RESEARCH ARTICLE

The protein–protein interactions required for assembly of the Tn*3* resolution synapse

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Abstract

The site-specific recombinase Tn*3* resolvase initiates DNA strand exchange when two *res* recombination sites and six resolvase dimers interact to form a synapse. The detailed architecture of this intricate recombination machine remains unclear. We have clarified which of the potential dimer–dimer interactions are required for synapsis and recombination, using a novel complementation strategy that exploits a previously uncharacterized resolvase from *Bartonella bacilliformis* ("Bart"). Tn*3* and Bart resolvases recognize different DNA motifs, via diverged C-terminal domains (CTDs). They also differ substantially at N-terminal domain (NTD) surfaces involved in dimerization and synapse assembly. We designed NTD-CTD hybrid proteins, and hybrid *res* sites containing both Tn*3* and Bart dimer binding sites. Using these components in in vivo assays, we demonstrate that productive synapsis requires a specific "R" interface involving resolvase NTDs at all three dimer-binding sites in *res*. Synapses containing mixtures of wild-type Tn*3* and Bart resolvase NTD dimers are recombination-defective, but activity can be restored by replacing patches of Tn*3* resolvase *R* interface residues with Bart residues, or vice versa. We conclude that the Tn*3*/Bart family synapse is assembled exclusively by *R* interactions between resolvase dimers, except for the one special dimer–dimer interaction required for catalysis.

KEYWORDS

binding sites, genetic recombination, helix turn helix motif, multiprotein complex, recombinase, transposon resolvase

1 | INTRODUCTION

The resolvases of the bacterial transposons Tn*3* and γδ are closely related DNA site-specific recombinases and founder members of the serine recombinase family (Rowland and Stark, 2005; Stark, 2014). They function to resolve cointegrate intermediates of replicative transposition (Grindley, 2002), and are characterized by stringent topological selectivity: recombination (resolution) is licensed only when two recombination sites (*res*) are oriented in direct repeat (head to tail) within a supercoiled DNA molecule. Each *res* has three binding sites for resolvase dimers (Figure 1a); one of these (site I, the crossover site) is centred on the bonds that are broken and rejoined during recombination. In a productive reaction, the two *res* sites and six bound resolvase dimers assemble into an interwound synapse
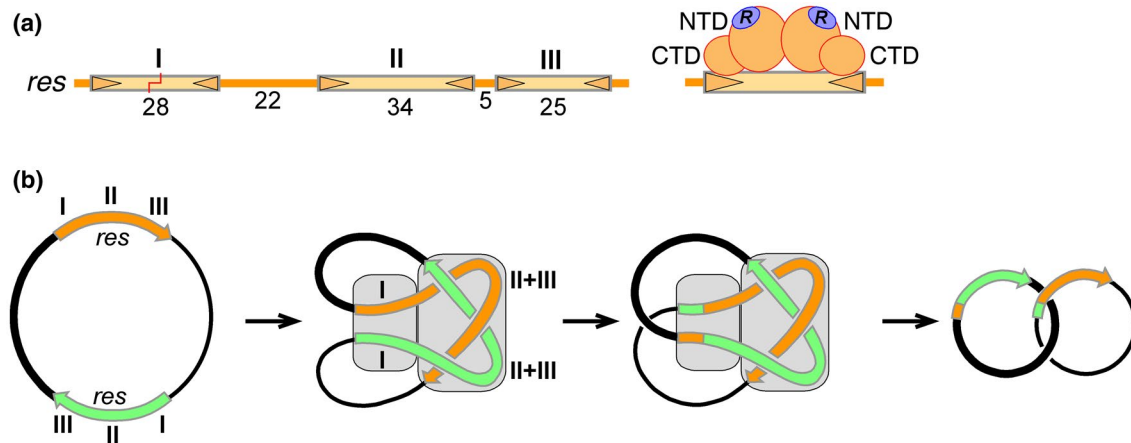
FIGURE 1 Site-specific recombination by Tn*3* resolvase. (a) The Tn*3 res* site (114 bp) contains three resolvase-binding sites (I, II, and III; the lengths of the sites and inter-site spacers are shown). Each binding site is recognized by a resolvase dimer (simplified cartoon on right). The NTDs, CTDs, and *R* interface regions are labeled. The staggered red line indicates the staggered break (with 2-nucleotide overhangs) when the DNA strands are cut during recombination. (b) Synapsis and recombination. Two *res* sites, directly repeated in a supercoiled plasmid, are brought together by interactions between *res*-bound resolvase dimers, forming a synapse. Three negative supercoil nodes are stabilized by interactions between *res* sites II and III (larger grey shape) and are trapped by interactions between the dimers bound at site I (smaller grey shape), forming a catalytic tetramer. This tetramer then catalyses recombination by breaking, exchanging and rejoining DNA strands. The DNA product is a 2-noded catenane

(Figure 1b). Within this complex, the dimers at site I interact and undergo large conformational changes to form a tetramer, which catalyses DNA strand exchange by a rotational mechanism (Stark *et al.*, 1989; Li *et al.*, 2005; Rice, 2014). Four more resolvase dimers, bound at sites II and III (the accessory sites), do not participate in catalysis but have essential regulatory and architectural roles within the synapse. Resolvase can mediate synapsis of two sets of accessory sites when site I is deleted, whereas site I-bound wild-type (wt) resolvase dimers do not make a stable synapse, or promote recombination, in the absence of the accessory sites (Watson *et al.*, 1996; Grindley *et al.*, 2006). However, "activated" resolvase gain-of-function mutants have been characterized, which can synapse and recombine two copies of isolated site I (Arnold *et al.*, 1999; Sarkis *et al.*, 2001; Burke *et al.*, 2004; Olorunniji *et al.*, 2008; Rice, 2014).

Tn*3*/γδ resolvase (185/183 amino acid residues) has two domains (Figure 2). The ~45-residue C-terminal domain (CTD) recognizes and binds to sequence motifs at the ends of each dimer-binding site in *res*. The ~140-residue N-terminal domain (NTD) contains the active site for DNA strand cleavage and rejoining; it also contains all the surfaces currently known to mediate subunit interactions (Grindley, 2002). A specific interaction between NTDs, seen in crystal structures of γδ resolvase, was shown to form both the dimer that binds to *res* and the solution dimer (Sanderson *et al.*, 1990; Hughes *et al.*, 1993; Rice and Steitz, 1994a; Yang and Steitz, 1995; Figure 2a). Using activated resolvase variants (see above) several structures of the catalytic tetramer have been solved, without DNA or in complex with site I DNA that has been cleaved by the enzyme to create double-strand breaks (Nöllmann *et al.*, 2004; Li *et al.*, 2005; Kamtekar *et al.*, 2006; Figure 2d). Formation of the tetramer involves substantial rearrangement of the dimer structure and new contacts between the two dimers.

Several other types of interactions between resolvase subunits were observed in the crystal structures (Sanderson *et al.*, 1990), but only one of these has been shown to have biological relevance; here, we call it the *R* (for *R*egulatory) interface (previously referred to as the 2–3′ interaction/interface). The *R* interface is not required for binding of resolvase dimers to DNA, nor is it involved directly in catalysis at site I, but it is required for assembly of the synapse. Mutations at any of four key *R* interface residues (R2, R32, K54, and E56; Figure 2a,c) can abolish recombination (Hughes *et al.*, 1990), and there is evidence that activity of the catalytic tetramer at site I requires *R* interactions between the resolvase subunits bound at site I and subunits bound at the accessory sites (Grindley, 1993; Burke *et al.*, 2004; Li *et al.*, 2005). Previous analysis has defined relationships between the positions of resolvase subunits on the *res* DNA within the synapse and requirement for *R* proficiency (Murley and Grindley, 1998). A number of structural models of the synapse incorporating *R* interactions have been proposed (Rice and Steitz, 1994b; Murley and Grindley, 1998; Sarkis *et al.*, 2001; Grindley, 2002; Rowland *et al.*, 2002; Mouw *et al.*, 2008; Rowland *et al.*, 2009). However, the involvement of additional protein–protein interfaces has not been ruled out.

