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Recombineering Using RecTE from *Pseudomonas syringae*[▽]

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In this report, we describe the identification of functions that promote genomic recombination of linear DNA introduced into *Pseudomonas* cells by electroporation. The genes encoding these functions were identified in *Pseudomonas syringae* pv. syringae B728a based on similarity to the lambda Red Exo/Beta and RecET proteins encoded by the lambda and Rac bacteriophages of *Escherichia coli*. The ability of the pseudomonad-encoded proteins to promote recombination was tested in *P. syringae* pv. tomato DC3000 using a quantitative assay based on recombination frequency. The results show that the *Pseudomonas* RecT homolog is sufficient to promote recombination of single-stranded DNA oligonucleotides and that efficient recombination of double-stranded DNA requires the expression of both the RecT and RecE homologs. Additionally, we illustrate the utility of this recombineering system to make targeted gene disruptions in the *P. syringae* chromosome.

There are currently more than 1,500 completed or draft bacterial genome sequences available for public access. This data resource continues to grow rapidly and provides potential insights into the roles of individual genes and regulons. However, testing hypotheses based on sequence data requires direct experimental manipulation of each genome. While many established methods for modifying bacterial DNA can assist in genetic analysis of these organisms, they are often time-consuming and limited with respect to the types of changes that can be directed.

New advances in recombineering (genetic engineering by recombination) offer powerful alternative strategies for site-directed mutagenesis of genomic loci and provide methods for rapid and precise functional genomic analysis in some organisms (9, 29, 36–38, 41, 43). In these cases, recombineering is very efficient when phage-encoded recombinases are supplied, such that *in vivo* expression of these proteins enables direct genetic engineering of chromosomal and episomal replicons. These proteins catalyze RecA-independent recombination (21) of linear DNA substrates with homologous genomic target loci. The phage recombination functions typically involve the coordinated action of a 5′-to-3′ exonuclease (i.e., RecE or lambda Exo) and a single-stranded DNA (ssDNA)-annealing and strand invasion protein (i.e., RecT or lambda Beta), which we shall refer to as recombinases for brevity. The recombinase binds to 3′ ssDNA ends that are exposed by the action of the exonuclease, forming a protein-DNA filament, which protects the substrate DNA and promotes annealing with the homologous genomic sequence (4, 17, 19, 24). The recombinases are sufficient to facilitate recombination of ssDNA oligonucleotides, presumably because the oligonucleotides resemble the 5′-end-resected double-stranded DNA (dsDNA) substrate (11). Most of the recombinase proteins that have been shown to facilitate recombination are located in operons and are adjacent to the exonuclease-encoding genes, although there are cases where functional recombinase proteins have been identified without an accompanying exonuclease (9).

Recombineering technologies have great potential in functional genomic applications and have worked exceptionally well in a few species, but adapting current systems to different bacteria is often problematic. Evidence suggests that these recombination systems have narrow species specificity such that a given system may catalyze robust recombination in one species and be essentially nonfunctional when expressed in another (9, 37). The reasons for this are not known but may be due to a requirement for specific interactions between the recombinase and host-encoded factors (9). Although there is a need to apply recombineering techniques to *Pseudomonas* species, only marginal success using the characterized phage recombination systems has been reported (14, 23). Most notably, recombinant strains of *Pseudomonas aeruginosa* were generated using long-homology substrates in the presence of plasmids expressing the lambda Red genes, but the relative influence of the Red genes was not reported (23).

Here, we describe the identification of new recombineering proteins that function in a pseudomonad. The genes that encode proteins with similarity to the RecE/RecT proteins of the Rac prophage and lambda Red Exo and Beta were identified in *Pseudomonas syringae* pv. syringae B728a. These proteins promote efficient homologous recombination between genomic loci and linear DNA substrates introduced directly into *P. syringae* pv. tomato DC3000 cells by electroporation. These findings provide a foundation for more efficient site-directed mutagenesis of chromosomal loci in *P. syringae* and serve as a strategy for identifying similar proteins for recombineering in other bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Pseudomonas syringae* strains were grown at 30°C in King's B (KB) medium (18) or on KB medium solidified with 1.5% (wt/vol) agar. Gentamicin and streptomycin were used at 10 μg/ml and 100 μg/ml, respectively. *Escherichia coli* DH5α was used as the host for subcloning and other plasmid manipulations used in this work. *E. coli* was grown at 37°C in

