

Functional Compromises among pH Tolerance, Site Specificity, and Sequence Tolerance for a DNA-Hydrolyzing Deoxyribozyme[†]

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ABSTRACT: We recently reported the identification by in vitro selection of 10MD5, a deoxyribozyme that requires both Mn^{2+} and Zn^{2+} to hydrolyze a single-stranded DNA substrate with formation of 5'-phosphate and 3'-hydroxyl termini. DNA cleavage by 10MD5 proceeds with $k_{obs} = 2.7 h^{-1}$ and rate enhancement of 10^{12} over the uncatalyzed P–O hydrolysis reaction. 10MD5 has a very sharp pH optimum near 7.5, with greatly reduced DNA cleavage rate and yield when the pH is changed by only 0.1 unit in either direction. Here we have optimized 10MD5 by reselection (in vitro evolution), leading to variants with broader pH tolerance, which is important for practical DNA cleavage applications. Because of the extensive Watson–Crick complementarity between deoxyribozyme and substrate, the parent 10MD5 is inherently sequence-specific; i.e., it is able to cleave one DNA substrate sequence in preference to other sequences. 10MD5 is also site-specific because only one phosphodiester bond within the DNA substrate is cleaved, although here we show that intentionally creating Watson–Crick mismatches near the cleavage site relaxes the site specificity. Newly evolved 10MD5 variants such as 9NL27 are also sequence-specific. However, the 9NL27 site specificity is relaxed for some substrate sequences even when full Watson–Crick complementarity is maintained, corresponding to a functional compromise between pH tolerance and site specificity. The site specificity of 9NL27 may be restored by expanding its “recognition site” from $ATG^{\wedge}T$ (as for 10MD5) to $ATG^{\wedge}TT$ or larger, i.e., by considering 9NL27 to have reduced substrate sequence tolerance relative to 10MD5. These findings provide fundamental insights into the interplay among key deoxyribozyme characteristics of tolerance and selectivity, with implications for ongoing development of practical DNA-catalyzed DNA hydrolysis.

DNA catalysts (deoxyribozymes, DNAzymes) have been identified by in vitro selection for many reactions, most commonly for oligonucleotide substrates (1–3). The largest rate enhancement currently reported by a deoxyribozyme is by 10MD5, which we recently found to catalyze Mn^{2+}/Zn^{2+} -dependent DNA phosphodiester bond hydrolysis with formation of 5'-phosphate and 3'-hydroxyl groups (Figure 1A) (4). Because uncatalyzed P–O bond hydrolysis has a half-life of 30 million years (5), and 10MD5 functions with k_{obs} of $2.7 h^{-1}$ ($t_{1/2}$ of 15 min), the rate enhancement of 10MD5 is 10^{12} . 10MD5 has several useful characteristics for a practical DNA-cleaving catalyst. In particular, 10MD5 is both sequence-specific (i.e., hydrolyzes one particular DNA sequence rather than other sequences) and site-specific (i.e., cleaves its DNA substrate at a particular phosphodiester bond rather than at nearby linkages). 10MD5 also has reasonably high substrate sequence tolerance, in that outside of a short $ATG^{\wedge}T$ “recognition site”, any substrate nucleotides are tolerated as long as Watson–Crick base-pairing interactions are maintained between the deoxyribozyme and its substrate.

Even with the identification of 10MD5, there is room for significant improvement of DNA-catalyzed DNA hydrolysis. One suboptimal feature of 10MD5 is its relatively sharp pH

dependence. The optimal pH value is 7.5 (as measured for the 1 M HEPES buffer stock solution); the 10MD5 rate and yield both decrease sharply as the pH is changed to either lower or higher values. For example, at pH of either 7.3 or 7.7, the k_{obs} value falls by over an order of magnitude to $< 0.2 h^{-1}$. For practical reasons such as concern over modest variations in buffer pH values, this poor pH tolerance is undesirable.

In the present study, we used reselection (in vitro evolution) with appropriate selection pressures to optimize the 10MD5 cleavage activity with respect to pH tolerance. One new 10MD5 variant, 9NL27, was investigated in detail with regard to pH tolerance, site specificity, sequence tolerance, and sequence specificity. 9NL27 is much more pH tolerant while retaining utility with regard to the other characteristics. However, a trade-off was identified between pH tolerance on the one hand and either site specificity or sequence tolerance on the other hand. These findings offer insight into the operation of a highly efficient deoxyribozyme and calibrate our ongoing efforts to establish deoxyribozymes as practical DNA hydrolysis catalysts.

EXPERIMENTAL PROCEDURES

Buffers and Metal Ions. All buffers for selection and assay procedures were prepared at room temperature. Stated pH values refer to the 1 M HEPES stock solutions, corresponding to the pH values that would be most readily measured by an investigator seeking to use deoxyribozymes for practical DNA cleavage. As described (4), there is an 0.2–0.3 pH unit offset between 1 M

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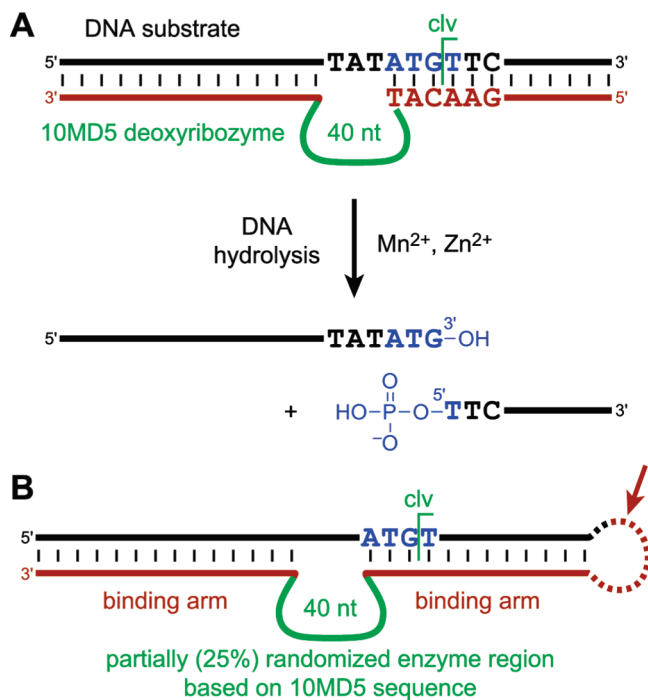