Productive synapsis by Sin resolvase, a related serine recombinase (~30% identity; Rowland *et al.*, 2002), has been shown to require a similar *R* interface, involving two residues (F52 and R54) that align with Tn*3*/γδ resolvase *R* interface residues K54 and E56 (Mouw *et al.*, 2008; Rowland *et al.*, 2009; Rice, 2014). The Sin and γδ resolvase *R* interfaces are structurally remarkably similar, despite the different amino acid residues involved. Sin-mediated synapsis of *resH* recombination sites was shown to require yet another specific interface, between the CTDs of Sin dimers. Sin mutagenesis and crystallographic data led to the first detailed structural model
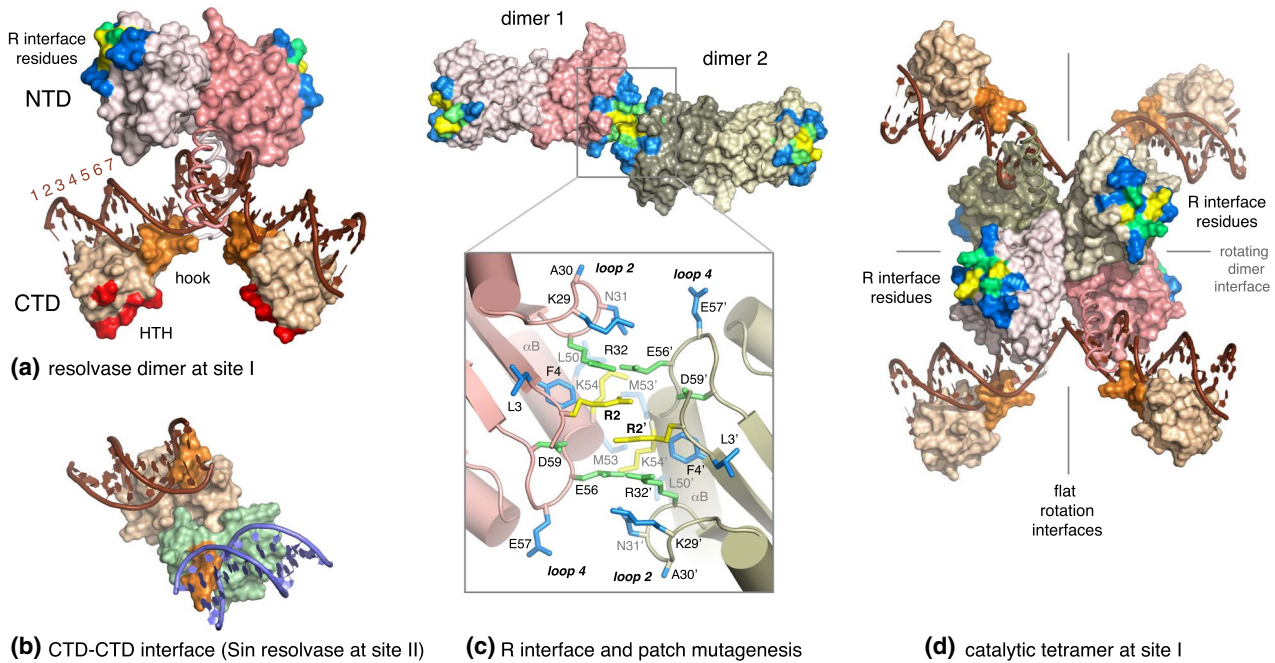
(a) resolvase dimer at site I

(b) CTD-CTD interface (Sin resolvase at site II)

(c) R interface and patch mutagenesis

(d) catalytic tetramer at site I

FIGURE 2 Interfaces between resolvase subunits. (a) Resolvase dimer bound at site I of *res* (pdb 1GDT; Yang and Steitz, 1995). The NTDs of the two resolvase subunits are colored salmon and light pink; the NTD arm regions (119-140) are rendered as backbone. *R* interface residues are colored yellow, green, and blue (see part C). The CTDs are colored to distinguish the "hook" (gold; residues 141-145) from the helix-turn-helix (HTH) module (light orange; residues 146-183). CTD surface residues mutated to rule out a hypothetical CTD–CTD interface are highlighted in red (149, 150, 153, 157, 167; further details in Figure S1). The numbering (1–7) of the DNA base pairs bound by the light pink subunit corresponds to that shown in Figure 3c (see also Figure S1b). (b) Sin resolvase CTD–CTD synapsis interface at site II (pdb 2R0Q; Mouw *et al.*, 2008). The CTD hook (gold; residues 147–151) is distinguished from the HTH module (light orange or green; residues 152–200), as in part A. The NTDs are not shown. (c) The *R* interface between two γδ resolvase dimers (salmon and light pink, light and dark olive; pdb 2RSL; Rice and Steitz, 1994a). The inset shows the main sidechains involved in the *R* interface. The canonical *R* interface tetrad is shown in yellow (R2, K54) and green (R32, E56). Ten surface residues (colored yellow and blue) were replaced to switch the interface specificity to mimic Bart resolvase (for details, see text and Figure S5). The three residues colored green (R32, E56, and D59) were not altered, as these are identical in Tn3/γδ and Bart resolvase. (d) γδ resolvase catalytic tetramer in a cleavage complex with two copies of site I (pdb 1ZR4: Li *et al*, 2005). Note that the *R* interface residues (colored as in part c) do not overlap other functional interfaces in the tetramer. Subunits are colored and rendered as in a and c

of a complete recombination synapse, incorporating CTD-CTD interactions (Figure 2b) between Sin dimers bound at the accessory site (site II) and *R* interactions between subunits at site I and site II (Mouw *et al.*, 2008; Rowland *et al.*, 2009). The Sin and Tn3/γδ catalytic tetramers are structurally similar (Keenholtz *et al.*, 2011; Trejo *et al.*, 2018), so synaptic site I - site I interactions were modeled using the γδ resolvase-site I synaptic structure 1ZR4. A speculative model of the Tn3/γδ synapse of two full *res* sites incorporating a similar CTD–CTD interface was suggested (Mouw *et al.*, 2008), but there is no current experimental evidence for this type of CTD interaction in the Tn3/γδ system (Hughes *et al.*, 1990; Grindley, 1993).

High-resolution structural analysis of the complete synapse for any regulated site-specific recombination system has not been achieved to date. Biochemical characterization of the network of protein–DNA and protein–protein interactions in such complexes is also very challenging. For the Tn3 resolution synapse, there are 12 identical resolvase subunits which form six dimers (three dimers bound to each *res*). To probe the resolvase architecture in the synapse, we need to be able to identify the dimers bound at two or more specific sites, to assess whether and how they interact. Our

chosen strategy required two types of resolvase that form structurally similar dimers on their binding sites in *res*, but are otherwise "orthogonal"; that is, each resolvase dimer recognizes only its own binding site sequences in a two-*res* substrate plasmid, and can make interactions with dimers of its own type, but not with the other type. By assaying for recombination proficiency, we would then be able to determine whether any particular arrangement of dimers can form the network of interactions needed to build a productive synapse.

We therefore sought a recombinase which is similar to Tn3 resolvase in its structure and synapse architecture, but which differs substantially in its DNA sequence recognition specificity and in surfaces implicated in synapse assembly. A previously uncharacterized resolvase from the sandfly-borne human pathogen *Bartonella bacilliformis* (GenBank ABM45303.1; hereinafter called Bart resolvase) was selected as being potentially suitable. Bart and Tn3 resolvases have only 49% of amino acid identity, but can be aligned with no gaps (Figure 3a) along with other related proteins that together form a "Tn3/γδ/Bart family" of serine resolvases (Figure S2). The Bart and Tn3 *res* sites are similarly arranged, but the sequence motifs predicted to be recognized by the resolvase CTDs are very different (Figure 3b,c), suggesting that
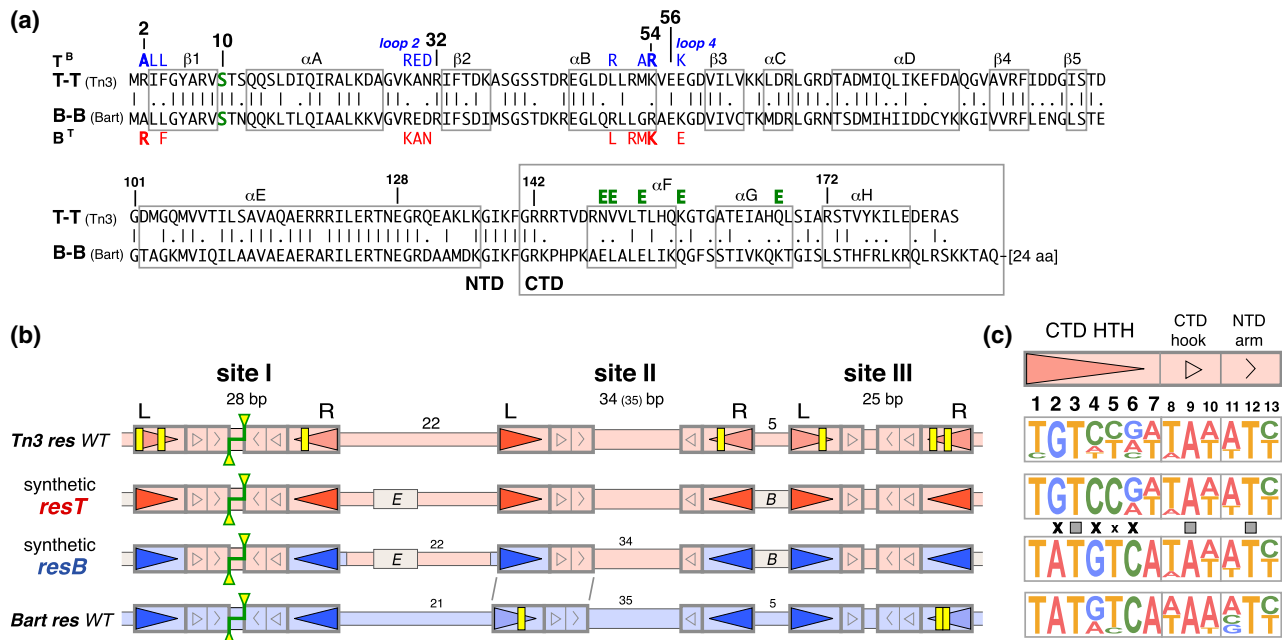
FIGURE 3 Tn*3* and Bart resolvases and *res* sites. (a) Amino acid sequence alignment of Tn*3* and *Bartonella* (Bart) resolvases. The 24 C-terminal residues of the natural Bart resolvase (Genbank ABM45303.1) were deleted in the Bart resolvase used in all our experiments (Figure S1). Secondary structure elements are indicated by narrow boxes and labels above the sequences, and the CTDs are in a wide box. Mutations of Tn*3* resolvase CTD residues that were made in order to test for CTD synapsis are indicated in green. The Tn*3* resolvase *R* interface residues R2, R32, K54, and E56, and the catalytic serine S10, are denoted by bold numbers above the sequences. The T[B] and B[T] NTDs have altered residues at the ten positions indicated by blue or red letters, respectively (see Results section and Figure S5). (b) *res* sites. *resT* and *resB* are synthetic sites for Tn*3* and Bart resolvases. They differ only in the six motifs (bold red or blue arrows) recognized by the helix-turn-helix (HTH) part of the CTD. Other sequences derive from WT Tn*3 res* (pale red), except the restriction sites used to construct the various *resT/resB* hybrids (boxes labeled E: EcoRI, and B: BglII) and two base pairs flanking site II in *resB* (pale blue). The full sequences of the sites are in Figure S3. To reduce off-target binding, *resT* and *resB* motifs were made fully uniform at positions 1–5, by substituting all the noncanonical bases (yellow bars) present in the WT sites. The DNA motifs contacted by the NTD arm and the CTD hook modules (small open and closed arrowheads, respectively) are similar in the Tn*3* and Bart sites (see part C and Figure S3). (c) CTD target selectivity. The sequence logos compare base frequencies (linear scale) in the six half-sites of the synthetic and natural *res* sites (excluding positions 11–13 of site IIR and site IIIL; see Figure S3). Crosses indicate bases that distinguish the *resT* and *resB* motifs: the corresponding single base change from γδ to Bart at these positions reduces CTD affinity by ≥100-fold (positions 2, 4, 6) or 9-fold (position 5) (Rimphanitchayakit and Grindley, 1990). Filled squares indicate bases present in all motifs

