicate a complete switch in target-site specificity when changing from the wild-type enzyme (which shows a more than 60-fold preference for methylation at GATC sites) to the variants, such as III-1, which shows a more than 20-fold preference for methylation at GATT, overall corresponding to a 1600-fold change in specificity. The activity of the variants is not largely affected, and the enzyme variants methylate the new target-site GATT with rates comparable to the rate of wild-type EcoDam at GATC sites. For example, the activity of mutant IV-1 at GATT sites is 20% of wild-type activity at GATC sites, indicating that mutant IV-1 is a good methyltransferase. At the same time, the mutants show much lower activity at the original GATC site than wild type has at GATT sites; variant III-1 is virtually inactive at GATC sites.

Molecular role of the selected mutations

All mutations identified in this work after three cycles of random mutagenesis and selection for methylation of GATT sites (Table 3) are located on the surface of the enzyme, which is not unexpected, since exchange of surface residues has a lower risk to disrupt the protein structure. Two mutated residues (K139 and G256) are located close to the DNA (Fig. 7a), suggesting that they may affect DNA interaction. Interestingly, these residues approach the DNA sequence in the 5′ direction of the GATC site and not close to the T4 of the GATT site, the recognition of which was altered here, suggesting that they are not directly involved in recognition of the T4. Other exchanges may affect protein structure and stability. For example, it has been reported that amino acid exchanges which introduce the consensus residue observed this position in related proteins often stabilize proteins.[28] We identify three such examples in our data set (Q75R, C87R, and S200G).

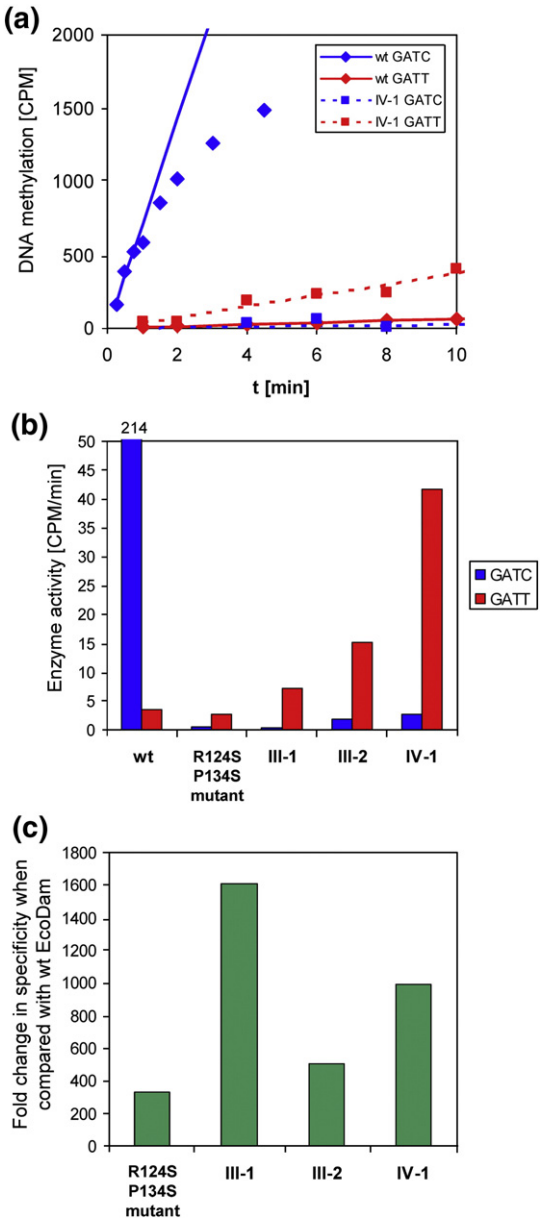

Fig. 6. Kinetic properties of wild-type EcoDam and some of its mutants. (a) Examples of DNA methylation kinetics with the purified wild-type EcoDam and mutant IV-1 tested using synthetic oligonucleotides containing a GATC or GATT site. All kinetics were carried out using 3 μM enzyme, except GATC methylation by wild-type EcoDam, which was done using 0.6 μM enzyme. (b) Comparison of the averaged initial slopes of reaction progress curves obtained from at least three independent methylation kinetics experiments. Deviations in slopes between the experiments were below ±20%. For this comparison, the slope of GATC methylation by wild-type EcoDam was multiplied by a factor of 5, to account for the lower enzyme concentration used in the assay. (c) The fold change in specificity of the mutants in respect to wild type was calculated using the normalized methylation rates shown in (b).