FIGURE 1: Deoxyribozymes for DNA hydrolysis. (A) Our initial report identified the 10MD5 deoxyribozyme (bottom strand), which binds its DNA substrate (top strand) via Watson–Crick base pairs and can cleave essentially any DNA sequence with multiple turnover as long as the ATG[^]T recognition site (blue) is present (4). (B) Reselection strategy, in which the DNA substrate is joined to the partially randomized deoxyribozyme pool by T4 RNA ligase (at site marked by arrow), with PAGE-shift selection for sequences that cleave the substrate and subsequent PCR amplification.

HEPES buffer and the standard incubation conditions of 70 mM HEPES, 20 mM MnCl₂, 1 mM ZnCl₂, and 150 mM NaCl; the diluted metal-containing solution has the lower pH value. All metal ion solutions were prepared as described (4).

Substrates, Reselection Procedure, and Cloning. All DNA substrates and all deoxyribozymes that were prepared by solid-phase synthesis were obtained from Integrated DNA Technologies (Coralville, IA) or prepared on an ABI 394 synthesizer and purified by 20% or 8% denaturing PAGE. The DNA cleavage substrate used during reselections had sequence 5'-AAAGTCTCATGTACTTATATGTTCTAGCGCgga-3', where the final three ribonucleotides (lowercase) enabled ligation to the deoxyribozyme pool by T4 RNA ligase (Figure 1B). The reselection procedure was performed essentially as described previously (6, 7), using a 25% partially randomized 10MD5 pool prepared using phosphoramidite ratios computed as described previously (8). Cloning and initial analyses of individual clones were also performed as described previously (6, 7).

Single-Turnover Cleavage Assays. The single-turnover cleavage assays of individual deoxyribozymes were performed using a DNA substrate with sequence 5'-AAAGTCTCATGTACTTATATGTTCTAGCGCGGA-3' (i.e., all-DNA version of the cleavage substrate used during reselections), with mutations as required for the particular experiment. The deoxyribozymes prepared by solid-phase synthesis had sequence 5'-CCGCGCT-AGAACAT-N₄₀-AGTACATGAGACTT-3', where N₄₀ refers to the initially random 40 nucleotides (i.e., enzyme region) as listed for each individual deoxyribozyme in the artificial phylogeny of Figure 4. The cleavage assays were performed as described previously (4). Briefly, the 5'-³²P-radiolabeled substrate (S)

was the limiting reagent relative to the deoxyribozyme (E). A 10 μL sample containing 0.2 pmol of S and 20 pmol of E was annealed in 5 mM HEPES, pH 7.5 (or other value as appropriate), 15 mM NaCl, and 0.1 mM EDTA by heating at 95 °C for 3 min, cooling on ice for 5 min, and heating at 37 °C for 2 min. The cleavage reaction was initiated by addition of stock solutions to a final volume of 20 μL containing 70 mM HEPES, pH 7.5 (or other value), 20 mM MnCl₂, 1 mM ZnCl₂, and 150 mM NaCl. Final concentrations for all single-turnover experiments were 10 nM S and 1 μM E. The metal Mn²⁺ was added from a 10× stock solution containing 200 mM MnCl₂. The metal Zn²⁺ was added from a 10× stock solution containing 10 mM ZnCl₂, 20 mM HNO₃, and 200 mM HEPES at pH 7.5 (or other value); this stock solution was freshly prepared from a 100× stock of 100 mM ZnCl₂ in 200 mM HNO₃. The metal ion stocks were added last to the final sample, which was divided into 2 μL aliquots that were all incubated at 37 °C. At appropriate times, aliquots were quenched with 5 μL of stop solution [80% formamide, 1× TBE (89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3), 50 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol]. Samples were separated by 20% PAGE and quantified with a PhosphorImager. Values of *k*_{obs} and final yield were obtained by fitting the yield versus time data directly to first-order kinetics, i.e., yield = $Y(1 - e^{-kt})$, where *k* = *k*_{obs} and *Y* = final yield. When *k*_{obs} was sufficiently low such that an exponential fit was not meaningful, the initial points were fit to a straight line, and *k*_{obs} was taken as the slope of the line.

Mass Spectrometry. Samples for mass spectrometry were prepared using 500 pmol of substrate as described (4). All MALDI mass spectra were obtained in the mass spectrometry laboratory of the UIUC School of Chemical Sciences.

RESULTS AND DISCUSSION

Reselections of 10MD5 for Improved pH Tolerance. To foster improved pH tolerance in new variants of 10MD5, reselections were performed with imposition of appropriate selection pressures. The 40-nucleotide enzyme region of 10MD5, located between its two fixed-sequence binding arms, was partially randomized to the extent of 25% (Figure 1B). That is, each of the 40 nucleotides had a 75% chance of retaining the particular A/T/G/C identity present in 10MD5 and a 25% chance of randomly being one of the other three nucleotides.