each CTD type (Tn*3* or Bart) would specifically recognize its cognate motif. Furthermore, the Tn*3* and Bart CTDs have very low amino acid sequence identity (22%), so it is likely that any synaptic CTD–CTD or NTD–CTD interactions would be disrupted by substitution with a CTD of the other type. The Bart and Tn*3* resolvase proteins also differ significantly at the *R* interface. Two of the key Tn*3* resolvase *R* interface residues (R2 and K54) are different in Bart resolvase (A2 and R54); one of these differences, when introduced into Tn*3*/γδ resolvase as an R2A mutation, is known to disrupt the interface and block recombination (Hughes *et al.*, 1990; Wenwieser, 2001). We therefore hypothesized that Bart and Tn*3* resolvases both make *R* interactions, but that their *R* interface surfaces would be incompatible. We further speculated that it might be possible to substitute the *R* interface residues of Tn*3* resolvase with the equivalent Bart resolvase residues and vice versa, thereby giving one resolvase the *R* interaction specificity of the other.

Here, we present experiments that rule out any required protein–protein interactions of the CTDs. We report the design and testing of a modular synthetic *res* site, which can be tailored to support either Tn*3* or Bart resolvase-mediated recombination by changing the

DNA sequence motifs recognized by the CTDs. Using hybrid *res* sites containing both Tn*3* and Bart sequence motifs, and hybrid Tn*3*/Bart resolvases, we demonstrate a requirement for specific *R* interface interactions by resolvase dimers at each of the three *res* binding sites (I, II, and III), and conclude that these, together with the interaction at site I that forms the catalytic tetramer, are necessary and sufficient for productive synapse assembly. We anticipate that the technologies developed in this work will help us to map the full network of protein–protein interactions in the Tn*3* resolution synapse.

2 | RESULTS

2.1 | Synapsis-defective mutants of Tn3 resolvase

Several types of interfaces between resolvase subunits and dimers have been hypothesized in published models of the Tn*3*/γδ resolution synapse (see Introduction). In preliminary experiments to identify amino acid residues that might contribute to synaptic

interfaces, we screened a library of Tn*3* resolvase random mutants, using *E. coli* assays similar to those described previously for isolating synapsis mutants of Sin (Mouw *et al.*, 2008; see Experimental Procedures). These assays identify mutants that are proficient in binding at site I, but defective in synapsis of sites II and III (and thus defective in recombination). In contrast to our analogous Sin screen, which identified synapsis-defective mutations at the CTD synaptic interface but not at the NTD *R* interface (see Introduction), all the effective Tn*3* resolvase mutations mapped to the NTD––to the four canonical *R* (2-3′) residues R2, R32, K54, and E56, and also the non-*R* residue E128 (Supplementary Information SI 1). The synapsis defect of the E128K mutant might be because it fails to bind properly at *res* site III, as has been shown for the γδ resolvase E128K mutant (Hatfull *et al.*, 1987) and which we have observed in binding assays in vivo (data not shown). In summary, these mutagenesis-screening results are consistent with an essential role for the *R* interface in assembling the Tn*3* resolution synapse, and provided no evidence that CTD synaptic interactions are important. Further evidence against synaptic CTD interactions is presented below.

2.2 | A uniform res site design for Tn3 and Bart resolvases

The strategy outlined in the Introduction, to probe inter-subunit interactions in the synapse, requires a uniform *res* site that can be customized to bind Tn*3* or Bart CTDs at specific positions simply by altering the CTD-binding DNA sequence motifs. The predicted natural Bart *res* site is very similar to that of Tn*3 res* in its organization of binding sites and "spacer" sequences (Figure S3). We therefore designed two synthetic 114-bp *res* sites, *resT*, and *resB*, for the Tn*3* and Bart resolvases, respectively, which differ only in the 7-bp sequences recognized by the helix-turn-helix (HTH) motifs of the CTDs, the other sequence elements all having Tn*3* character (Figure 3b). The design included sites for restriction endonucleases to facilitate the construction of hybrid *res* sites. The Bart and Tn*3* CTD-binding motifs differ at four positions (Figure 3c). Previous analysis indicates that the Tn*3* resolvase CTD should not recognize the Bart motifs (Rimphanitchayakit and Grindley, 1990), and we predicted that likewise, the Bart resolvase CTD would not recognize the Tn*3* motifs.

2.3 | Activities of Tn3 and Bart resolvases on resT and resB substrates

The alignable parts of the Tn*3* and Bart resolvase CTDs are only 22% identical, and the Bart CTD is 28 residues longer at its C-terminus (Figure 3a). This C-terminal extension is not needed for recombination activity, and we truncated it by 24 amino acids, making the two CTDs similar in size. All of the experiments described below used this truncated Bart CTD.

Recombination activity was assayed in *E. coli*. The resolvase proteins were expressed in cells containing a two-*res* substrate plasmid with Tn*3*- and/or Bart-type binding sites (Figure 4a). The two *res* sites flank a *galK* indicator gene that is deleted by resolvase-mediated recombination (resolution) (Burke *et al.*, 2004; Rowland *et al.*, 2009). Efficient (>90%) resolution results in white colonies on MacConkey/galactose agar indicator plates, whereas red colonies indicate lower efficiency (or no) resolution (Arnold *et al.*, 1999; Burke *et al.*, 2004; Rowland *et al.*, 2005). The extent of resolution can be determined more accurately by gel electrophoresis of the plasmid DNA, or by transforming *E. coli* strain DS941 with the recovered plasmid DNA (selecting for the antibiotic resistance marker on the recombination test plasmid only), and counting red and white colonies on MacConkey/galactose agar plates (see Experimental Procedures).

Tn*3* resolvase efficiently recombined a substrate containing two *resT* sites, and Bart resolvase efficiently recombined a substrate containing two *resB* sites (Figure 4b, lines 1 and 2). The activity of Tn*3* resolvase on *resT* sites and on wild-type (wt) Tn*3 res* sites was indistinguishable by this assay. As expected, Tn*3* resolvase had no measurable activity on a substrate containing two *resB* sites, Bart resolvase was similarly inactive on a *resT* substrate, and a substrate containing one *resT* and one *resB* was not resolved when either one or both resolvases were expressed in the cells (Figure 4b, lines 1–3). Recombination of a *resT* or *resB* substrate (by Tn*3* or Bart resolvase, respectively) was abolished by deleting the accessory binding sites (II and III) from one of the two *res* sites (data not shown), demonstrating that wt Bart resolvase, like wt Tn*3* resolvase, requires two complete *res* sites for activity. From these results we draw the following conclusions. (1) Bart resolvase is active and efficient, and uses a synapse whose architecture is like that of Tn*3* resolvase. (2) Tn*3* and Bart resolvases specifically recognize their cognate *res* sites. (3) The sequences common to *resT* and *resB* support resolution by both Tn*3* and Bart resolvases, suggesting that DNA-binding specificity is conferred primarily by the resolvase CTDs. The Tn*3*-derived central sequences of the binding sites (which the NTDs are predicted to contact) support high resolution activity by both Tn*3* and Bart resolvases. (4) Tn*3* and Bart resolvases cannot complement each other to resolve a *resT* × *resB* substrate (presumably because they cannot make required synaptic protein–protein interactions).