Conclusions

By combining directed evolution and rational design, it was possible to redesign the target sequence of an enzyme that specifically interacts with DNA and generate a new enzyme with high activity and specificity not present in nature. Epigenetic systems control important processes in bacteria including regulation of gene expression, phase variation, and

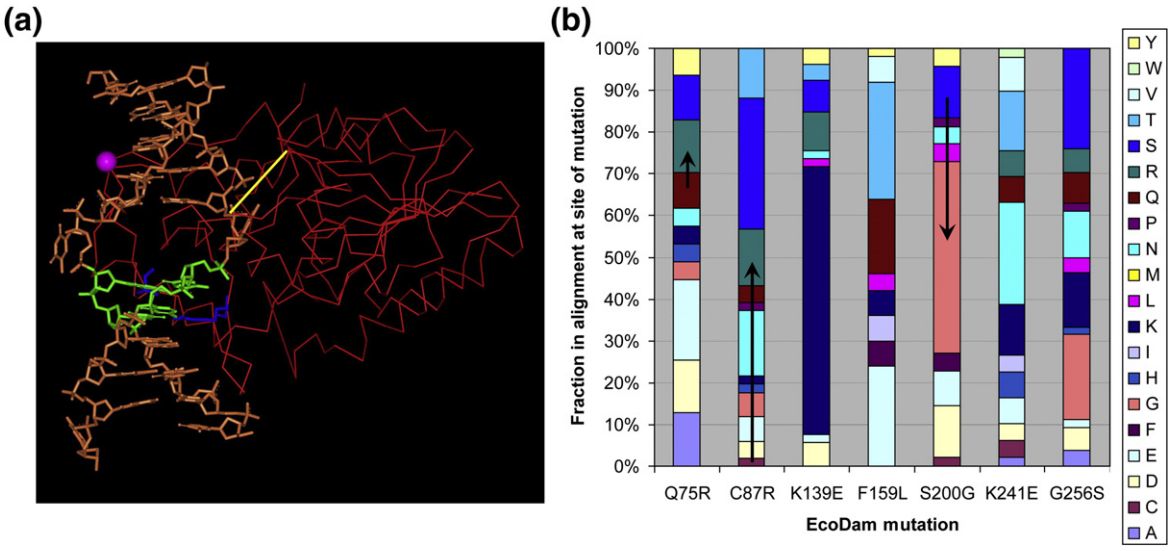

Fig. 7. Molecular interpretation of the identified mutations. (a) Structure of EcoDam in complex with specific DNA. The DNA is shown in orange with the third and fourth base pair of the GATC sequence highlighted in green. The enzyme is shown as C^{α} trace in red. R124 and P134 are shown in blue; the C^{α} position of K139 is highlighted by a pink sphere, and the position of the disordered loop containing G256 is shown in yellow. (b) Conservation of the mutated residues in an alignment of 54 EcoDam homologues. In three cases, we observed mutations that change the residue originally present in EcoDam to one more often observed in the multiple sequence alignment (Q75R, C87R, and S200G, indicated by arrows). The alignment quality was not good enough to draw conclusions on the conservation of EcoDam residues E84, P104, M231, S274, and A276.

pathogenicity.[9,11] The generation of 6-methyladenine changes the structural properties of the DNA and influences DNA–protein interactions,[29] providing a general biological readout of the modification in the context of a new target sequence. DNA MTases with novel specificities might allow to construct new regulatory epigenetic systems in bacteria or constitute an artificial restriction–modification system.[19]

Materials and Methods

Site-directed and site-saturation mutagenesis

The RP protein library and the GANN substrate library were generated using randomized oligonucleotides by site-directed mutagenesis and PCR-megaprimer methods as described previously.[22] Primers were purchased in purified form from MWG (Ebersbach, München). Mutagenesis was confirmed by restriction marker site analysis and DNA sequencing. Primers for the protein library contained NNB sequences at the sites of randomization (with N = A, G, C, or T and B = G, C, or T), which encode for all amino acids but exclude two of the three possible stop codons: CGGTTACAACGGCCTGTGTNNBTACAATCTCCGCGGTGAGTTTAACGTGNNBTTCGGCCGCTA.

The sequences of primers used to generate the GANN substrate library was as follows:
primer 1, AACTATATCCGGANDGGCGAATGGGACGCGCCCTG (with D = A, G, or T)
primer 2, AACTATATCCGGAVCGGCGAATGGGACGCGCCCTG (with V = A, G, or C).

Primers 1 and 2 were used in 4:1 molar ratio to obtain an equal mixture of all GANN sites but exclude the GATC sequence.

