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Distinct Facilitated Diffusion Mechanisms by *E. coli* Type II Restriction Endonucleases

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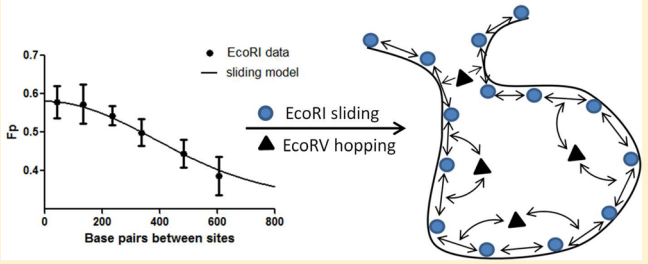
Supporting Information

ABSTRACT: The passive search by proteins for particular DNA sequences involving nonspecific DNA is essential for gene regulation, DNA repair, phage defense, and diverse epigenetic processes. Distinct mechanisms contribute to these searches, and it remains unresolved as to which mechanism or blend of mechanisms best suits a particular protein and, more importantly, its biological role. To address this, we compare the translocation properties of two well-studied bacterial restriction endonucleases (ENases), EcoRI and EcoRV. These dimeric, magnesium-dependent enzymes hydrolyze related sites (EcoRI ENase, 5′-GAATTC-3′; EcoRV ENase, 5′-GATATC-3′), leaving overhangs and blunt DNA segments, respectively. Here, we demonstrate that the extensive sliding by EcoRI ENase, involving sliding up to ∼600 bp prior to dissociating from the DNA, contrasts with a larger reliance on hopping mechanism(s) by EcoRV ENase. The mechanism displayed by EcoRI ENase results in a highly thorough search of DNA, whereas the EcoRV ENase mechanism results in an extended, yet less rigorous, interrogation of DNA sequence space. We describe how these mechanistic distinctions are complemented by other aspects of these endonucleases, such as the 10-fold higher *in vivo* concentrations of EcoRI ENase compared to that of EcoRV ENase. Further, we hypothesize that the highly diverse enzyme arsenal that bacteria employ against foreign DNA involves seemingly similar enzymes that rely on distinct but complementary search mechanisms. Our comparative approach reveals how different proteins utilize distinct site-locating strategies.



Bacterial restriction endonucleases are broadly dispersed and provide a robust safeguard against various foreign nucleic acids.[1] Although other strategies contribute toward this protection (e.g., CRISPR,[2] RecBCD,[3] and abortive infection systems[4]), the diversity of endonucleolytic-based strategies speaks to their importance. Many endonucleases (ENases) are coupled with a methyltransferase, which together form a Type II restriction modification (R−M) system, one of four types of R−M systems.[1,5] The ENase(s) recognize and cleave unmethylated sites in invading DNA, whereas the methyltransferase(s) protect those sites on the bacterial genome. A delicate interplay between these two competing enzyme types is necessary for this protection, preventing, for example, the ENase from cutting genomic DNA, yet allowing certain foreign DNA elements to be incorporated into the bacterial genome, providing genetic diversity. Among the superfamily of ENases, Type II's, which typically cut 4−8 base pair (bp) sites, are the most diverse and abundant.[6] Here, we compare two well-studied Type II ENases, EcoRI ENase (EcoRI) (5′-GAATTC-3′) and EcoRV ENase (EcoRV) (5′-GATATC-3′), both of which are Mg^{2+}-dependent homodimers found in *Escherichia coli*.[6] We are interested in defining the mechanistic distinctions between these ENases as well as how any distinctions may help to understand why most bacteria contain several seemingly redundant R−M systems.[1]

The vast majority of DNA binding/modifying proteins move along DNA in search of their recognition sites without relying on an external energy source, a process called facilitated diffusion.[7−9] Some proteins are present at a few copies per cell (<100), whereas other proteins are present at very high levels (e.g., > 300 000 per cell);[10−12] moreover, some proteins locate sites that occur frequently (e.g., every 20−100 bp), whereas others locate sites separated by millions of base pairs.[12,13] Distinct mechanisms are therefore likely to contribute to these facilitated diffusion processes (Figure 1); however, experimental evidence clearly segregating or directly comparing these mechanisms is limited. Processive catalysis, as measured by the efficiency of modifications involving a second site, provides insight into the underlying translocation mechanism.[14−16] ENases that cut both sites during one binding event, where the enzyme travels to the second site without returning to the bulk solution following catalysis at the first site, are considered processive. How processivity (F_p) changes with different site-to-site (intersite) spacings between the two sites is critical for deducing translocation mechanisms and remains a powerful technique to do so.[14−16] Sliding is one such mechanism, where proteins linearly diffuse along the contour of DNA (Figure 1). Processive (F_p) sliding, defined as the probability of sliding from one site to another, depends on the rate constant of a

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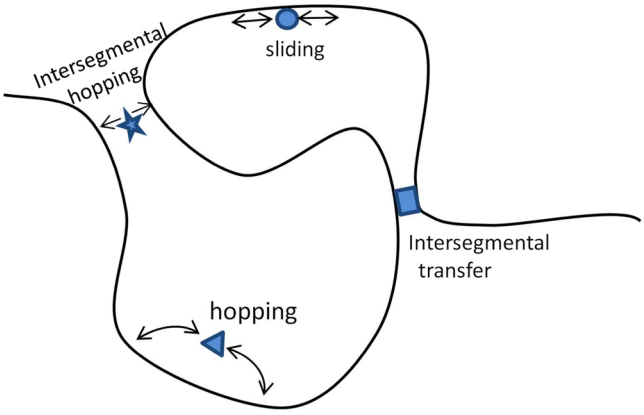

Figure 1. Known facilitated diffusion mechanisms for DNA binding/modifying proteins. The circle shows sliding, where the protein makes a series of single base pair shifts, usually redundantly sampling small regions of DNA. The triangle represents hopping, where the protein undergoes a series of disassociation and reassociation steps, allowing larger areas of the DNA to be sampled. The star shows intersegmental hopping, where the looping of DNA captures the hopping protein, allowing it to transfer between distal DNA segments. The square depicts intersegmental transfer, where the protein transfer involves simultaneous binding of two DNA segments.

single base pair slide (k_{slide}), the rate constant of the protein dissociating from DNA (k_{off}), and the number of base pairs (n) between the two sites (eq 1)

$$F_p = (k_{slide}/(k_{slide} + k_{off}))^{n^2} \quad (1)$$

This makes processivity at, for example, >250 bp increasingly unlikely (although our work here brings this into question).[8,14] Processive (F_p) sliding can also be approximated by an exponential decay function (Supporting Information, Figure 1). Hopping involves rapid dissociation and reassociation within a poorly defined region surrounding the DNA (Figure 1). Hopping scales as the inverse of r, which is the intersite distance defined by the worm-like chain model, and a, which is the distance the protein slides between each hop.[14,16]

$$F_p = a/r \quad (2)$$

Processivity is typically less sharply dependent on the intersite spacings for hopping than sliding. Because hopping involves larger displacements along the DNA than sliding, it is often proposed to lead to a more efficient long-distance search mechanism.[8] Importantly, studies of protein sliding and hopping largely rely on intersite spacings of ∼10 to several hundred base pairs ,[8,9,14,15] where *in vivo* search events often involve thousands of base pairs between sites.