2.4 | NTD–CTD hybrid resolvases: CTD interactions are not required for synapsis

To dissect the role of the resolvase CTD in synapsis, we made two Tn*3*-Bart hybrid resolvase proteins, each with the NTD of one resolvase and the CTD of the other (sequences shown in Figure 3a). The hybrid with a Tn*3* NTD and a Bart CTD (abbreviated to T-B resolvase) is predicted to recognize *resB* but not *resT*, and accordingly recombined the *resB* × *resB* but not the *resT* × *resT* substrate (Figure 4b, line 4). Likewise, the Bart NTD–Tn*3* CTD hybrid (B–T resolvase) resolved the *resT* × *resT* but not the *resB* × *resB* substrate (line 5). The high activity of these
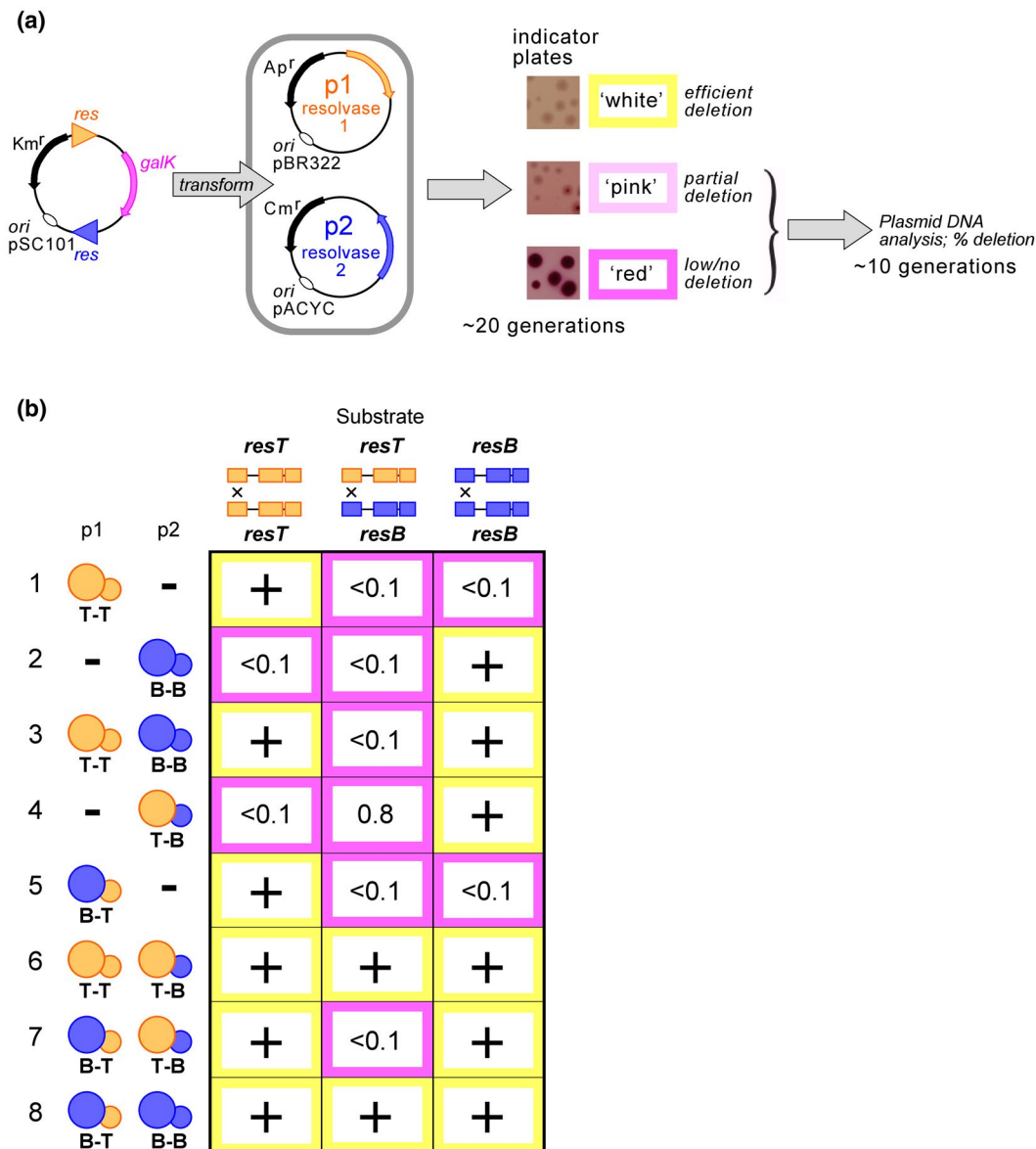
FIGURE 4 Recombination of *resT* and *resB* substrates. (a) Assay for recombination activity in *E. coli*. The test substrate plasmid contains two *res* sites (*resT*, *resB*, or modified versions as described in the text) in direct repeat, flanking a *galK* marker gene. In *E. coli* cells (strain DS941; *galK⁻*) containing appropriate resolvase expression plasmids, recombination between the two *res* sites creates two product circles. The circle containing *galK* has no origin of replication and is lost during subsequent cell divisions. Colonies in which recombination (deletion) was efficient (approaching 100%) lack GalK and are thus uncolored ("white") on galactose-MacConkey agar indicator plates, whereas colonies with inefficient or zero recombination retain GalK activity and are red. Intermediate recombination efficiency gives pink colonies. Cells from a pool of >100 red or pink colonies were cultured further and plasmid DNA was recovered to measure % recombination, as described in Experimental Procedures. These % values therefore report on a later time point, when the substrate has been exposed to the resolvases in the cells for a further ~10 cell generations. The indicator plate pictures show assays 6, 10, and 7 from Figure 6a ("white," "pink," and "red" colonies, respectively). (b) Specificity of recombination of *resT* and *resB* substrates by Tn*3*, Bart, and hybrid resolvases. Tn*3* and Bart resolvases efficiently resolve only their fully cognate substrates (lines 1 and 2). They do not complement each other to resolve a *resT × resB* substrate (line 3). Hybrid resolvases B-T and T-B efficiently resolve substrates recognized by their CTDs (lines 4 and 5). The *resT × resB* substrate is resolved efficiently when two resolvases with the same type of NTD, but different CTDs are expressed (lines 6–8). Enzymes are named according to the identities of the NTD and CTD domains, in the order NTD-CTD. For example, B-T resolvase has a Bart NTD (B) attached to a Tn*3* CTD (T). "+" in pale yellow boxes indicates pale-colored ("white") colonies in the in vivo assay, and thus, 100% or near-100% resolution of the test substrate; magenta boxes indicate red colonies, for which % recombination was estimated as described in the Experimental Procedures section (numerical values in the boxes; see Figure 4a)

hybrid resolvases argues strongly that there are no essential synaptic NTD–CTD interactions, because the Tn*3* NTD should be incapable of functional interactions with the Bart CTD, and vice versa (see above).

Each NTD–CTD hybrid resolvase had only a very low level of activity on the *resT × resB* substrate (Figure 4b, lines 4 and 5), as expected (each resolvase should recognize only the site compatible

with its CTD). Nor did the two hybrid resolvases complement each other to recombine this substrate (Figure 4b, line 7), as expected (the Tn*3* and Bart resolvase NTDs should not interact productively). However, the *resT* × *resB* substrate was recombined efficiently by a hybrid resolvase together with the wt (non-hybrid) resolvase with the same type of NTD; that is, T–B resolvase plus Tn*3* resolvase (T–T), or B–T resolvase plus Bart resolvase (B–B) (Figure 4b, lines 6 and 8). In these experiments, the *resT* site should be exclusively occupied by Tn*3* CTDs and the *resB* site by Bart CTDs. We conclude that inter-site CTD–CTD interactions are not essential for productive synapsis, whereas inter-site NTD–NTD interactions are essential (in agreement with our mutagenesis experiments; see above).

To confirm these conclusions, we created two more hybrids, with the Tn*3* resolvase NTD linked to the CTDs of two other well-characterized resolvases, Tn*21* and Tn*552*. These CTDs interact weakly with Tn*3* DNA sequence motifs, despite having low amino acid sequence similarity to Tn*3* resolvase (Halford *et al.*, 1985; Rowland and Dyke, 1989; Grindley, 2002; Figures S2 and S4). The hybrid resolvases (with Tn*3* NTD and either Tn*21* or Tn552 CTD) act like wt Tn*3* resolvase in our assays; they recombine *resT* × *resT* but not *resB* × *resB*, and they complement T–B resolvase to recombine the *resT* × *resB* substrate (Figure S4). Any essential Tn*3* CTD-CTD interactions would have to be replaceable with Tn*3*-Bart, Tn*552*-Bart, or Tn*21*-Bart interactions in the above experiments, which seems very unlikely.