In the comprehensive reselection strategy, selection rounds were performed as described previously (4) at varying pH values of either 7.2, 7.5, or 7.8 (70 mM HEPES, 20 mM MnCl₂, 1 mM ZnCl₂, and 150 mM NaCl at 37 °C; all pH values represent those measured for the 1 M HEPES buffer stock solutions). The first three rounds of all selection experiments were performed at pH 7.5 with 14 h incubation time; 16% cleavage yield for the pool was observed at round 3. In one reselection thread (Figure 2A), starting at round 4 the pH was then varied, first decreasing to 7.2 and then increasing to 7.8. In the second reselection thread (Figure 2B), the roles of pH 7.2 and 7.8 were swapped; i.e., the pH was first increased to 7.8 and then decreased to 7.2. In both cases, the cleavage activity of the pool initially decreased sharply when the pH was changed from 7.5. Selection rounds were continued, decreasing the incubation time to 2 h and then 10 min while continuing to vary the pH. Individual deoxyribozymes were cloned from round 8 of the reselection thread for which pH changes began with pH 7.2 (33% pool yield in 2 h at pH 7.2) as well as round 9 of the reselection thread that began changes with

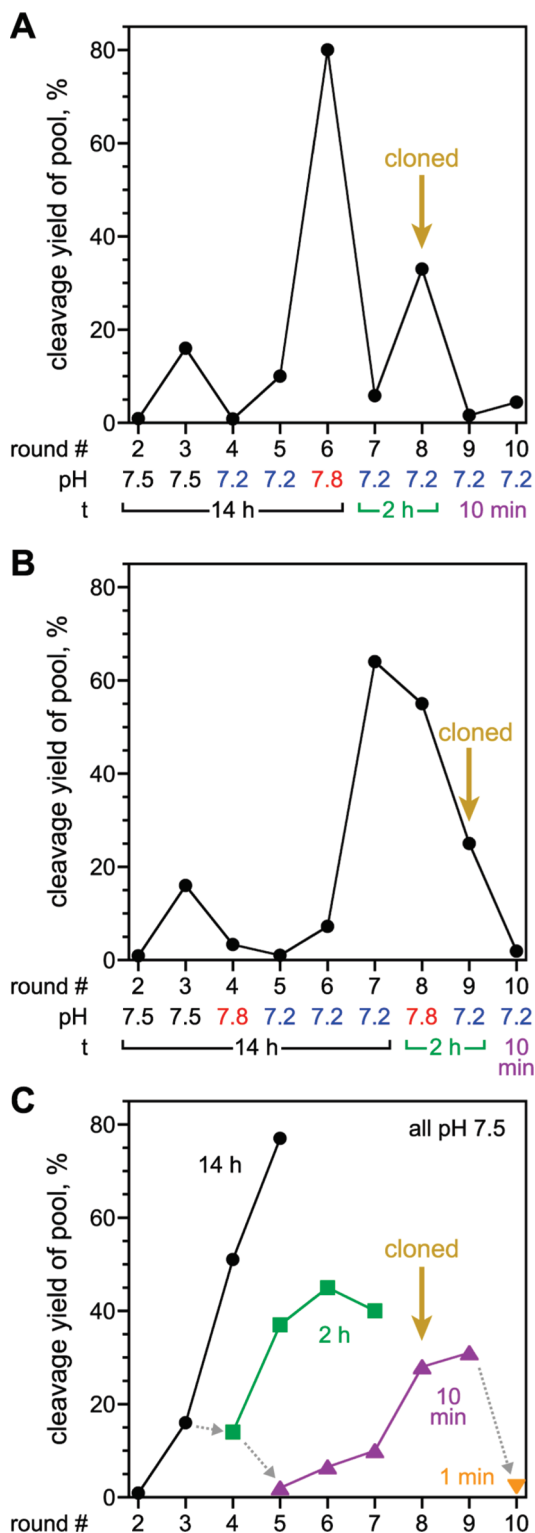


FIGURE 2: Activity profiles for the reselection experiments. Gold arrows denote the rounds for which cloning was performed. (A) First reselection thread, in which the pH began at 7.5 and was first decreased to 7.2. (B) Second reselection thread, in which the pH was first increased to 7.8. (C) Third reselection thread, conducted at constant pH of 7.5.

pH 7.8 (25% pool yield in 2 h at pH 7.2). In general, the pool yields were higher at pH 7.8 than at pH 7.2 for the same incubation time. This likely occurred because deoxyribozyme activities generically increase with increasing pH for reactions such as phosphodiester cleavage by hydrolysis or transesterifica-

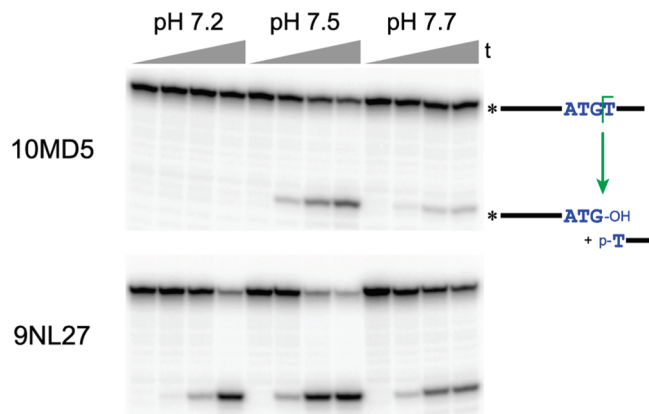


FIGURE 3: Initial survey of individual deoxyribozyme clones. The PAGE images show DNA cleavage activity for the original 10MD5 deoxyribozyme and the reselected 9NL27 variant at pH 7.2, 7.5, and 7.7 under the standard incubation conditions of 70 mM HEPES, 20 mM $MnCl_2$, 1 mM $ZnCl_2$, and 150 mM NaCl at 37 °C. $t = 0, 15$ min, 2 h, and 22 h. Std, 5'- ^{32}P -radiolabeled oligonucleotide standard corresponding to substrate cleavage at the ATG^T site.

tion, where nucleophilic attack of water or a substrate 2'-OH group (e.g., during DNA-catalyzed RNA cleavage) is assisted by deprotonation.

Reselection of 10MD5 for Faster Rate at pH 7.5. In a third reselection thread, rounds were performed at constant pH of 7.5 with decreasing incubation time in successive selection rounds. This approach allowed us to assess whether faster DNA hydrolysis rates could be achieved by 10MD5 variants without simultaneously seeking improved pH tolerance. The incubation time in each round was progressively decreased from 14 to 2 h to 10 to 1 min, with the activity profile shown in Figure 2C. Deoxyribozymes were cloned from round 8 (28% pool yield in 10 min).