Evidence has been recently reported for a translocation mechanism that relies on hopping but includes a distal DNA segment being positioned in proximity to the protein, allowing it to transfer between DNA duplexes when hopping and providing a means to efficiently translocate between DNA segments[17−20] (Figure 1). We deemed this intersegmental hopping, although this can be considered a subtype, or particular circumstance, of the well-established hopping mechanism,[7−9,14−16] which, in many ways, it is similar to.[17] Importantly, intersegmental hopping is distinct from intersegmental transfer, which requires an intermediate where proteins or protein complexes simultaneously bind two DNA segments.[21,22] Also, intersegmental hopping does not refer to movement along the trajectory of the helix. Intersegmental hopping occurs optimally when the segments within the same strand are separated by 500−600 bp, as shown by our previous work.[17] This base pair dependence corroborates well with the probability of DNA loop formation of sites.[23,24] We suggested that the relative reliance on sliding, hopping, and/or intersegmental hopping of a particular protein is driven by its particular biological context, including target site density, cellular protein (of interest) levels, and the relative consequence of site finding.[17] For example, *E. coli* DNA adenine methyltransferase (Dam) primarily utilizes intersegmental hopping as only ∼100 Dam enzymes methylate ∼20 000 sites along the entire bacterial genome. Since skipping sites is relatively inconsequential for Dam (see Discussion), sliding/hopping short distances would be unnecessarily redundant and indeed is not significantly utilized.[17] Here, we are interested in whether EcoRI and EcoRV, both of which recognize and cut 6 base pair recognition sites, display distinct search mechanisms and if those distinctions can further elucidate details concerning translocation mechanisms and help to understand a coordinated host defense strategy.

Extensive studies of EcoRI and EcoRV reveal unresolved questions about their translocation properties.[14,25,26] Modrich et al. found that EcoRI is more processive with circularized versus linear DNA.[27] These results have been interpreted to involve hopping or 3D translocation (possibly related to intersegmental hopping),[14] but extensive sliding cannot be dismissed. Rau et al. used DNA segments with under 100 bp intersite spacings as a basis for extrapolating a sliding mechanism out to ∼400 bp.[28] EcoRV utilizes a combination of short-range sliding and longer-range hopping mechanisms, as deduced by standard processivity experiments and equations (eqs 1 and 2).[14] Although EcoRV can carry out what we call intersegmental hopping,[18,19] the relative importance of this translocation mechanism in comparison to sliding and hopping mechanisms remains inconclusive. Interestingly, both EcoRI and EcoRV can translocate hundreds of base pairs as part of their search strategy.[14,27] Here, we investigate how they differ in the particular blend of the underlying site-finding mechanisms. Both the extent and the comparative nature of our experiments provide several new insights to these well-studied systems.

MATERIALS AND METHODS

Processivity Assays. EcoRI was diluted into the following storage buffer: 10 mM KPO_4, 300 mM NaCl, 0.1 mM EDTA, 200 μg/mL BSA, 10 mM DTT, 0.15% Triton X, and 50% glycerol. Reactions were done in the EcoRI reaction buffer (20 mM Tris, pH 7.4, 5 mM $MgCl_2$, 0.2 mM EDTA, and 50 μg/mL BSA) with 400 nM DNA and a total of 65 mM NaCl at 37 °C. Reactions were initiated by the addition of 1 nM EcoRI, and time points were quenched in 50 mM EDTA.

EcoRV was diluted into the following storage buffer: 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 200 μg/mL BSA, and 50% glycerol. Reactions were done in EcoRV reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.25 mM EDTA, and 50 μg/mL BSA) with 400 nM DNA and a total of 28 mM NaCl at 37 °C. Reactions were initiated by the addition of 1.6 nM EcoRV, and time points were quenched in 50 mM EDTA.

Salt Study. Reactions were carried out exactly as above with NaCl levels modulated. For EcoRI, 50, 65, and 90 mM NaCl were used. For EcoRV, 15, 28, and 50 mM NaCl were used.

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Chase Assays. EcoRV (240 nM) was incubated on 400 nM DNA with buffer (above) at 4 °C for 30 min at the designated salt concentrations. The reaction was initiated with a mixture of 10 mM Mg^{2+} and chased (0−30 μM) as indicated. Reactions were quenched in 50 mM EDTA.

EcoRI (100 nM) was incubated on 400 nM DNA with buffer (above) at 4 °C for 30 min at 65 mM NaCl. The reaction was initiated with a mixture of 10 mM Mg^{2+} and chased (0−4 μM) as indicated. Reactions were quenched in 50 mM EDTA.

Enzymes. EcoRI was purchased from NEB. Purified EcoRV was kindly provided for by Dr. John Perona. The purification protocol can be seen in Hiller et al.[29]

Data Analysis. The reaction products were analyzed using PAGE (20−5% (depending on substrate length) 29:1 acrylamide/bis(acrylamide)) at 300 V for 1−3 h. Gels were scanned on a Typhoon Phosphoimager (GE). Densitometry was done on the provided software (GE). The processivity values were derived from a least-squares fitting using Microsoft Excel.

DNA Substrates. See the Supporting Information.

RESULTS

Processivity Assay. Our processivity values for EcoRI are derived using a multiple turnover steady-state assay with 400 nM DNA and 1 nM EcoRI enzyme (dimers) to minimize situations where two enzymes are bound to a single DNA molecule, as was shown previously in Pollak et al.[17] Each two-site substrate is symmetric with respect to the amount of DNA flanking each site to avoid kinetic site preferences. The DNA fragments are separated and quantified by PAGE. The fluorescein label is on only one end of the DNA to simplify the analysis (particularly for PAGE with longer substrates). Processivity depends on the relative populations of uncut, singly cut (Sc), and doubly cut (Dc) DNA over time, where smaller accumulations of singly cut DNA correspond to higher processivity. Processivity is ultimately based on the relative densities of the top band (Uc), the middle band (Sc′), and the bottom band (Dc*) at each time point from the gel (Figure 2). Subsequent manipulations of these band densities (eqs 3−5) provides the relative amounts of uncut, singly cut (Sc), and doubly cut (Dc) DNA. Singly cut (Sc) corresponds to the percentage of singly cut DNA at a particular time (t); doubly cut (Dc) corresponds to the percentage of doubly cut DNA at a particular time (t) (eqs 4−7). Our analysis accounts for the presence of unlabeled "ghost bands" (bands that are invisible due to their lack of fluorescein label). For example, only one of the two DNA fragments associated with singly cut DNA is visualized. Sc′ is therefore doubled to account for the unseen singly cut ghost band (eqs 3 and 4). Dc* contains a portion of density corresponding to singly cut DNA and therefore Sc′ is subtracted from Dc* (eqs 3 and 5).[17]