As a further test, we introduced multiple mutations on the solvent-accessible (non-DNA-binding) surface of the Tn*3* resolvase CTD (Figures 2a and 3a). Pairs or triplets of amino acid residues were mutated to the negatively charged residue glutamate, which should disrupt any CTD–CTD interactions by charge repulsion as well as surface non-complementarity (Supplementary Information SI 2; Figure S1). However, these mutants were all indistinguishable from wt Tn*3* resolvase in *E. coli* assays for recombination and for binding at the individual sites I, II, and III (data not shown).

We conclude that, unlike the Sin synapse (see Introduction), the Tn*3*/Bart synapse does not involve synaptic interactions between the CTDs.

2.5 | Resolvase dimers targeted to specific binding sites

To test whether resolvase dimers with Tn*3* or Bart resolvase CTDs can be targeted to specific binding sites within *res*, we made hybrid *res* sites containing all six possible arrangements of Tn*3* and Bart resolvase dimer–binding sites. These are denoted as **btt**, **tbt**, etc. The letters in each triplet indicate binding sites I, II, and III in that order; **t** represents a dimer-binding site with Tn*3* motifs, and **b** represents a dimer-binding site with Bart motifs.

We then constructed four test substrate plasmids, which are similar to the *resT* × *resT* substrate except that one or both *res* sequences are hybrids with one **b** site instead of a **t** site (**btt**, **tbt**, **ttb**;

Figure 5a). Tn*3* resolvase (T–T) resolved these substrates poorly, presumably because the Tn*3* CTD did not bind strongly to the **b** binding sites (line 1). Likewise, the hybrid B–T resolvase (which has a Tn*3* CTD) gave low levels of resolution (line 2). However, when B–T resolvase was complemented with Bart resolvase (B–B) which binds strongly to the **b** site(s), efficient resolution was restored (line 3).

Analogous results were obtained with substrates that are similar to *resB* × *resB* except that one or both *res* sequences are hybrids containing a **t** binding site (Figure 5b). However, some of these hybrid *res* substrates were fully or substantially resolved when just a single resolvase with Bart CTDs (B–B or T–B) was expressed (lines 1 and 2). This suggests that the Bart CTD can bind weakly to **t** sites, aided by the extensive cooperative NTD interactions within the synapse. Such relaxed sequence specificity has been proposed to be a general feature of protein–DNA interactions in nucleoprotein complexes (Hall and Halford, 1993).

We also used these hybrid-*res* substrates to target catalytically defective resolvase mutants to specific dimer-binding sites. The active site serine (S10 in Tn*3* resolvase) attacks the scissile DNA phosphodiester at the center of *res* binding site I during strand exchange; mutating it to alanine abolishes recombination activity while preserving DNA binding and subunit interactions (Olorunniji and Stark, 2009). Therefore, resolution should be inhibited if S10A NTDs are targeted to one or both copies of site I, but should remain efficient if S10A NTDs are targeted to sites II or III. This was the observed result with both Tn*3* and Bart resolvase S10A mutants (Figure 5a, line 4; Figure 5b, line 4). These data also clearly indicate that when Tn*3* CTDs are present, they out-compete, and thus exclude, Bart CTDs that may bind weakly at non-cognate **t** sites in the hybrid-*res* substrates (see above).

In summary, resolvase dimers can be targeted to specific binding sites in hybrid *res* substrates, according to the identity of their CTDs. Resolution remains efficient when catalysis-defective S10A mutant NTDs are targeted to sites II and/or III, but is inhibited by S10A NTDs targeted to site I, confirming targeting specificity.

2.6 | All resolvase dimers in the synaptic complex must make R interactions

A key justification for our choice of Bart resolvase was the prediction that Bart resolvase NTDs would be unable to make effective *R* (2–3′) interactions with Tn*3* resolvase NTDs (see Introduction). *R* interactions between catalytic and regulatory dimers (i.e., dimers bound at site I and the accessory sites II–III, respectively) are required for resolution by Tn*3*/γδ resolvase (Hughes *et al.*, 1990; Grindley, 1993; Wenwieser, 2001; Burke *et al.*, 2004). In our experiments, resolution efficiency was greatly reduced when the NTDs bound at site I and at the accessory sites were different (see Figure 6b, line 4), supporting the hypothesis that the Tn*3* and Bart *R* surfaces are functionally incompatible.
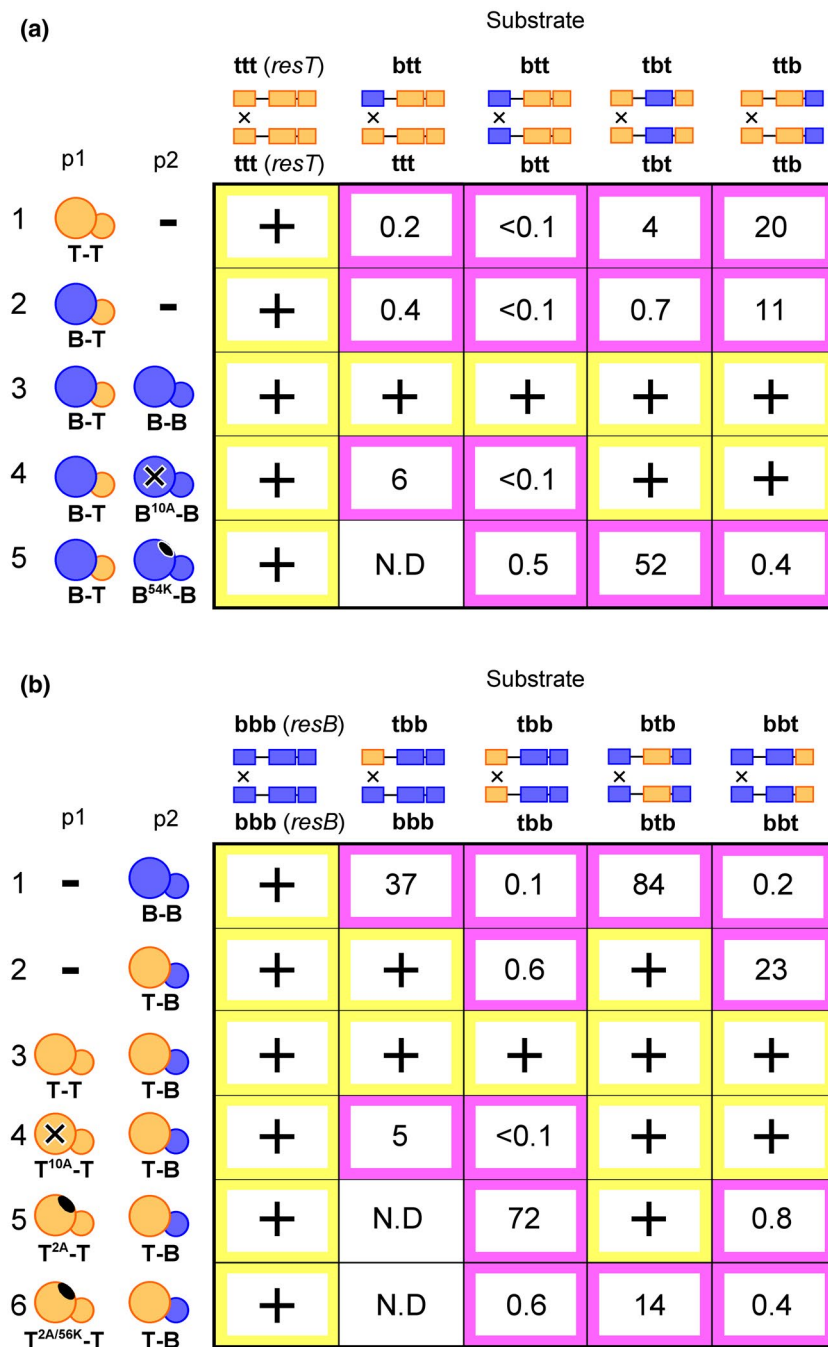
(a)

(b)

FIGURE 5 Targeting of NTDs to specific dimer-binding sites in *resT/resB*. Substrates with hybrid *res* sites, containing dimer-binding sites recognized by Bart (B) or Tn*3* (T) CTDs, were tested with different combinations of resolvases. The *res* sites are each named by three lower-case letters representing the binding sites I, II, and III (**t** = Tn*3* CTD-binding site, **b** = Bart CTD-binding site). In part a, all or most of the binding sites are **t**-type; in part b most sites are **b**-type. The results are depicted as described in the Figure 4 legend. Single-residue mutations are indicated by superscripts on the relevant NTD labels, and a black patch or black cross in the cartoons. For further details and interpretation, see main text

To examine further the role of the *R* interface in synapse assembly, we made *R* interface mutants. The Tn*3* resolvase *R* interface mutant R2A (Hughes *et al.*, 1990; Wenwieser, 2001) has no resolution activity on the *resT* × *resT* substrate (see Figure 6a, line 2). Using hybrid-*res* substrates, we targeted this mutant resolvase (T²ᴬ-T) to **t**-type binding sites I, or II, or III in both *res* sites, with T–B resolvase (wt Tn*3* resolvase NTDs) at the remaining **b** sites (Figure 5b, line 5). Recombination was inhibited compared to the wt Tn*3* resolvase (T–T) control (line 3) when the R2A mutant NTDs were at binding site I or III, but no loss of activity was detected when they were at site II. However, a double mutant NTD R2A E56K, which was shown previously to be more defective than the R2A single mutant in *R* interactions (Wenwieser, 2001) strongly

inhibited recombination when it was targeted to site I, site II, or site III (Figure 5b, line 6).