Initial Survey of Clones from the First Two Reselection Threads for pH Tolerance. Individual clones from the first two reselection threads were assayed for DNA hydrolysis activities at pH 7.2, 7.5, and 7.7. Each clone was initially tested as a PCR product prepared from miniprep DNA and separated from its complementary strand by PAGE (the complementary strand was longer due to a nonamplifiable spacer incorporated at the 5'-end of the corresponding PCR primer). For clones from the first thread, 10 out of 11 clones functioned well at all three pH values (the remaining clone is discussed below). For clones from the second thread, the same broad pH tolerance was observed for 18 out of 18 clones. Certain individual deoxyribozymes were sequenced and independently prepared by solid-phase synthesis, and their catalytic activities were confirmed. Data for the 9NL27 deoxyribozyme from the second selection thread (as compared with the parent 10MD5 deoxyribozyme) is representative (Figure 3).

Initial Survey of Clones from the Third Reselection Thread for Faster Rate. None of the 31 clones from the third thread had substantial improvement in k_{obs} relative to 10MD5 itself (data not shown), despite the selection pressure to function with shorter incubation times at constant pH of 7.5. This observation indicates that faster variants of 10MD5 are inaccessible in sequence space by this reselection approach.

Hydrolysis Sites for the New Clones. With a single exception, all of the round 8 clones from the first reselection thread, as well as all of the round 9 clones from the second thread and the round 8 clones from the third thread, led to DNA cleavage products that migrate on polyacrylamide gel at the same

	1	10	20	30	40	
10MD5	CGCTAGATAA	GTGGGTGCGT	TTGCTATAGC	TGTCCCTCAA		
8NLJ4	. . . C G . . . C C . . . T G		first thread
8NLJ9 AA . . . T C . . . T T		
8NLJ11	. . . A A . . . C T		
8NLJ13 G . TT C . . . T		
8NLJ14 G . . . T G . . . T TC		
8NLJ15	. . . A G . . . C T T . . . T . . .		
9NL1	. . . AC AGA . T C . . . T TCT		
9NL2	. . . A G . . . C G G		
9NL5	. . . A A . . . C T G		
9NL8	. . . C A . . . C A . . . T		
9NL10	. . . A C T TA . G		
9NL15	. . . A . . . G G . AT C . . . T G		
9NL17	. . . AC A . . . C G . . . T		
9NL19	. . . A A . . . C T		
9NL20 A . . . T T		
9NL26	. . . A A . . . T C . . . T		
9NL27	. . . A A . . . C C . . . T		
9NL29	. . . C A . AT C . . . T		
9NL33	. . . A A . . . C T G		
8NLE1	. . . CT AAC . T T . A		second thread
8NLE2	. . . C AG . T T		
8NLE3 C TA . G		
8NLE4	. . . AC AA . T C . . . T T . . . C . . .		
8NLE5 G . . . T T		
8NLE6	. . . A A . . . C		
8NLE7	. . . C G . TT G A . C . . T . . .		
8NLE8	. . . A . . . G AG . T TCT		
8NLE9	. . . A A . AC G		
8NLE10	. . . A C TA . G		
8NLE11	. . . C A . T C TA		
8NLE12	. . . G . . . T AGC . T TC		
8NLE16	. . . A G		
8NLE17	. . . AA AA . AT C T . T		
8NLE18	. . . G AG . T T . T		
8NLE20	. . . C G . TC C . C . . . T A . C		
8NLE21 G . TT A . C . C		
8NLE22 A . A TA		
8NLE23	. . . T AGC . T C T . . . C . . .		
8NLE24	. . . A A . C A		
8NLE25	. . . A A . T A TA		
8NLE27	. . . AC A . C A		
8NLE28	. . . G A . T TA		
8NLE29	. . . T . . . G A . T A TA . . T		
8NLE30 A . A TA . . G		
8NLE31	. . . TC A . T C TA . . G		
8NLE33	. . . C A . T A TA . . G		
8NLE34	. . . C . . . G AAC . T C T		
8NLE35	. . . AC A . AC G		
8NLE36	. . . C A . T TA . G		
8NLE37 AGC . T G . T T		

FIGURE 4: Artificial phylogeny of reselected 10MD5 variants, using all unique sequences from the three reselection threads. Shown is the 40 nt enzyme-region sequence for each deoxyribozyme. The gray boxes denote the three variable regions (2, 5, and 5 nt) identified within the enzyme region. Colored red are the T16 and G19 positions, which were uniformly mutated in clones from the two reselection threads that experienced selection pressure for pH tolerance. Colored green is the C30 position, which was consistently mutated to T in these clones as well. The 40 nt enzyme regions for the two deoxyribozymes not included in the phylogeny were 8NLJ1, 5'-CGATCGATGACTGTGCGCGGTTCCCTCA-AGAGTATTAGCCA-3', and 9NL12, 5'-GCCTCGCATAGTGGGGGTCTTTGCCATAGTTGTCTCCCA-3' (differences from 10MD5 are underlined).

position as the product from 10MD5 itself (Figure 3). These data strongly suggest that all of these clones hydrolyze the DNA substrate at the same site as does the parent 10MD5 deoxyribozyme. More detailed characterizations of the DNA cleavage products were performed as described below.