$$\text{total density per lane: } T = \text{Uc} + 2\text{Sc}' + \text{Dc}^* - \text{Sc} \quad (3)$$

$$T = \text{Uc} + \text{Sc}' + \text{Dc}^* \quad (3a)$$

$$\text{singly cut (Sc)} = 2\text{Sc}'/T \quad (4)$$

$$\text{doubly cut (Dc)} = (\text{Dc}^* - \text{Sc}')/T \quad (5)$$

These values (Sc and Dc from several time points) are simultaneously fit to a sequential reaction mechanism,[30] revealing k_1 (the first-order rate constant from uncut to singly cut), k_2 (the first-order rate constant from singly cut to doubly cut), and, finally, F_p (eqs 6−8).[17]

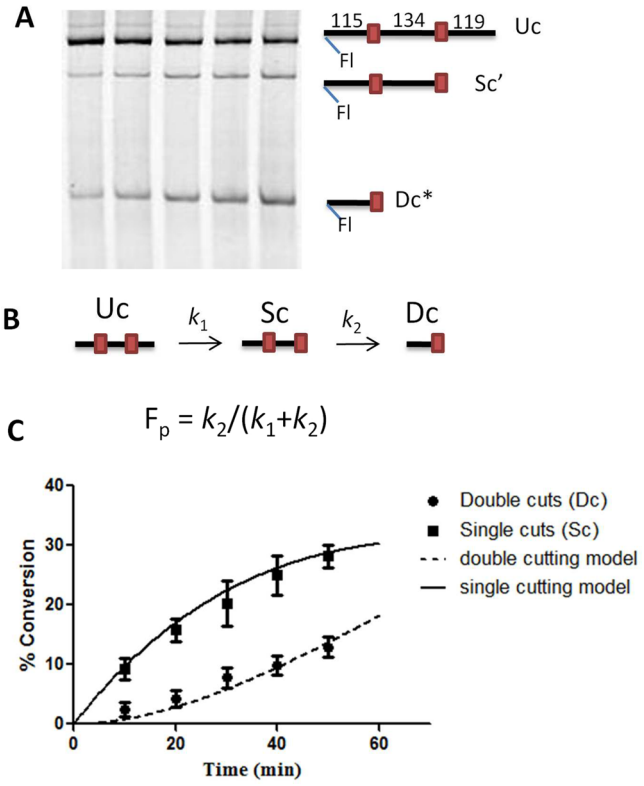

Figure 2. ENase processivity assay. (A) Nondenaturing PAGE gel showing the separation of products of a flourescien-labeled two-site symmetric substrate with specific sites for either EcoRI or EcoRV. An EcoRI substrate with a 134 bp intersite separation is shown. (B) Uc, Sc′, and Dc* are further processed (text) revealing uncut (Uc), singly cut (Sc), and doubly cut (Dc) DNA, which is the basis for the k_1, k_2, and, ultimately, processivity (F_p). (C) Plot of Sc and Dc from gel in panel A.

$$\text{singly cut (Sc)} = \frac{k_1}{(k_2 - k_1)} \times (e^{-tk_1} - e^{-tk_2}) \quad (6)$$

$$\text{doubly cut (Dc)} = 1 + \frac{1}{(k_1 - k_2)} \times (k_2 e^{-tk_1} - k_1 e^{-tk_2}) \quad (7)$$

$$F_p = \frac{k_2}{k_1 + k_2} \quad (8)$$

For unprocessive catalysis, each cutting event is uncorrelated and involves association of the enzyme from the bulk solution back to the DNA. The steps subsequent to the enzyme binding to the DNA limit the kinetics of binding to the specific site, and there are twice as many available sites for single cutting events as for double cutting events. Therefore, $k_2 = k_1/2$ for completely unprocessive catalysis, resulting in $F_p = {}^1/_3$ as the lower limit of processivity. For processive catalysis, $k_2 > k_1/2$ as the enzyme remains associated with the DNA following the single cut and preceding the second cut. Highly processive catalysis is associated with a relatively small accumulation of singly cut intermediates. For $k_2 \gg k_1$, $F_p = 1$. However, endonucleases indiscriminately associate with either fragment generated after a DNA cleavage.[14,27] Therefore, only half of the cleavage events will result in the enzyme associating with the fragment with the second site. Given the lower limit of

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processivity, this means maximal processivity for endonucleases is F_p = 0.66 not 1.

EcoRI and EcoRV Display Different Intersite Processivity Dependencies. How processivity changes with respect to changes in intersite distance is the basis for elucidating translocation mechanisms (eqs 1 and 2). Here, we use a series of two-site substrates with flanking sequences held constant at 115 and 119 bp and the spacing between sites ranging from 42 to 605 bp for EcoRI (Figures 2,3A). We observe a processivity value of F_p = 0.58 ± 0.03 with 42 bp between the two sites, meaning that EcoRI is largely processive with this substrate, as processivity is maximally F_p = 0.66.[14,27] Importantly, the inclusion of 65 mM NaCl is responsible for the slight lowering of processivity from the theoretical maximum, consistent with prior work.[27] Furthermore, processivity remains largely unchanged with intersite distances up to 134 bp and then only gradually decreases with further increases in intersite distances. With our substrates here, we show that EcoRI is processive with an intersite distance of up to 605 bp (Figure 3B).