Structural analysis suggested that the Bart resolvase residue R54 would be a key contributor to its equivalent of the *R* interface (see Experimental Procedures). Consistent with this idea, the mutation R54K (that is, switching to the Tn*3* resolvase residue at this position) abolished all detectable Bart resolvase activity on the *resB* × *resB* substrate (see Figure 6a, line 7). Using our hybrid substrates we targeted this mutant (B⁵⁴ᴷ-B) to sites I or II or III of *res*, and the hybrid B-T resolvase (with wt Bart resolvase NTDs) to the remaining sites (Figure 5a, line 5). Resolution of all three substrates was inhibited compared to the wt Bart resolvase (B-B) control (Figure 5a, line 3), but again placing the mutant at site II had the smallest inhibitory effect. The results are

strikingly comparable to the "reverse" experiment with mutant Tn*3* resolvase NTDs described above.

In summary, our assays indicate that resolvase dimers at all three binding sites in *res* make *R* interactions, but the effect of *R* interface mutants at site II is less pronounced than at sites I and III. We also conclude that Bart and Tn*3* resolvases may use similar *R* interfaces to build their respective synapses.

2.7 | Swapping the R interaction specificities of Tn3 and Bart resolvases

We predicted that, if only *R* interactions are involved in building the synapse, it might be possible to make the Tn*3* NTD function like a Bart NTD, and vice versa, by changing only *R* interface residues.
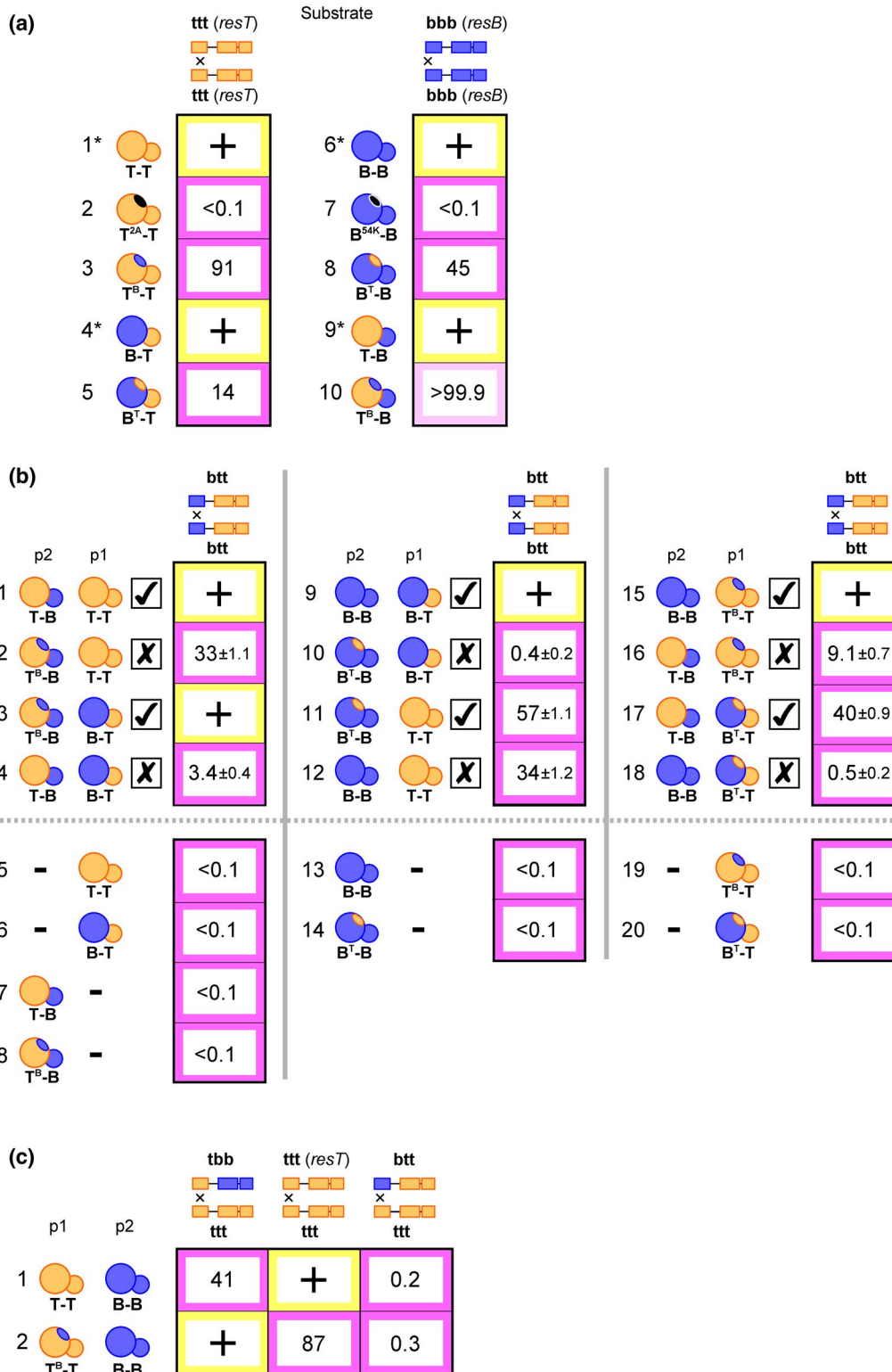
FIGURE 6 Recombination by resolvases with R interface patch mutations. (a) Resolvases with R interface specificity-swap patch mutations, T^B-T and B^T-B, resolve *resT* and *resB* substrates, respectively, indicating restoration of functional R interfaces (lines, 3, 5, 8, and 10). In contrast, single-residue "swap" mutations at the R interface abolish recombination (lines 2 and 7). The four relevant "white colony" results from Figure 5 are shown here again for comparison (lines marked with *). (b) R interactions between dimers at the crossover sites and at the accessory sites of *res*. NTDs attached to a Bart CTD (p2, left-hand cartoon in each pair) are targeted to the crossover site (site I), whereas NTDs attached to a Tn*3* CTD (p1, right-hand cartoon in each pair) are targeted to the accessory sites II and III, in a **btt** × **btt** substrate. The two-resolvase data are arranged into three sets of four (rows 1–4, 9–12, and 15–18, above the dashed grey line), to facilitate informative comparison of reactions; in each set, each resolvase pair differs from the one above or below by substitution of one NTD. The small boxes (ticked or crossed) indicate whether or not the R interfaces targeted to the crossover sites have the same "flavour" (i.e., Tn*3* or Bart) as the R interfaces targeted to the accessory sites, and are therefore predicted to make a functional interaction. The "swapped specificity" T^B and B^T NTDs function preferentially together with Bart and Tn*3* NTDs, respectively. One-resolvase controls are below the dashed grey line in the relevant column. See main text for further details and interpretation. The data are from single assays, all conducted in parallel under identical conditions (except controls 5–8 and 13). The error values represent the intrinsic RMS sampling error in determining % recombination by counting (see Experimental Procedures). Triplicate assays (three independent experiments) for two examples gave mean ± standard error as follows: 3.28 ± 0.09% (reaction 4) and 31.3 ± 2.3% (reaction 12). (c) No evidence for essential non-R synaptic interactions between dimers at sites II/III. T^B resolvase complements B–B resolvase to restore resolution activity on a **tbb** × **ttt** substrate. There is no rescue with a **btt** × **ttt** substrate. See text for details

Starting with no concrete information about the critical residues and interactions of the Bart R interface, we made systematic profile comparisons of Tn*3*-like and Bart-like resolvase sequences, relating these to the structure of the γδ resolvase R interface (Figure 2c). We then designed mutations of residues on a small surface patch of each protein, comprising the canonical R interface residues and other nearby residues predicted to be involved. For each protein, these comprise a total of 10 substitutions in parts of three surface loops near the R interface, sparing adjoining residues that pack in the core of the NTD (see Figure 2c, Figure S5, Experimental Procedures and Supplementary Information SI 2). The Tn*3* NTD with a Bart patch is designated T^B, while the Bart NTD with a Tn*3* patch is designated B^T.