One specific clone, named 8NLJ1 and derived from the first reselection thread, clearly had a different DNA hydrolysis site than 10MD5 and all of the other newly identified variants. 8NLJ1 was also unique in having its optimum activity at pH noticeably lower than 7.5 (Supporting Information Figure S1). By comparing the migration rate of the 8NLJ1 product with that of standard oligonucleotides (Supporting Information Figure S1) and as

determined by MALDI mass spectrometry (see section below), 8NLJ1 was shown to cleave the DNA substrate at a position six nucleotides to the 5'-side of the original 10MD5 cleavage site, leaving a 3'-phosphate rather than a 5'-phosphate. Sequencing showed that 8NLJ1 differs from 10MD5 at 19 out of the 40 enzyme-region nucleotides, although these 40 nucleotide positions were only 25% randomized (see Figure 4 caption for 8NLJ1 sequence; note that 25% randomization is only an average value, and individual sequences in the partially randomized pool have a wide distribution centered on an average of 10 out of 40 differences). These findings indicate that 8NLJ1 is essentially unrelated to 10MD5 and evolved opportunistically

from the partially randomized 10MD5 pool to cleave at the new substrate site.

Artificial Phylogeny of the 10MD5 Variants. All of the active clones from all three reselection threads were sequenced. In particular, 11 clones from the first thread, 18 clones from the second thread, and 31 clones from the third thread were sequenced, leading to 7, 14, and 31 nonidentical sequences, respectively (a total of 52 sequences). Aside from 8NLJ1 from the first thread (as described above) and 9NL12 from the second thread (for which 14 out of 40 nucleotides were different from 10MD5), all other clones had 11 or fewer mutations relative to the parent 10MD5 deoxyribozyme. The combination of sequence relationship and uniform retention of hydrolysis site within the DNA substrate strongly suggested that all of these new deoxyribozymes are functionally related. Using all sequences except those of 8NLJ1 and 9NL12, an artificial phylogeny was constructed (Figure 4), revealing conserved and nonconserved nucleotides of the 40 nt enzyme region.

Inspection of the artificial phylogeny led to some intriguing observations. One 2 nt region and two 5 nt regions (12 nucleotides in all; see gray boxes in Figure 4) showed substantial variability, whereas most other nucleotides of the 40 were highly conserved. Although clones from all three reselection threads collectively shared variations at the 12 nonconserved positions, changes among these 12 nucleotides were nonarbitrary with respect to nucleotide position and pH dependence. For clones from the third thread, which lacked selection pressure for pH tolerance, all 12 positions varied substantially with no obvious correlations (bottom portion of Figure 4). However, for the 19 unique sequences from the other two reselection threads during which the pH was repeatedly changed, two nucleotides, both of which are in the central 5 nt nonconserved region, were always mutated (red nucleotides in top portion of Figure 4): T16 and G19 of the 40-nucleotide enzyme region. T16 was always changed to a purine (T16R), and G19 was always mutated to a pyrimidine (G19Y). The sole exception, 9NL10, had T16C but retained parent nucleotide G19, and it also had the poorest activity at pH other than 7.5. In addition, for 20 out of 21 of the clones evolved for pH tolerance, a single C→T mutation was observed among the otherwise conserved nucleotides (C30T; green nucleotides in Figure 4). In contrast, position C30 was unmutated in all but three of the clones from the third thread, which lacked selection pressure for pH tolerance.

In summary of the artificial phylogeny, the alignments revealed three nonconserved sequence regions encompassing 12 nt out of the 40 nt in the entire enzyme region. The alignments also suggested that mutations at three nucleotide positions, T16R, G19Y, and C30T, are strongly correlated with improved pH tolerance during DNA hydrolysis.

Block Deletions and Mutations on the Basis of the Artificial Phylogeny. The artificial phylogeny prompted us to investigate the three nonconserved sequence blocks of 2, 5, or 5 nt in more detail (data not shown). Deleting all nucleotides of any of the three blocks led to <4% cleavage yield after 20 h. Mutating the 2 nt block from CT → AC (as suggested by the most commonly observed changes in the phylogeny) led to poor cleavage activity (10% in 20 h). Mutation of the first 5 nt block from GTGCG → AGCTT led to retention of good (51% in 20 h) cleavage yield, but with substantially reduced rate ($k_{\text{obs}} = 0.18 \text{ h}^{-1}$ versus 2.7 h^{-1} for 10MD5). Mutation of the other 5 nt block from CTCAA → TATGG led to only 1.5% cleavage in 20 h. We concluded that, despite the clear lack of phylogenetic conservation