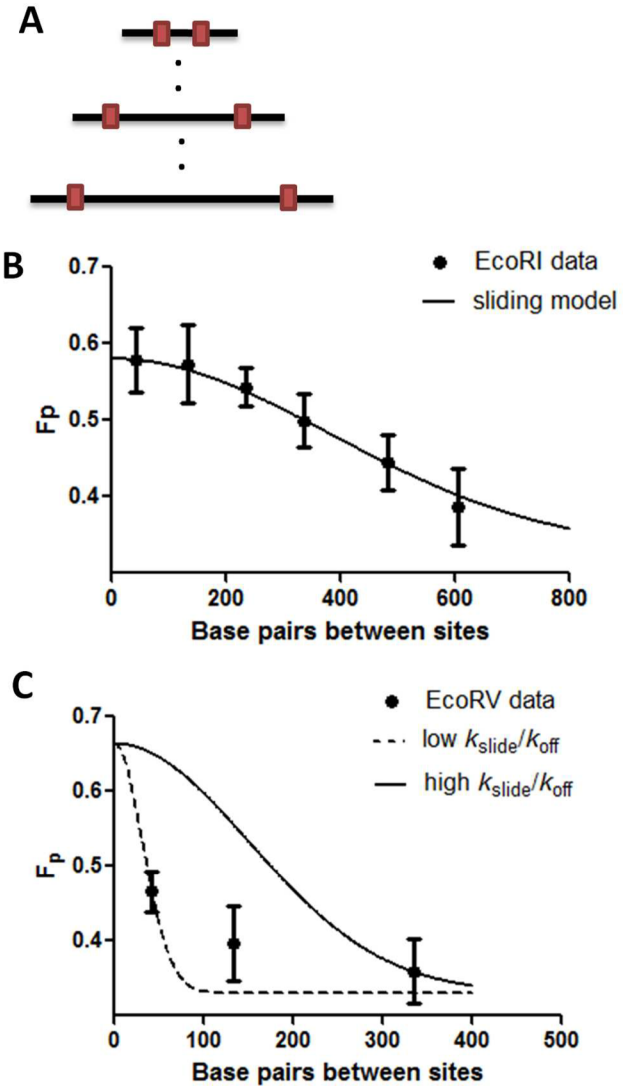

Figure 3. Processivity data for EcoRI ENase and EcoRV ENase shows different intersite spacing dependencies. (A) Schematic of substrates with variation of intersite distances. (B) Processivity data for EcoRI includes 1 nM enzyme, 400 nM DNA, 5 mM $MgCl_2$, 65 mM NaCl, and buffer at 37 °C. Processivity trend is fit to the sliding model (eq 9), where each sliding step is orders of magnitude more probable than a dissociation step (k_{slide}/k_{off} of 290 000:1). (C) Processivity data for EcoRV includes 1.6 nM enzyme, 400 nM DNA, 10 mM $MgCl_2$, 28 mM NaCl, and buffer at 37 °C. Processivity decreases more sharply as a function of the spacing between the two sites than that in panel B. Fit to a higher k_{slide}/k_{off} of 45 000:1 (solid line) and a lower k_{slide}/k_{off} of 2100:1 (dashed line). The processivity trend cannot fit to the sliding model, and the data is therefore consistent with a combination of sliding and hopping mechanisms.

On the basis of our results from Figure 3B, we extrapolate that EcoRI has a theoretical processivity value of ~0.58 when the two sites are directly next to each other (separated by 0 bp) under these conditions, and eq 1 is normalized to reflect this, resulting in eq 9. Under lower salt levels, processivity is slightly higher, approaching the theoretical maximum (below). Equation 9 also accounts for the fact that the maximum level of processivity for an endonuclease is lowered by the random partitioning between the products of the first cleavage event (see above), overall defining the range of our processivity assay under these experimental conditions at F_p = 0.33−0.58.

$$F_p = [(k_{slide}/(k_{slide} + k_{off}))^{n^2}]/4 + 1/3 \quad (9)$$

The processivity data set fits the sliding model remarkably well (Figure 3B) with extensive sliding vs dissociation kinetics, requiring an extremely high k_{slide}/k_{off} ratio of ~290 000:1. Others have proposed that such a high ratio is unlikely and therefore invoke that hopping makes a significant contribution to the translocation mechanism.[8,14] However, the excellent fit to the sliding model suggests that this is not the case. We next applied the same measure of processivity to explore the translocation mechanism used by EcoRV, which has been extensively studied, and our intention here was to essentially replicate the results of Stanford et al. with our assay (Figure 3C).[14] Importantly, many prior studies of EcoRI and EcoRV and other processive nucleic acid modifying enzymes have relied on different assays, making direct comparisons between related enzymes difficult. Here, the series of intersite distances span from 42 to 335 bp. The salt concentration was lowered to 28 mM to maximize the processivity values (below). The most obvious distinction between the two enzymes is that processivity decreases when increasing the site spacing from 42 to 134 bp for EcoRV, whereas the processivity remains essentially unchanged for EcoRI in this intersite distance range (Figure 3B). Importantly, the processivity trend observed for EcoRV cannot be reconciled solely with the sliding model, either using high or low k_{slide}/k_{off} ratios (Figure 3C). This suggests that EcoRV uses a combination of sliding and hopping, consistent with prior results.[14] Also, a significant reliance on an intersegmental hopping mechanism would result in processivity increases starting at ~200 bp separations,[17] which is not the case here or in prior work using longer intersite spacings.[14]

The Greater Salt Dependence by EcoRV than That of EcoRI Suggests Distinctive Translocation Mechanisms. The influence of salt concentration on processivity reveals important details concerning translocation mechanisms.[15] Salt screens the interaction between the negatively charged DNA polymer and the positively charged DNA binding cleft on the protein. Importantly, ENase processivity measurements determine how likely a second site is to be cut following an initial cutting event, limiting the read out of salt's impact only to the protein moving between the two sites. Decreases in processivity

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associated with increases in salt concentration more strongly implicate a hopping mechanism, as salt disrupts the ability of the protein to reassociate with the DNA as it hops (Figure 4A).

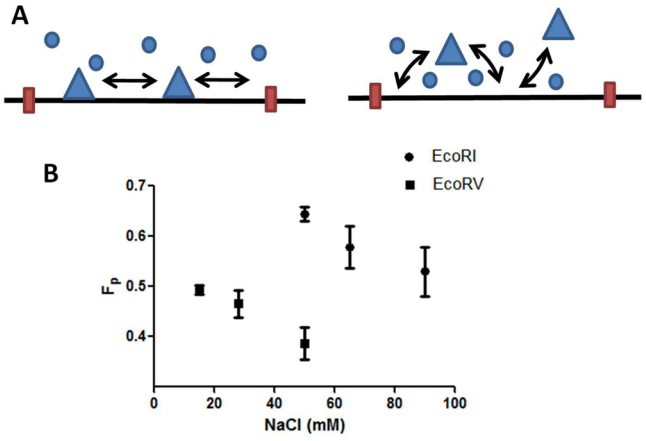

Figure 4. Differential salt dependencies for EcoRI and EcoRV. (A) Salt (circles) effects the processivity of a hopping enzyme (right) more dramatically than a sliding one (left). (B) Processivity assays with different amounts of salt for a substrates with 42 bp between the sites. For EcoRV, 15, 28, 50 mM NaCl; for EcoRI, 50, 65, 90 mM NaCl. EcoRV's processivity is more sensitive to salt, consistent with a hopping mechanism. EcoRI's processivity is less sensitive to salt, which is consistent with a sliding mechanism. EcoRI is more likely to closely interrogate short distances along DNA under conditions of high salt (*in vivo*) than is EcoRV.