The T^B and B^T NTDs were attached either to Tn*3* or Bart CTDs. Whereas Tn*3* and Bart resolvases with just a single mutation of a key R interface residue (R2A and R54K respectively) were completely inactive on their respective *resT* or *resB* substrates (see above), all four remodeled-interface proteins (T^B-T, T^B-B, B^T-B, and B^T-T) had significant resolution activity (Figure 6a). To test whether the T^B and B^T modifications had actually switched the specificity of the NTDs, we used the hybrid-*res* substrate **btt** × **btt** (Figure 6b). The accessory sites were loaded with either wt Tn*3* resolvase NTDs (using T–T resolvase; lines 1 and 2; 11 and 12) or wt Bart resolvase NTDs (using the hybrid B–T resolvase; lines 3 and 4; 9 and 10). According to our hypothesis, T^B NTDs placed at site I (using T^B-B resolvase) should interact preferentially with Bart resolvase NTDs placed at the accessory sites II–III. The results confirm this prediction: T^B NTDs at site I plus Tn*3* NTDs at the accessory sites gave a low level of resolution, but high resolution efficiency was restored when Bart NTDs were at the accessory sites (Figure 6b, compare lines 2 and 3). Similarly, B^T NTDs at site I interact preferentially with Tn*3* NTDs at the accessory sites (compare lines 10 and 11).

We also performed these experiments "in reverse"; that is, T^B or B^T NTDs were targeted to *res* binding sites II and III, and unmodified Tn*3* or Bart NTDs were targeted to site I, with similar results (Figure 6b, lines 15–18).

In summary, the patch mutations switched the specificity of the resolvase NTDs with respect to their essential synapse-building functions. The recombination-blocking Tn*3* R2A and Bart R54K mutations at the R interface of the respective NTDs are rescued by the many further mutations we introduced, to convert the surface of one NTD to the character of the other. The success of this strategy supports the idea that the surfaces of the two resolvases in the vicinity of these residues are functionally equivalent, but non-complementary.

Some early models of the synapse (Krasnow *et al.*, 1983; Boocock *et al.*, 1986; Sarkis *et al.*, 2001; Rowland *et al.*, 2002) proposed that dimers bound at the *res* accessory sites (II and III) might mediate synaptic interactions via the "catalytic" dimer–dimer interface (as at site I) or another type of NTD–NTD interface. To test these proposals, we used the substrates **tbb** × **ttt** and **btt** × **ttt** (Figure 6c). Bart NTDs placed at the **b** sites of these substrates are predicted not to interact with Tn*3* NTDs placed at the **t** sites, so recombination efficiency should be low, as observed (Figure 6c, line 1). If the only interface involved in synaptic interactions by dimers at sites II and III is the R interface, it should be possible to restore efficient recombination of **tbb** × **ttt** by targeting patch-mutated Tn*3* NTDs (T^B) to the **t** sites (using T^B-T). However, it should not be possible to rescue recombination of **btt** × **ttt** in this way, because of the additional NTD interactions required to assemble the catalytic tetramer at site I. Our results confirm these predictions (Figure 6c, compare lines 1 and 2).

We conclude that, apart from the well-characterized catalytic tetramer-forming interactions at site I of *res*, the productive synapse is assembled using only R interactions between resolvase dimers.

3 | DISCUSSION

We used Tn*3* and Bart resolvases, and Tn*3*-Bart hybrids, to target distinct types of resolvase dimers to distinct binding sites in the resolvase recombination site *res*, allowing us to map protein–protein interactions within the synaptic recombination intermediate. Our results lead us to conclude that the architecture of the productive synapse is achieved by resolvase subunits making three types of essential protein–protein interactions. (1) At each binding site of *res*,

the CTDs of two resolvase subunits recognize and bind to DNA sequence motifs at the ends, and the NTDs interact to make a dimer (Figure 2a). (2) The dimers bound at two interacting *res* sites make *R* interactions with each other (Figure 2c), thus, intertwining the two DNA sequences and holding them together. (3) The two dimers bound at the two copies of site I interact and rearrange, forming a catalytic tetramer (Figure 2d).

The two key innovations that allowed us to reach these conclusions were our characterization of the Bart resolvase recombination system and our "patch mutagenesis" strategy to remodel the *R* interface.

The Bart resolution system was chosen for this work because of its potential as an "orthogonal" yet structurally similar system to that of Tn*3* (see Introduction). A crucial success in our experimental strategy was the achievement of almost complete DNA sequence specificity switching of Tn*3* and Bart resolvases by swapping their CTDs. There had been previous attempts to alter the DNA sequence specificity of serine recombinases related to Tn*3* resolvase, by mutations or substitution of the CTD with one from a different recombinase (Ackroyd *et al*., 1990; Avila *et al*., 1990; Grindley, 1993; Boocock *et al*., 1995; Murley and Grindley, 1998; Schneider *et al*., 2000), but none of these variant proteins had completely switched specificity from the original target DNA sequence to a new one. More complete specificity changes have been obtained by replacing the resolvase CTD with structurally unrelated zinc finger or TALE DNA-binding domains (Akopian *et al*., 2003; Gaj *et al*., 2013; Stark, 2014), but the substantially altered structures/sizes of these hybrid proteins make them entirely unsuitable for mapping interactions within the natural Tn*3* synapse.

Mutation of just one amino acid residue at the *R* interface of either Tn*3* or Bart resolvase to the other's residue at that position (R2A and R54K respectively) was sufficient to render the resolvase inactive; but remarkably, for each resolvase, activity was restored by adding a further nine mutations, to create a Bart-like *R* surface on Tn*3* resolvase, and a Tn*3*-like *R* surface on Bart resolvase. When the recombination synapse contained mixtures of Tn*3* and Bart resolvase NTD dimers, these patches of mutations were sufficient to give one type of resolvase NTD the functionality of the other. Our results thus imply that the *R* interface is solely responsible for holding the two *res* sites together in the synapse, until formation of the catalytic resolvase tetramer at site I.

We note that our conversion of the Tn*3* resolvase *R* interface to Bart specificity (T^B) was more effective than conversion of the Bart *R* interface to Tn*3* specificity (B^T) (e.g. Figure 6a, compare lines 3 and 5; 8 and 10). This is unsurprising to us because the mutations of Tn*3* resolvase were informed by high-resolution crystal structures (see Introduction), whereas we have no structural data for Bart resolvase. The interface mutations were entirely based on a priori structural analysis and prediction; the effectiveness of the switching could undoubtedly be improved by iterative mutagenesis-screening methods.

Synapsis of the accessory sites (II and III; Figure 1a) can occur in the absence of site I and is thought to precede the site I-site I interaction (Boocock *et al*., 1986; Watson *et al*., 1996). This II–III synaptic complex has also been used in synthetic recombination systems, to impose topological selectivity on other recombinases (Kilbride *et al*., 1999, 2006; Grainge *et al*., 2000; Olorunniji *et al*., 2012).

Involvement of "catalytic-type" tetramers (Figure 2d) in synapsis at sites II and III was previously ruled out by protein crosslinking data (Murley and Grindley, 1998; Wenwieser, 2001), and our new results imply that only *R* interactions between DNA-bound dimers are required.

Sin resolvase is distantly related to Tn*3*/γδ resolvase and has a very different organization of protein-binding sites in its *res* sequence (Rowland *et al*., 2002). Biochemical and crystallographic analysis of Sin-DNA complexes revealed a synaptic CTD–CTD interface at the *res* accessory sites (Figure 2b) and essential *R*-type contacts between subunits bound at the accessory sites and site I. It was also shown that the *R* interface plays no part in accessory site synapsis. These features were incorporated into a structural model of the complete synapse (Mouw *et al*., 2008; Rowland *et al*., 2009). It was speculated at that time that all serine resolvases might share common features of synapse architecture including a synaptic CTD–CTD tetramer. Recombination by Hin DNA invertase (a related serine recombinase with similar domain structure) also involves a crucial role for the CTD in synapsis (McLean *et al*., 2013). However, the results presented here rule out any essential role for CTD protein–protein interactions in the Tn*3* resolvase synapse (and by implication the synapses formed by Bart, γδ and other closely related resolvases with similar *res* sequences).

Previous studies with γδ resolvase identified essential *R* interactions within the synapse, and models of the synapse architecture postulated *R* interactions between pairs of dimers bound at sites II and III in the same *res* site (Grindley, 1993; Murley and Grindley, 1998; Sarkis *et al*., 2001). Our results support crucial synaptic roles for the *R* interface, involving dimers bound at all three sites in *res*, and furthermore imply that the *R* interface is the only "new" interface made in the synapse by dimers bound at sites II and III. Thus, we deduce that the *R* interface makes the primary bridging contacts between the two *res* sites in the synapse.

The effects of *R* interaction deficiency at site II are generally less than at site I or site III, consistent with earlier proposals that the role of the resolvase dimers bound at site II might be primarily "architectural" (including bending the path of the DNA double helix; Blake *et al*., 1995; Soultanas *et al*., 1995), whereas the *R* interaction between sites I and III might be critical for events leading to catalysis of DNA strand exchange. The site I–site III *R* interaction might simply position the site I-bound resolvase dimers optimally for strand exchange, or it might have an additional allosteric role to stimulate catalytic activity (see for example Grindley *et al*., 2006).