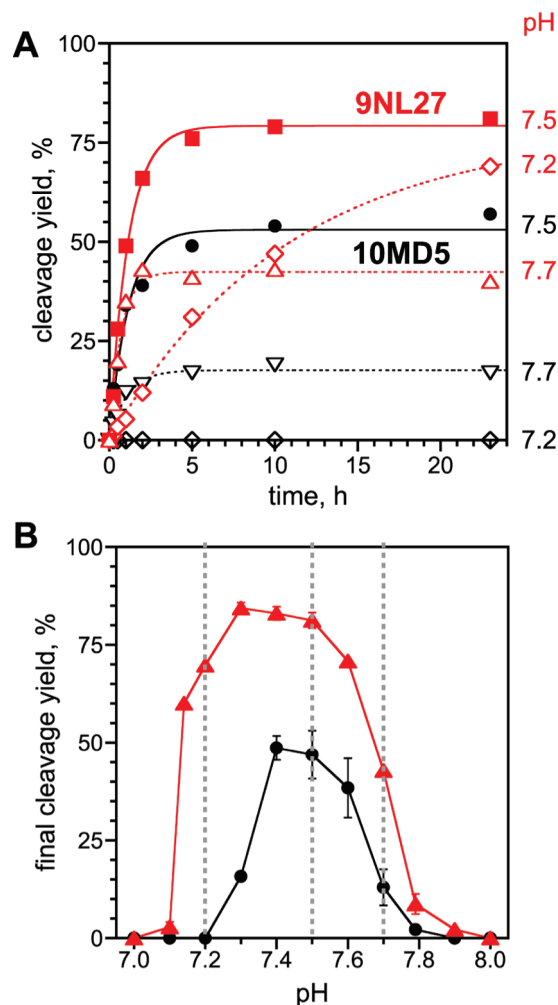


FIGURE 5: Establishing the broader pH tolerance of 9NL27 as compared with the parent 10MD5 deoxyribozyme. (A) Kinetic plots for 10MD5 (black) and 9NL27 (red) at pH 7.20, 7.50, and 7.70, each under the standard incubation conditions of 70 mM HEPES, 20 mM MnCl_2 , 1 mM ZnCl_2 , and 150 mM NaCl at 37 °C. k_{obs} values for 9NL27 at pH 7.20, 7.50, and 7.70 were 0.093, 0.88, and 1.43 h^{-1} , respectively; k_{obs} for 10MD5 at pH 7.50 was 0.87 h^{-1} . The stated pH values were measured for the 1 M HEPES buffer; the actual pH values measured for the three 70 mM HEPES solutions containing Mn^{2+} and Zn^{2+} were 6.93, 7.25, and 7.42 (4). (B) Final cleavage yield from curve fits as a function of pH. Error bars represent the range of values from two independent experiments. The yield at 23 h was used in cases where the kinetic plot did not clearly turn over by the final time point. The vertical dashed lines mark the three pH values from which the data in panel A were derived. The cleavage yields observed here for 10MD5 are modestly lower than those reported for 10MD5 in our initial study (4); the yields now observed are reproducible using current reagents.

(Figure 4), none of these nucleotide blocks is dispensable for catalysis, and individual deoxyribozyme variants should be investigated in more detail.

Initial Analysis of Four New 10MD5 Variants. We evaluated the importance of the nucleotides T16, G19, and C30 by examining several deoxyribozymes in which these nucleotides were mutated. Four individual deoxyribozymes with enhanced pH tolerance were chosen for further investigation: 9NL1, 9NL12, 9NL27, and 9NL33, each of which has a different combination of nucleotide mutations, but in all cases consistent with T16R, G19Y, and C30T (Figure 4). All four of these deoxyribozymes were prepared by solid-phase synthesis and assayed for DNA cleavage activity at several pH values, confirming their broad pH tolerance

and also revealing similar rates and yields (Figure 3 and data not shown). 9NL27, which has only five mutations relative to 10MD5 (C3A, T16A, G19C, T25C, and C30T), was chosen as the focal point for all remaining efforts in this study.

Characterization of 9NL27 for pH Tolerance. A more comprehensive examination of the pH tolerance of 9NL27 was performed, at pH values ranging from 6.8 to 8.1. Although 10MD5 had a sharp activity optimum at pH near 7.4–7.5, 9NL27 was noticeably more pH tolerant (Figure 5), consistent with the selection pressure applied during its identification. The 9NL27 activity fell sharply below pH 7.2, which was the lowest pH value at which reselection rounds were conducted; nevertheless, 9NL27 was much more active than 10MD5 at pH < 7.5. Similarly, 9NL27 was more effective than 10MD5 at pH > 7.5, up to ~7.8 when physical precipitation of Zn²⁺ was clearly evident. Overall, the results in Figure 5 indicate that the selection pressure for improved pH tolerance was effective, leading to, among many additional deoxyribozymes, 9NL27 as a variant of 10MD5 that has only five mutations yet tolerates a much wider pH range.

Characterization of 9NL27 and Other Variants for Site Specificity and Establishment of an Expanded Recognition Site (Reduced Sequence Tolerance). In our previous report, we found that 10MD5 can be used sequence-specifically to cleave

any DNA substrate sequence when the substrate's ATG[^]T recognition site is maintained (4). Because 9NL27 is merely a quintuply mutated version of 10MD5, we initially presumed that 9NL27 would share with 10MD5 the ATG[^]T recognition site. When substrate nucleotides outside ATG[^]T were systematically changed (A↔T, G↔C) and the 9NL27 binding arms were covaried to maintain Watson–Crick base pairing, substantial DNA cleavage activity was still observed, but unexpectedly with markedly relaxed site specificity (Figure 6). The “miscleavage” of the DNA substrate (56% of the combined cleavage product) occurs one nucleotide to the 5'-side of the original ATG[^]T site, i.e., at AT[^]GT with formation of 5'-phosphate and 3'-hydroxyl, as assessed by gel migration position and confirmed by mass spectrometry (see below).

We then examined 9NL27 activity when additional substrate nucleotides beyond ATG[^]T were retained unmutated (Figure 7). Most of the site specificity was restored when the recognition site was expanded by merely one nucleotide to ATG[^]TT, although a small amount of the miscleavage product (~6%) remained clearly evident. Retaining one additional substrate nucleotide, TATG[^]TT, led to complete site specificity (<0.5% miscleavage, similar to 10MD5), and broader pH tolerance was maintained (Supporting Information Figure S2). Together, these data indicate that 9NL27 suffers from reduced site specificity relative to 10MD5 unless its substrate recognition site is expanded. We established that, like 10MD5, 9NL27 is fully sequence-specific outside of its expanded recognition site, by verifying systematic covariation of all other substrate nucleotides to all three alternative nucleobase identities (Supporting Information Figure S3). Similar to 9NL27, the 9NL1, 9NL12, and 9NL33 deoxyribozymes were also found to have substantially relaxed site specificity that was ameliorated by modest expansion of their recognition sites from ATG[^]T (Supporting Information Figure S4).