During sliding, in contrast, the protein remains associated with the DNA throughout the translocation, resulting in an attenuated response to salt than that in hopping. However, salt may also affect the dissociation of the protein from nonspecific DNA while sliding, although this was shown not to significantly affect EcoRI or other proteins that undergo sliding.[28] We challenged both EcoRI and EcoRV with increasing salt (NaCl) using a substrate with 42 bp between the sites.

EcoRI's processivity under low salt concentrations (F_p= 0.64 ± 0.01) (Figure 4B) is near the maximum level (F_p = 0.66), consistent with the defined limitations of our assay (see above). (This further corroborates the legitimacy of eq 9.) EcoRV's processivity does not extrapolate to the maximum level at low salt, suggesting that, unlike EcoRI, intersite spacings shorter than those investigated here might reveal higher processivity values, further suggesting that EcoRV's sliding is less extensive than that of EcoRI. The greater sensitivity to salt concentration by EcoRV is consistent with EcoRV being dissociated from the DNA for longer periods of time, presumably due to a greater reliance on a hopping mechanism. This is further evidence that the two ENases use distinct facilitated diffusion mechanisms. Our results suggest that under physiological conditions (~100 mM NaCl) EcoRV will show poor processivity and is therefore unlikely to closely interrogate short segments of DNA, as has been shown in prior work.[14] Interestingly, van den Broek et al. showed that EcoRV undergoes intersegmental hopping by DNA looping at 100 mM NaCl (but not at 25 or 150 mM NaCl).[19] The van den Broek et al. report contrasts with Gowers et al.,[18] who initially showed that movement between plasmid concatamers, presumably by the same mechanism, is possible only under low salt conditions and does not occur when NaCl is ≥100 mM. We sought to further probe the conditions and extent to which EcoRV (and EcoRI) utilizes intersegmental hopping.

Chase Assay: EcoRV Utilizes Intersegmental Hopping to a Greater Extent than Does EcoRI. Intersegmental hopping involves protein movement between two distal DNA segments that come into sufficient proximity (Figure 1). Importantly, we distinguish this from a similar process involving a protein or protein complex that simultaneously binds the two DNA segments (intersegmental transfer).This simultaneous binding is not possible for the two ENases studied here.[17] We use a chase assay to probe if EcoRV (and EcoRI) manifests intersegmental hopping under low (6 mM) and/or high (100 mM) NaCl conditions.

The chase assay relies on the fact that the enzyme incubated with the DNA generates cleaved product much faster than during subsequent steady-state catalytic cycles, where the enzyme releases product, returns to bulk solution, and cuts again.[31] This generates a burst of product formation resulting from the use of relatively high enzyme concentrations combined with a ~1.5-fold excess of DNA (400 nM). EcoRV is first incubated on a single-site substrate in the absence of metal, and cleavage is initiated by Mg^{2+} addition with various amounts of unlabeled competitor DNA chase. In the absence of divalent metal, EcoRV binds to specific DNA only ~5-fold tighter than to nonspecific DNA,[32] and previous reports estimate even less discrimination.[33] Therefore, prior to initiation by Mg^{2+}, EcoRV exists as three dynamic populations: bound to nonspecific DNA surrounding the site, bound to the specific site, and protein that has dissociated from nonspecific or specific DNA (Figure 5).

The burst value corresponds to the asymptote to the *y* axis from the line fit to the steady-state portion of product formation (Figures 6A–C). This burst therefore relates to the extent of the initial cutting event only. This is seen for each amount of chase from Figures 6A–C, where the percent cut (% cut) here refers to the perecent of input DNA cut. The percent

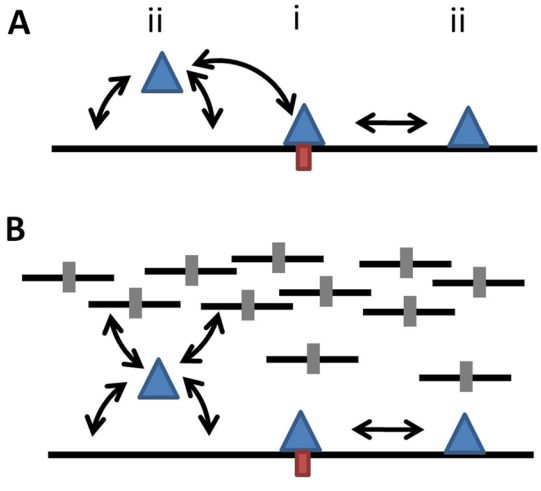

Figure 5. Schematic of the chase assay. Enzyme (triangle) is in multiple dynamic populations bound to a single-site (rectangle) substrate in the absence of metal or chase, poised for catalysis: (i) it remains bound to the site, (ii) it can hop or slide to the site, and (iii) it leaves the DNA (not shown). (A) The initiation of cleavage in the absence of chase allows the enzyme to cut, which includes hopping or sliding to the site. (B) The initiation of cleavage with excess chase. Sufficient quantities of chase will capture the population of hopping enzymes by way intersegmental hopping.

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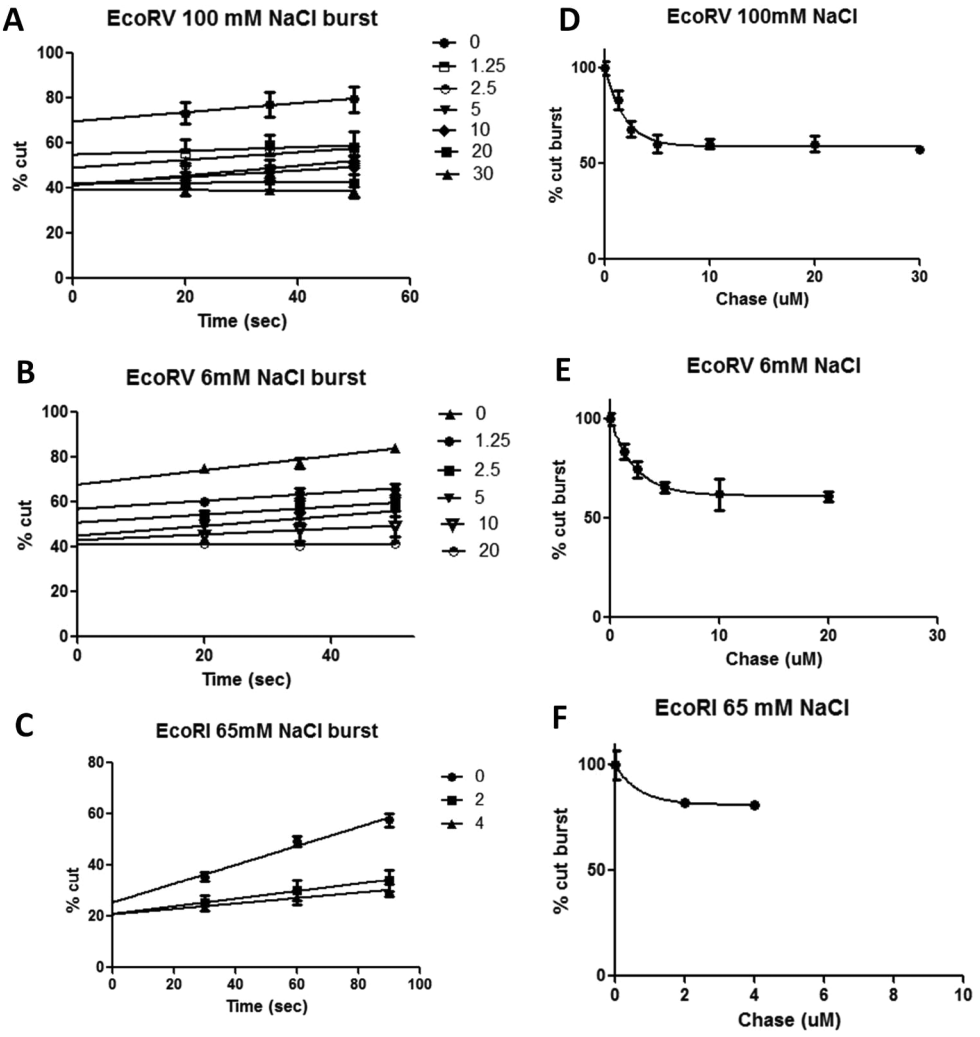