Our results have significantly clarified the nature of the resolvase interactions in the recombination synaptic complex, but do not yet allow us to build a complete structural model that is fully supported by experimental evidence. Completion of this task will require an "interaction map" for each resolvase subunit within the synapse, and will therefore require resolvase subunits (rather than dimers) to be targeted to specific loci in substrate *res* sites. This is likely to be more challenging because of the highly cooperative protein–protein interactions in the synapse, but we are confident that the tools and methodologies presented here will allow us to achieve the necessary targeting specificity.

4 | EXPERIMENTAL PROCEDURES

4.1 | Recombination substrates

Recombination sites *resB* and *resT* (synthesized by GeneArt) were cloned in the polylinker vector pMTL23 (Chambers *et al.*, 1988), and hybrid sites were constructed by fragment swaps using the EcoRI and BglII restriction sites between sites I, II, and III (Figure 3b, Figure S3). The in vivo resolution substrates are low copy number plasmids (pSC101 origin) similar in construction to pGal (*res × res*) (Burke *et al.*, 2004; see also Prorocic, 2009). The parent plasmid (lacking *res* sites) is pMS183Δ (confers kanamycin resistance; Supplementary data SI 4; Prorocic, 2009). All plasmid sequences and further details of their construction are available on request.

4.2 | Resolvase expression plasmids

Expression plasmids for resolvases with a Tn*3*, Tn*21* or Tn*552* CTD are similar to pMA5811 (confer ampicillin resistance; pBR322 origin; Burke *et al.*, 2004). All these plasmids ("p1" in Figures) contain the following sequence upstream of the resolvase start codon: T<u>TAAGAAGGAG</u>ATATACAT*ATG* (homology to consensus ribosome binding site underlined; resolvase start codon in italics). Expression plasmids for resolvases with a Bart resolvase CTD are based on pACYC184 (confer chloramphenicol resistance). The resolvase gene was inserted (as part of a promoterless ~1 kbp fragment) into the tetracycline resistance gene (*tet*), in the opposite orientation to *tet* transcription (cf. pLysS, designed to express T7 lysozyme; Studier, 1991). All these plasmids ("p2" in Figures) contain the following sequence upstream of the resolvase start codon: CTTT<u>G</u>TTATATACAT*ATG*. The upstream sequences containing the ribosome binding site in the p1 and p2 plasmids were different so as to give compatible resolvase expression levels. Tn*3* resolvase coding sequences are as described (Burke *et al.*, 2004). The original Bart resolvase sequence (GenBank ABM45303.1) is from *Bartonella bacilliformis* KC583. The Bart resolvase coding sequences used here were synthesized (by GeneArt). Both Tn*3* and Bart resolvase coding sequences have restriction sites placed at regular intervals to allow for future fragment exchanges. Random mutations in resolvase reading frames were introduced by PCR-based methods (Burke *et al.*, 2004).

4.3 | In vivo recombination assays

The complementation assay has been described (Rowland *et al.*, 2005; Mouw *et al.*, 2008). In brief, chemically competent cells of the *E. coli* strain DS941 containing one or two resolvase expression plasmids (see above) were transformed with substrate plasmid DNA, and colonies were grown on selective MacConkey/galactose agar indicator plates (Arnold *et al.*, 1999; Burke *et al.*, 2004). Pale-colored ("white") colonies indicate near-complete resolution; that is, the *galK* gene has been deleted from all or most (typically >90%) copies of the multi-copy substrate plasmid (confirmed by gel electrophoresis and/ or transformation of recovered plasmid DNA for key controls). When colonies were white (indicated by pale yellow boxes with a + sign in the Figures), no further analysis was carried out. If a greater fraction of the substrate plasmid molecules remains unresolved, so that most cells retain some copies of the *galK* gene, colonies are red (or in rare cases, an intermediate "pink" phenotype). When red or pink colonies were observed, pooled colonies (>100) were grown overnight in liquid culture (~10 generations). Plasmid DNA prepared from these cultures contained the resolvase expression plasmid(s), plus any unrecombined substrate, and resolution product plasmids, as observed by gel electrophoresis (data not shown). To provide more accurate quantitation of extent of recombination (resolution), this plasmid DNA was used to transform *E. coli*, selecting for the substrate/product plasmid (i.e., kanamycin resistance) on MacConkey/ galactose indicator plates. To prevent co-transformation with a resolvase expression plasmid (potentially resulting in resolution of unrecombined substrate), we used a strain (derived from *E. coli* DS941) that already maintained plasmids with pBR322 and pACYC184 origins (expressing catalytically inactive (S10A) mutants of Tn*3* and Bart resolvases, respectively). Percent resolution was determined by counting the red and white colonies (typically >2,000 total) resulting from transformation with unrecombined substrate plasmid or resolution product plasmid respectively. In the Figures, "<0.1%" resolution means that we counted >1,000 red colonies and ≤1 white colonies; ">99.9%" resolution means >1,000 white colonies and zero red colonies. In Figure 6b, ± sampling errors (SE) are given, as: SE (%) = $100\sqrt{(p.(1-p)/n)}$, where *r* and *w* are the counts of red and white colonies, respectively, $p = w/(w+r)$, and $n = w + r$. Quantitative comparisons discussed in the text are from sets of assays done in parallel (using identical growth conditions, etc.), including all data in Figure 4b (except rows 3,7), 5a (except row 5), 6a, 6b (except rows 5–8,13), and 6c. The standard error in nine representative triplicate assays done as independent experiments was between 0.1% and 2.4% recombination (% recombination in the range 0.8%–71%).

4.4 | Design of R interface patch mutations

The aim was to modify a small surface patch of the Tn*3* or Bart NTD, to switch the interface specificity (Figure 2 and Figure S5). The two mutants described (T^B and B^T) represent an initial test of a structure-informed "one-step" interface redesign; they are not products of stepwise optimization or selection. No structures are available for Bart resolvase; our redesign of the interfaces relied only on structures for the γδ resolvase interface (Figure 2c; Rice and Steitz, 1994a) and the very similar Tn*3* resolvase interface (Montaño and Rice, unpublished). Bart resolvase differs from Tn*3*/γδ resolvase at two of the four canonical *R* interface residues: R2 is replaced by A2, and K54 by R54. R2 is at the heart of the Tn*3* and γδ *R* interfaces: it contacts D59′ across the interface, and stacks between R2′ and R32 (Figure 2c). Although we hypothesize that the role of R54 in the Bart *R* interface might be similar to that of R2 in the Tn*3* resolvase

interface (and analogous to the role of R54 in the Sin *R* interface), our designs assume only that the NTDs share similar core structures. To evaluate candidate residues for mutation, we systematically inspected side-chain contacts in the γδ resolvase interface (Figure 2c), and compared profiles of residue frequencies in groups of resolvases similar to Bart (A2, R54) or to Tn3/γδ resolvase (R2, K54). These two groups seem to represent two primary "flavours" of *R* interface in the diverse Tn*3*/Bart resolvase family (Figure S2).

The profile comparisons highlighted additional residues with a potential role in interface specificity (Figure S5). A key difference between Bart and Tn3/γδ resolvases is in surface loops 2 and 4 (positions 29–32 and 55–58). In γδ resolvase, only R32 and E56′ make close contact across the interface, while residues 29–31 and 57′ make limited contacts and were not highlighted in loss-of-function screens (Figure 2c). Bart-group resolvases retain R32 and E56, but have long conserved side-chains at positions 29–30 and 57 (Figure S5), suggesting that these residues might engage in the interface. At position 53, the Bart group typically have small residues (Ala or Gly), while in γδ resolvase, two bulky M53 sidechains pack on the interface (Figure 3c). A smaller sidechain might allow closer approach of the B-helices, shifting the entire interface. We replaced M53 with A53 (as in the Bart group profile), not G53 (from Bart resolvase), to minimize any risk of destabilizing the α-helix. Following similar logic, we replaced a total of ten surface residues, with zero net change in the predicted surface charge (Figure S5). Changes were made only at residues directly involved in the γδ 2–3′ interface, residues supporting these (I3 and F4), or residues assigned as potential Bart interface contacts (29–31); the underlying foundation of NTD core residues is mostly conserved.

For the patch mutant Bart NTD (B^T), we made ten changes (all to Tn*3* residues: Figure S5). Eight of these changes are the exact reverse of those made in the Tn*3* NTD (B^T). One extra change was made (L52R, to preserve amphipathic character in helix B, breaking a long run of hydrophobic residues––LQLL<u>L</u>MK––and adding one unit of charge). At L3 no change was justified. Neither of these residues is likely to make any direct interface contacts (see Figure 2c).

4.5 | Bioinformatics

Sequence alignments and profile analyses made extensive use of Jalview (Waterhouse *et al.*, 2009),
and standard NCBI tools.

AUTHOR CONTRIBUTIONS
S.-J.R. carried out the experimental work along with M.E.B. S.-J.R., M.R.B., P.A.R., and W.M.S. planned and designed the project. M.R.B. analyzed and redesigned the resolvase and *res* site sequences. S.-J.R., M.R.B., P.A.R., and W.M.S. all contributed to interpretation of the data and preparation of the manuscript. The authors all confirm that they have no conflict of interest concerning the contents of this manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.