Whole-gene randomization by epPCR

Whole-gene randomization was performed using epPCR by employing biased nucleotide composition, high Mg^{2+}, and addition of Mn^{2+}.[23] In order to get a high mutational load, we used 1 pg of template DNA and carried out 35 cycles of epPCR reaction. The epPCR reaction mixture (50 µl) contained 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 7 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.2 mM each of deoxyadenosine triphosphate and deoxyguanosine triphosphate, 1 mM each of deoxycytidine triphosphate and deoxythymidine triphosphate, 25 pmol each of the oligonucleotide primers (forward, CAT CAC AGC AGC GGC CTG GTG CCGC; reverse, TTT GTT AGC AGC CGG ATC CAG TG), 1 pg of template DNA, 1% dimethyl sulfoxide, and 2.5 U of Taq DNA polymerase (NEB). PCR conditions were 1× 94 °C for 4 min; 35× 94 °C for 45 s, 60 °C for 35 s, and 72 °C for 3 min; and, finally, 1× 72 °C for 10 min. The epPCR product was excised from agarose gel and re-amplified by normal semi-nested PCR using 100 pg epPCR product (100 pg in 50 µl) as template, forward primer (TTA ACT TTA AGA AGG AGA TAT ACC) and reverse primer (TTA GAG GCC CCA AGG GGT TAT G) (25 pmol each), deoxyribonucleotide triposhpate (0.2 mM each), 10× ThermoPol buffer (NEB) (5 µl), and Taq DNA polymerase (NEB) (2.5 U). PCR conditions were 1× 94 °C for 4 min; 30× 94 °C for 45 s, 60 °C for 35 s, and 72 °C for 1 min; and, finally, 1× 72 °C for 10 min.

DNA shuffling

The third-generation mutant library was generated by *in vitro* DNA shuffling.[24] The EcoDam gene (1 kb) was PCR amplified from selected second-generation mutants (II-1, II-2, II-3, and II-4) using specific primers. DNA

(4 μg) containing equimolar amounts of all PCR products was digested with 0.15 U of DNaseI (NEB) in 100 μl of 50 mM Tris/HCl, pH 7.4, and 1 mM $MgCl_2$ for 10–20 min at room temperature. Fragments of 10–50 bp were purified from a 2% low melting agarose gel using the QIAEX II Gel Extraction Kit (QIAGEN). The purified fragments were resuspended in PCR mixture (0.2 mM each dNTP, 2.2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris/HCl, pH 9.0, and 0.1% Triton X-100) at a concentration of 10–30 ng/μl. No primers were used at this point. *Taq* DNA polymerase (2.5 U) was added per 100 μl reaction mixture. A PCR program of 94 °C for 1 min, 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s (45 times) and 72°C for 5 min was used to assemble full gene products. Finally, a conventional PCR was set up to amplify the EcoDam gene using a semi-nested primer set.

Protein expression and purification

EcoDam gene was cloned as His_6-fusion protein in pET28a (Clontech) expression vector with T7 inducible promoter. Wild-type EcoDam and all its variants were expressed in *E. coli* HMS174(DE3) (*F-recA 1 hsdR*($r^-_{K12}m^+_{K12}$) *Rif*^{r}) (Novagen) and purified using Ni-NTA agarose (Qiagen) as described previously.[30]

Restriction protection analyses

For restriction protection analysis, each transformed mutant was grown overnight in LB media in the presence of kanamycin (25 μg/ml) at 37 °C with shaking at 200 rpm. Overnight culture was diluted 1:100 into fresh LB media containing kanamycin and grown until an OD_{600} (optical density at 600 nm) of 0.7. The culture was induced by addition of 1 mM IPTG and grown for 2 h, and the plasmid was isolated using Macherey–Nagel plasmid purification kit. Plasmids (1 μg) were digested with 10 U BspEI (New England BioLabs) in buffer recommended by the supplier. The reaction products were separated on agarose gels, DNA was stained with ethidium bromide, and images were taken using a gel documentation system. Images were analyzed with AIDA Image Analysis software (Raytest, Straubenhard, Germany) for semiquantitative assessment of the methylation levels.

DNA methylation kinetics

DNA methylation was analyzed under single-turnover conditions in 50 mM Hepes (pH 7.5), 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5 mM DTT, and 0.2 g/l bovine serum albumin containing 0.76 μM [methyl-^{3}H]AdoMet (NEN) at 37 °C as described previously[31] using 0.5 μM oligonucleotide substrate and 3 μM enzyme. Due to fast reaction rates, methylation of GATC sites by wild-type EcoDam was assayed using 0.6 μM enzyme. Initial turnover rates were derived by linear regression of the initial part of the reaction progress curves. The sequences of the 20-mer oligonucleotide substrates used for kinetics are as follows:
GATC-substrate, 5′-tatatccggatcggcgaatg-3′/5′-Bt-cattcgccgMtccggatata-3′
GATT-substrate, 5′-tatatccggattggcgaatg-3′/5′-Bt-cattcgccaatccggatata-3′
(with M = N6-methyladenosin and Bt = biotin).

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