Figure 6. EcoRV undergoes intersegmental hopping under low (6 mM) and high (100 mM) NaCl. (A) 240 nM EcoRV is incubated on 400 nM DNA with buffer at 4 °C and 100 mM NaCl. Initiation with Mg^{2+} and chase (0−30 μM) causes a burst of product formation commensurate with the amount of enzyme, which is indicated by the asymptote to the y axis from the slower steady-state cleavage trace. Chase decreases the burst level. Percent (%) cut refers to amount of cut input DNA for panels A−C. (B) Burst assay with EcoRV and 6 mM NaCl. Initiation is with Mg^{2+} and chase (0−20 μM). (C) Burst assay with 100 nM EcoRI (note that this is less enzyme than that for the EcoRV experiments) incubated on 400 nM DNA with buffer at 4 °C and 65 mM NaCl. (D) Burst levels in panel A (from the asymptote to y axis, where 100% cut burst corresponds to no chase for panels D−F) are depicted as a function of chase. This shows a clear plateau, signifying that a portion of the enzyme population can be captured by the chase by undergoing intersegmental hopping. (E) Burst levels in panel B are depicted as a function of chase, with a similar outcome as that in panel D. (F) Bursts levels in C are depicted as a function of chase. Most of the enzyme is not captured by chase through intersegmental hopping.

cut burst (% cut burst) (Figure 6D−F) corresponds to the percent of the burst retained with each amount of chase added, with 100% referring to burst with no chase. Our detected burst level in the absence of chase indicates very little enzyme dissociates completely away from the DNA (Figure 6A, B). The population of enzyme that is not bound to the site but that can return to it will do so by hopping or sliding (Figure 5).

The extent of inhibition by the chase is dependent on the fraction of the protein that hops during the initial phase of the reaction. The addition of the chase DNA will capture the fraction of enzyme that has separated from the DNA, likely during a hop, but that in the absence of chase returns and generates product. Thus, the extent to which the chase DNA decreases the burst magnitude is directly related to the fraction of protein that dissociates but normally reassociates.[34] An enzyme that hops but cannot undergo intersegmental hopping will be unaffected by the chase.

In comparison to the burst level with no chase, a 5-fold excess of chase DNA causes a clear decrease in the burst magnitude, consistent with a portion of the enzyme being captured during intersegmental hopping events (Figure 6D,E), either under low or high salt levels. Importantly, the effect of the chase on the burst plateaus with increasing chase concentrations, meaning the chase can capture only roughly 40% of the enzyme population, suggesting that the remaining enzyme population is poised on the specific site or is able to move undisturbed (likely by sliding) from the nonspecific sites to the specific site during this time period. The plateau suggests that chase is saturating. A similar approach and interpretation was recently reported by Stivers and co-workers.[35]

Under similar conditions, EcoRI's burst is less affected by chase than that of EcoRV (Figure 6F), consistent with a smaller fraction of the enzyme (~20%) utilizing intersegmental hopping. This is also consistent with prior similar EcoRI chase binding assays.[15,36] This may be due in part to EcoRI's

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~1000-fold discrimination for specific over nonspecific DNA[37] or its heavy reliance on sliding. A similar, yet noncatalytic, assay used by Siderova et al.[34] also detects intersegmental hopping for EcoRI. Importantly, this chase assay is useful in determining whether an enzyme can utilize intersegmental hopping (similarly to others using single-site substrates[18,19,34]), but it is uninformative as to the relative extent that other possible translocation mechanism are employed. EcoRV is capable of intersegmental hopping at salt concentrations where it is unlikely to be processive, suggesting that this mechanism can contribute to site finding *in vivo*.

Evidence for EcoRV Reliance on Intersegmental Hopping: Systematic Variation in Flanking DNA Length. The processivity trend from a series of substrates where the intersite spacing remains the same (42 bp) but the flanking DNA length changes can be informative regarding translocation mechanism (Figure 7A).[17] For example, we recently showed

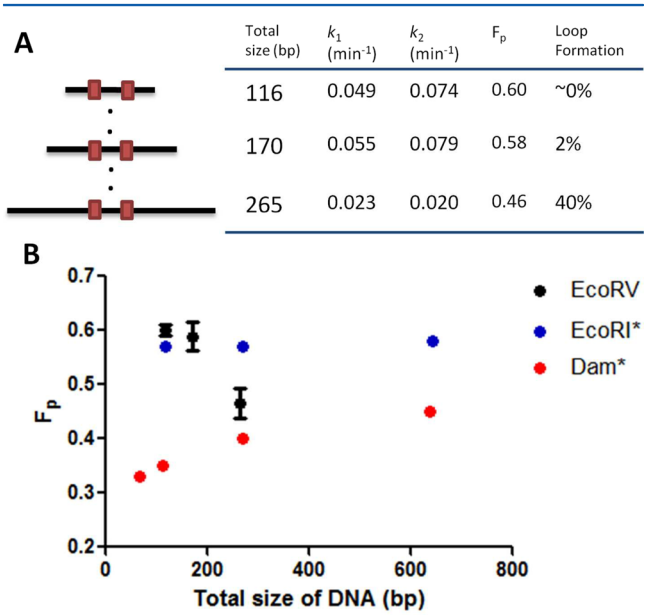

Figure 7. Processivity trends with changes to flanking DNA amounts. (A). Processivity data for EcoRV on DNA substrates that have identical intersite spacings. Loop formation probability (%) is based on the looping equation,[24] where DNA under ~200 bp is unlikely to undergo looping. Here, DNA > 500 bp has a 100% loop formation probability. (B) Processivity data for EcoRV, black dots. Loss of EcoRV processivity is consistent with intersegmental hopping "distracting" from sliding or hopping. EcoRI* does not utilize looping, and its processivity trend is not altered within this set of substrates. Dam* can utilize only intersegmental hopping, and its processivity slightly increases. * indicates replotted from ref 17. Copyright 2014 Elsevier.