Characterization of 10MD5 for Site Specificity. The unexpected observation of relaxed site specificity for 9NL27 and the other reselected 10MD5 variants prompted us to evaluate in more detail the site specificity of 10MD5 itself. In our previous report on 10MD5, the DNA substrate always retained ATG[^]T, and only one cleavage site was ever observed (4). Here we briefly examined the impact of changes to ATG[^]T, while either retaining or dispensing with Watson–Crick base pairs between these nucleotides and the deoxyribozyme. Changes to individual nucleotides of ATG[^]T while retaining Watson–Crick base pairing still permitted good cleavage activity, with retention of site specificity (Figure 8A, sets 1–4). However, when changes were made at all three nucleotide positions at the same time (ATG[^]T to TAC[^]T),

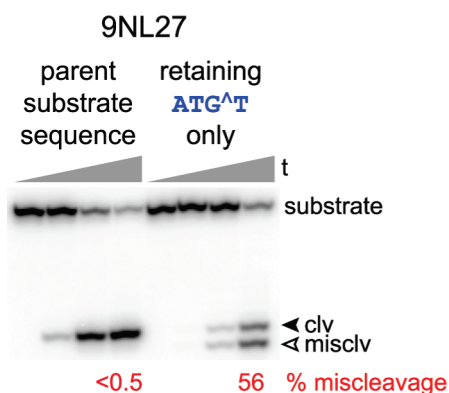


FIGURE 6: Relaxed site specificity observed for 9NL27 when substrate nucleotides outside ATG[^]T are systematically changed, even though Watson–Crick base pairing to the deoxyribozyme is maintained. The substrate sequence was either the parent sequence used during selection (left) or the sequence in which ATG[^]T was retained and all other nucleotides were changed (A↔T, G↔C) with corresponding changes to the deoxyribozyme to maintain base pairing (right). “% miscleavage” refers to the percentage of the total product that corresponds to miscleavage. *t* = 0, 15 min, 2 h, and 22 h under the standard incubation conditions at pH 7.5 (see Figure 3).

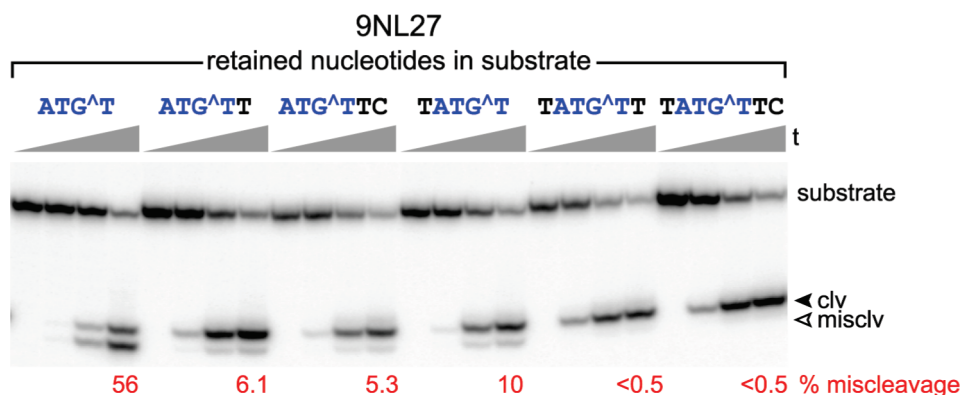


FIGURE 7: Expansion of the recognition site beyond ATG[^]T (i.e., reducing the sequence tolerance) restores site specificity to 9NL27. *t* = 0, 15 min, 2 h, and 22 h. Sequence changes were analogous to those described in Figure 6.

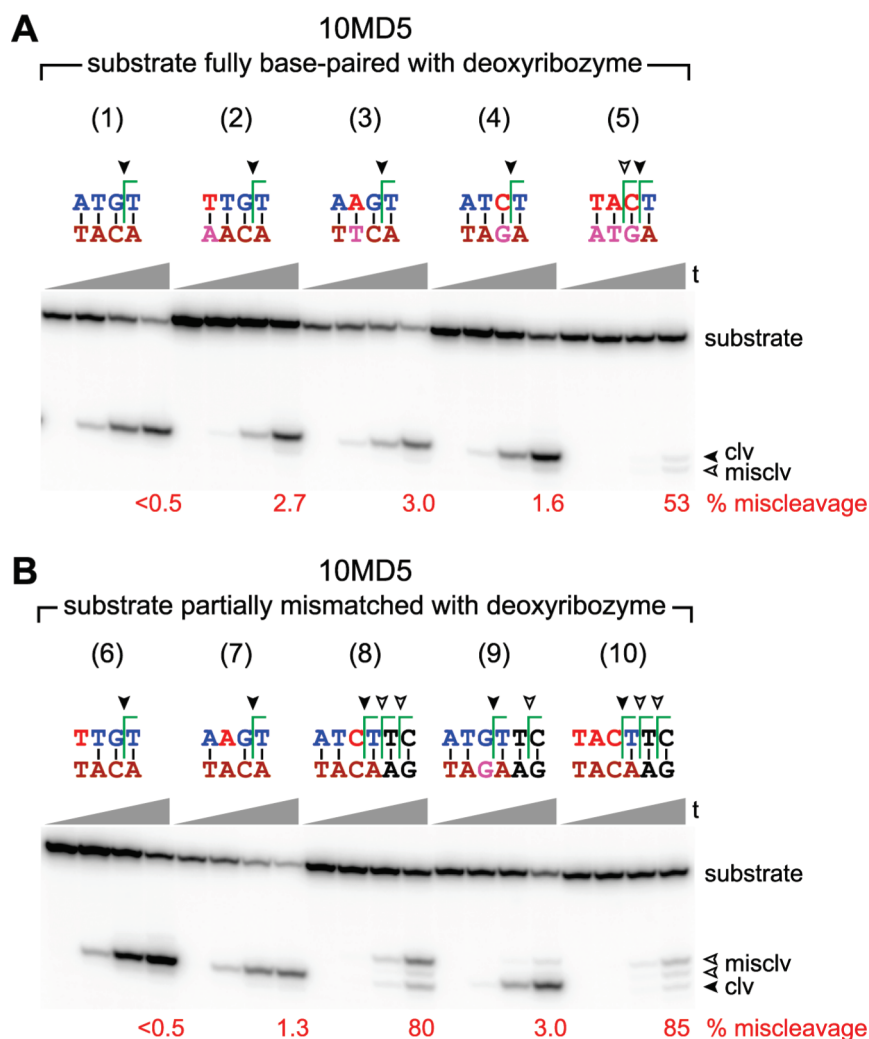


FIGURE 8: Evaluation of 10MD5 site specificity. (A) Complete site specificity is observed when Watson–Crick base pairing to the substrate is maintained as individual $\text{ATG}^{\wedge}\text{T}$ nucleotides are changed; however, both activity and site specificity are decreased considerably when all three $\text{ATG}^{\wedge}\text{T}$ nucleotides are changed simultaneously. (B) Watson–Crick mismatches between substrate and deoxyribozyme (from either side) erode the site specificity, at least for changes directly at the cleavage site or when several changes are made at once. All miscleavage events result in 5'-phosphate and 3'-hydroxyl termini, as verified by MALDI mass spectrometry. $t = 0, 15 \text{ min}, 2 \text{ h}, \text{ and } 22 \text{ h}$.