that EcoRI's processivity is insensitive to increases in the amounts of flanking DNA (Figure 7B, replotted from ref 17. Copyright 2014 Elsevier). This lack of responsiveness is consistent with eq 9 (which depends only on the amount of DNA between the sites) and therefore is further suggestive of a translocation mechanism that relies significantly on sliding.[17] Importantly, the size regime of the total length of the substrates coincides with a dynamic range of DNA loop formation probability, where DNA under ~200 bp will not loop but is increasingly likely to do so between 200 and 600 bp.

Unlike EcoRI, EcoRV's processivity is modulated with changes in flanking DNA lengths. The two shorter substrates (116 and 170 total bp size, Figure 7) are more processive than the longer substrate (265 total bp size, Figure 7, replot from Figure 3). A compelling explanation for this trend involves the fact that intersegmental hopping proceeds though DNA loops on DNA > 200 bp.[17] The smaller two substrates are therefore highly unlikely to enable the protein to use intersegmental hopping due to their size and therefore the enzyme can only slide or hop, which it appears to do quite efficiently given the high processivity values (Figure 7A). The longer substrate is interestingly less processive (Figure 7B). Since the sites are only 42 bp apart, intersegmental hopping may represent a distraction, guiding the enzyme away from sliding or hopping to the adjacent second site and instead transferring it to a distal DNA segment. This notion is corroborated considering the individual rate constants of the data set, where the longer substrate is slower than the two smaller ones (Figure 7A). Because the search for the sites following the association between the enzyme and the DNA is rate-limiting, movement between loops slows the reaction. Importantly, the 265 bp substrate follows the trends from Figures 3 and 4 (it is all the same data point) and therefore is unlikely to be an outlier.

DISCUSSION

Facilitated diffusion on DNA is a ubiquitous cellular process employed by diverse classes of proteins, including transcription factors, DNA methyltransferases, restriction endonucleases, and DNA repair enzymes. That these proteins passively locate their largely undisclosed sites of action, which can differ from nontarget sites by as little as a few atoms, under biological time scales is impressive, yet not fully understood. While sliding, hopping, and intersegmental hopping mechanisms have been identified and characterized, examples of how these mechanisms are coupled to biological processes are seldom considered. Here, we continue to explore if particular proteins utilize different combinations of these mechanisms to accommodate their cellular function(s). We previously showed that Dam primarily uses intersegmental hopping in comparison to sliding and hopping mechanisms, which supports the necessary large movements each enzyme must make along the genome. In contrast, repair enzymes, such as uracil DNA glycosylase (UNG), are effective at moving short distances along DNA using hopping and sliding but are poorly processive when sites are separated by more than ~100 bp. Repair enzymes have high cellular copy numbers (~10^5) and excise damaged bases that are very rare (1 every 10^6 bp).[12,15] Therefore, *in vivo* processive catalysis is unlikely, and a scenario where each enzyme is confined to shorter stretches of genomic DNA seems probable. Furthermore, Dam readily skips sites (methylation marks separated by 1 kb are sufficient for mismatch repair, whereas each site is typically separated by only ~250 bp), whereas the repair of each target by UNG is more critical, and rigorous and repetitive searching of local regions is necessary. Biological context is clearly connected to the combination of translocation mechanisms that a particular protein will use, and our study here reveals this for two closely related enzymes.

EcoRI and EcoRV display distinct translocation properties, and we are interested in whether this helps to explain the enormous biological investment in the diversity and coexistence of distinct Type II systems. We provide several lines of evidence that EcoRI relies on an extreme sliding mechanism, where it can make ~290 000 single base pair movements within a single binding event before dissociation (Figure 3B). The

excellent fit of our processivity data to the theoretical sliding model is the initial basis for this conclusion. Importantly, our EcoRI processivity results are consistent with several prior reports. For example, Modrich and colleagues demonstrated that EcoRI processivity is only slightly less when two sites are 377 bp apart compared to that with a 51 bp separation.[27] Extrapolations by Rau and co-workers suggest that EcoRI uses a sliding mechanism and is capable of sliding 400 bp, on average, prior to dissociation.[28] Recent computational work further invokes EcoRI's propensity for sliding.[38] Single molecule and NMR experiments have also been used to clearly distinguish sliding from other mechanisms.[21,39,40] The calculated sliding lengths from these studies are generally much shorter for other proteins than what is seen here for EcoRI,[39,41] consistent with the notion that EcoRI's sliding is indeed extensive.

In contrast, the EcoRV processivity data does not fit the sliding model, consistent with results from Stanford et al.[14] This precludes us from estimating a sliding distance for EcoRV, but it invokes a mechanism involving a blend of sliding and hopping (Figure 3C). The minor dependence on salt concentration shown by EcoRI in comparison to that for EcoRV is further consistent with the former being more reliant on sliding, as each movement does not involve microscopic dissociation−reassociation steps as is seen in hopping. EcoRV's lack of processivity with short site spacing under high salt concentrations (Figure 4) is suggestive that EcoRV is unlikely to continuously interrogate short segments of DNA *in vivo* (~100 mM NaCl).[14] As cellular ionic strength conditions fluctuate up to 100-fold to respond to environmental changes,[42] enzymes that undergo the same task but respond differently to salt are likely to be of benefit to the cell. Our chase DNA experiments (Figure 6) show that EcoRV is more likely to fluidly move between distal DNA segments, thereby expanding the breadth of DNA that it can search. This is most dramatically highlighted considering Figure 7, where EcoRV is less effective in processively cleaving shortly spaced sites when the size of the DNA increases. Unlike Dam and EcoRI, which primarily rely on intersegmental hopping and sliding, respectively, EcoRV does not appear to dominantly use either and instead likely relies on a blend of mechanisms.

Our interpretation of the translocation mechanism used by EcoRV in Figure 7 is reconciled by comparisons with prior EcoRI and Dam data sets.[17] If sliding dominates translocation, then EcoRV's processivity will be insensitive to changes in flanks; therefore, sliding dominates for EcoRI but not for EcoRV. Importantly, our result is consistent with EcoRV utilizing intersegmental hopping to a greater extent than does EcoRI. This variation (Figure 7) of the standard processivity assay (Figure 3) succeeds in partitioning intersegmental hopping from other potential mechanisms and suggests that EcoRV uses intersegmental hopping in relative tandem with sliding and hopping mechanisms, without one being especially dominant. In contrast, EcoRI dominantly uses sliding. Reconciliation with prior Dam data is more convoluted: because Dam is nearly incapable of sliding/hopping, the addition of flanking DNA to provide looping actually increases its processivity. However, we imagine that the path resulting in processive catalysis is quite circuitous here. In contrast, EcoRV uses sliding/hopping to a greater extent than does Dam. Therefore, intersegmental hopping by flanking nonspecific DNA confounds processivity for EcoRV for these substrates (Figure 7B) considering that traveling the short distance between sites is much more likely to be productive using sliding/hopping. Previous reports show that intersegmental hopping further assists sliding/hopping mechanisms in site locating for EcoRV;[18,19] however, these reports concern long (several kilobase pair) substrates, where intersegmental hopping is likely to be helpful. In contrast, Figure 7 concerns movement between only 42 bp.

Relative protein expression levels may help to explain why EcoRI and EcoRV have different translocation properties but still can individually effectively cleave phage DNA. In *E. coli*, EcoRI has ~1000 dimer copies,[43,44] whereas EcoRV has only 100 dimer copies.[45] Due to its sliding mechanism, each EcoRI enzyme is confined to a smaller region of DNA; however, the abundance of protein copies extends its occupancy along DNA (Figure 8). Although rarer, each EcoRV enzyme can extend its footprint to more distant regions of DNA. These two distinct translocation mechanisms may function synergistically to ensure phage defense. Importantly, the drawback of one of these translocation mechanisms is mitigated by the other, creating a robust phage restriction strategy.

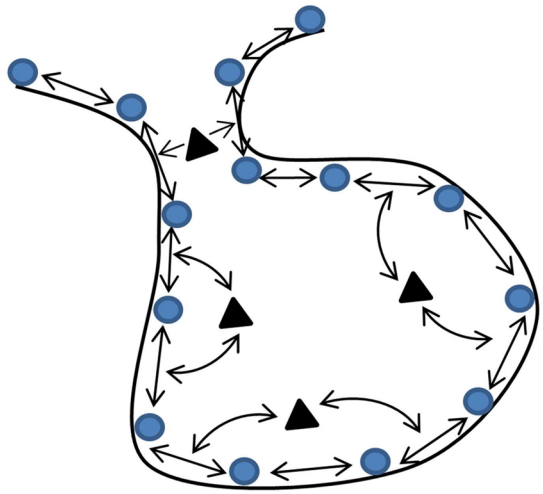

Figure 8. Relative protein levels explain translocation diversity. EcoRI's high cellular protein levels in comparison to that of EcoRV help to explain why both can effectively cleave phage DNA. EcoRI (circles) redundantly slides over smaller regions, but large cellular copy numbers allow it to search more of the DNA. EcoRV's (triangles) ability to undergo hopping and intersegmental hopping causes site skipping but affords a broader search of the DNA.

There are other distinctions between these two enzymes that make their differing translocation mechanisms seem more reasonable. EcoRI does not bend the DNA at its site, whereas EcoRV does.[29] As protein-mediated DNA bending can facilitate DNA looping,[46] this may be related to enhancing the ability of EcoRV to carry out intersegmental hopping. EcoRV leaves blunt ends, whereas those of EcoRI are sticky, leaving 4 overhanging nucleotides. These overhanging bases may facilitate the enzyme to stay more closely positioned to its cutting site following catalysis, as DNA binding proteins readily bind to sticky ends. In contrast, EcoRV leaves its site more readily following cutting, allowing it to be intercepted by proximal looping DNA. The blunt and sticky end distinction between EcoRI and EcoRV is also strong evidence that these enzymes evolved in different organisms[6] and therefore may have evolved separate translocation mechanisms. The extent of the distinction between these two enzymes may be surprising to those familiar with their similarities: their recognition sites

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are nearly identical, the proteins are similar in size, and they use the same cofactor and maintain the same oligomeric state for catalysis.[6] Structural classification suggests evolutionary divergence separates blunt end- and sticky-end generating ENases.[47,48] Interestingly, unlike their methyltransferase partners, there is very little homology or correlation between structure and function between ENases other than the conserved (D/E)XK catalytic motif.[49] Unfortunately, a structural basis for predicting translocation mechanism does not exist for these saddle proteins,[50] other than in examples of proteins with positively charged, disordered, DNA binding tails.[22]

An appreciation of ENase translocation mechanism diversity may help to explain why 80% of bacteria have multiple R−M systems. Given the high fitness cost of maintaining R−M systems,[1] acquiring several may seem superfluous. However, certain strains of bacteria contain as many as 16 R−M systems.[51,52] Phage anti-restriction mechanisms, other bacterial phage defense systems distinct from R−M systems (introduction), and the evolutionary advantage of acquiring foreign DNA all highlight the intricacies and extent of the arms race between bacteria and invading foreign nucleic acids.[1,5] Having several distinct R−M systems in a single cell is in part correlated with the propensity of R−M systems to act as "selfish elements" spreading through horizontal gene transfer, where their removal is avoided because it results in post-segregational killing.[44] Another explanation of replicate R−M systems relies on their involvement in the evolution of new strains, where differing recognition specificities can segregate species into distinct variants.[1,51] Interestingly, bacteria lacking RecBCD, which effectively cleaves invading phage DNA, contain more R−M systems,[51] suggesting multiple R−M systems can have an additive effect on phage defense. Overall, the prevalence and functional consequences of the redundancy of type II R−M systems remains a mystery,[51] which is also the case for the related Type II toxin−antitoxin systems.[53] On the basis of our results, we suggest that a plausible explanation for the existence of several restriction systems in a single organism is their mechanistic differences and that translocation mechanisms may contribute to this. Instead of redundancy, distinct site-finding strategies likely confer effective phage restriction and may contribute to the other functions of R−M systems.

■ ASSOCIATED CONTENT

Supporting Information

Comparison of processivity trends as predicted by the sliding model and an exponential decay model using a k_{slide}/k_{off} ratio of 99 999:1; details regarding the construction of the DNA substrates used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

ENase, restriction endonuclease; EcoRI, restriction endonuclease EcoRI; EcoRV, restriction endonuclease EcoRV; bp, base pair; kb, kilobase pairs; CRISPR, clustered regularly interspaced short palindromic repeats; R−M, restriction−modification system; Dam, DNA adenine methyltransferase; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; UNG, uracil DNA glycosylase

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