the cleavage rate and yield were substantially decreased, and the site specificity was relaxed for the small amount of product that was formed (set 5). When Watson–Crick mismatches were introduced at either $\text{ATG}^{\wedge}\text{T}$ position, good site specificity was maintained (Figure 8B, sets 6 and 7), whereas a Watson–Crick mismatch at the $\text{ATG}^{\wedge}\text{T}$ position introduced by a change in either the substrate or deoxyribozyme resulted in loss of site specificity (sets 8 and 9). When all three $\text{ATG}^{\wedge}\text{T}$ substrate nucleotides were mutated without Watson–Crick compensation in the deoxyribozyme (set 10), the site specificity was relaxed as well. In summary of these data, 10MD5 is, like its evolved 9NL27 variant, capable of exhibiting reduced site specificity, but doing so requires more substantial changes (relative to 9NL27) to either nucleotide identity or base pairing near the cleavage site.

Confirmation of DNA Cleavage Sites and Hydrolytic Cleavage Mechanism for 9NL27 and Other Variants. Mass spectrometry was used to confirm the DNA substrate cleavage sites as assigned on the basis of gel migration positions (Supporting Information Table S1). In particular, MALDI-MS assays were performed on the site-specific cleavage products using the parent substrate sequence along with each of the new deoxyribozymes 9NL27, 9NL1, 9NL12, and 9NL33, as well

as 8NLJ1 with its distinct cleavage site. In addition, non-site-specific cleavage products from both 9NL27 and 10MD5 were assayed. In all cases, the mass spectrometry data were fully consistent with hydrolytic DNA cleavage at readily assigned cleavage sites.

A Unified Perspective on the Functional Compromises, and Implications for Ongoing Development of DNA-Catalyzed DNA Cleavage. As a functionally relevant characteristic, sequence specificity in DNA cleavage by the 10MD5 family of deoxyribozymes is an inherent consequence of the overall Watson–Crick “binding arms” design (Figure 1). In contrast, the other three functional characteristics of pH tolerance, site specificity, and sequence tolerance are not automatically imposed by the overall catalyst architecture. Our data show that although 9NL27 is more pH tolerant than is the parent 10MD5 (Figure 5), as expected on the basis of the reselection design, this pH tolerance comes at the expense of site specificity (Figure 6). Furthermore, the site specificity of 9NL27 may be restored, but only by reducing its sequence tolerance, i.e., by expanding its recognition site (Figure 7). These observations can be used to develop a unified perspective on the functional compromises of DNA-catalyzed DNA cleavage, illustrating that the three

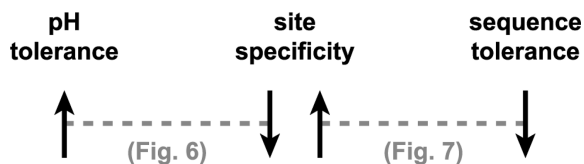


FIGURE 9: A unified perspective on the functional compromises among pH tolerance, site specificity, and sequence tolerance by the 10MD5 family of deoxyribozymes.

characteristics of pH tolerance, site specificity, and sequence tolerance are balanced among each other (Figure 9).

A key implication of our findings is that such functional compromises may force choices about which of the characteristics are most important in a new DNA-hydrolyzing catalyst. At least for 10MD5 and its evolutionarily accessible sequence variants such as 9NL27, site specificity is traded for either pH tolerance or sequence tolerance. This trade-off was also observed for the sequence-unrelated variant 9NL12, suggesting that the compromises of Figure 9 are a more general phenomenon. Because all three tolerance/specificity characteristics are of practical importance, in ongoing experiments we are seeking entirely new deoxyribozymes unrelated to 10MD5, to determine if even more favorable combinations of these characteristics may be embodied within particular DNA catalysts. Our efforts also seek to apply 10MD5, 9NL27, and other deoxyribozymes to cleavage of double-stranded rather than single-stranded DNA substrates.

CONCLUSION

A practical DNA hydrolysis catalyst should be favorable in terms of four key characteristics: sequence specificity, pH tolerance, site specificity, and sequence tolerance. In principle, these four characteristics have no necessary correlation. Our earlier report described the DNA-hydrolyzing 10MD5 deoxyribozyme, which is inherently sequence-specific by its overall design (4). 10MD5 is site-specific and has promising sequence tolerance (requiring only ATG[^]T near the cleavage site) but has rather narrow pH tolerance. Here we used in vitro evolution to systematically identify 10MD5 variants that have improved pH

tolerance, with 9NL27 (which has only five mutations relative to 10MD5, including at key positions T16, G19, and C30) as the representative example. The improvement in pH tolerance exhibited by 9NL27 and several other variants comes at the expense of relaxed site specificity, which may be regained at the counter-expense of reducing the sequence tolerance, i.e., expanding the recognition site from ATG[^]T of 10MD5. In addition to providing experimental data regarding important functional compromises for the initial family of DNA-hydrolyzing deoxyribozymes exemplified by 10MD5, these findings inform our ongoing efforts to identify new DNA-cleaving DNA catalysts.

SUPPORTING INFORMATION AVAILABLE

Additional experimental details. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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