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristic(s)	Reference
Strains		
P. syringae pv. tomato DC3000	Wild type	3
P. syringae pv. syringae B728a	Wild type	12
Plasmids		
pBS1-sacB	P_{BAD}::Gateway cassette with adjacent *sacB*	35
pBS47	P_{nptII}::Gateway cassette with adjacent *sacB*	This work
pUCP24	*E. coli-Pseudomonas* shuttle vector	40
pUCP24/47	pUCP24 derivative with the Gateway destination R1-R2 cloned into the multiple cloning site	This work
pUCP24/61	pUCP24 derivative with the P_{nptII} promoter cloned upstream of a multiple-cloning site; used as the empty vector control	This work
pUCP24/*recT*	pUCP24/47 derivative; P_{nptII}::*recT*	This work
pUCP24/*recTE*	pUCP24/47 derivative; P_{nptII}::*recTE*	This work
pBR322	Source of the tetracycline resistance gene	2

LB medium or LB medium solidified with 1.5% (wt/vol) agar. All bacteria used in these experiments are shown in Table 1.

Bioinformatics. *Pseudomonas*-associated RecT proteins were identified using PSI-BLAST, as described by Iyer et al. (16), except where noted. The National Center for Biotechnology Information (NCBI) nonredundant (NR) database (September 2009) was searched using the PSI-BLAST program (1). These searches were conducted using the PAM 30 matrix and run iteratively with an inclusion threshold of 0.01 until convergence was achieved. Convergence was achieved in 8 and 14 iterations for Red Beta and *E. coli* RecT, respectively. Then, information describing the subset of matches to sequences derived from organisms belonging to the *Pseudomonas* genus was manually collected (see Table 3).

RecT and RecE expression vector constructions. All expression vectors used in these experiments were based on pUCP24 (40) (GenBank accession no. U07167) and were constructed either by conventional methods or by Gateway cloning (Invitrogen, Carlsbad, CA). First, pBS47 was created from pBS1-sacB (35) by replacement of the arabinose-inducible P_{BAD} promoter with the constitutive *nptII* promoter. Next, the P_{nptII}::Gateway cassette and *sacB* gene from pBS47 were cloned as an NdeI/XbaI restriction fragment into HindIII/XbaI-digested pUCP24 to yield pUCP24/47. These restriction fragments were created by sequential digestion to permit the NdeI- and HindIII-generated overhangs to be filled in by treatment with the Klenow fragment prior to the XbaI digestions in order to facilitate blunt- and cohesive-end ligation of these fragment ends, respectively. The *P. syringae recT* ($recT_{Psy}$) and $recTE_{Psy}$ genes were then cloned into pUCP24/47 in two steps. First, the $recT_{Psy}$ and $recTE_{Psy}$ genes were PCR amplified from *P. syringae* pv. syringae B728a using primer pairs (oSWC1694/oSWC1695 for $recT_{Psy}$ and oSWC1694/oSWC2526 for $recTE_{Psy}$) and cloned into pENTR/SD/D-TOPO (Invitrogen) to create the entry vectors pENTR/*recT* and pENTR/*recTE*, respectively. Then, these genes were cloned from the entry vectors into the respective expression vectors by the Gateway LR reaction to create pUCP24/*recT* and pUCP24/*recTE*, as per the manufacturer's specifications. The resulting plasmids, pUCP24/*recT* (GenBank accession no. HM368667) and pUCP24/*recTE* (GenBank accession no. HM368666), were designed to provide constitutive expression of the *recT* and *recTE* genes, respectively. The negative-control plasmid, pUCP24/61 (GenBank accession no. HM368668), was constructed by ligation of the NdeI/XbaI fragment from pBS61 with similarly digested pUCP24. pBS61 was constructed from pBS47 by digestion with NheI and SpeI followed by ligation to delete the Gateway cassette. All of the expression vectors also encode the *Bacillus subtilis sacB* gene, which is used as a counter-selectable marker to expedite plasmid elimination from *P. syringae* and other cells when desired (3, 22, 33). We have confirmed that the RecTE expression vector can be successfully eliminated from *P. syringae* cells after recombination in order to prepare the recombinants for other experiments. Vector constructions were confirmed by DNA sequencing.

Recombineering substrates. The sequences of all oligonucleotides used are shown in Table 2. Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (IDT; Coralville, IA). Oligonucleotides were then diluted to 1 μg/μl in sterile double-distilled water, and the indicated amounts of oligonucleotides were added to electrocompetent cells. The *rpsL*(*K43R*) (13) mutations were directed by substrates containing four base changes (i.e., encoded by oSWC1255) to minimize potential negative effects of the methyl-directed mismatch repair system on recombination (7, 34).

PCR products were generated using the Expand High Fidelity PCR system (Roche, Basel, Switzerland). Long flanking homology (LFH) PCR was used to generate a substrate for the construction of a genomic deletion. LFH PCR was performed as described in references 27 and 39. PCR-generated substrates were confirmed by agarose gel electrophoresis, and the product was purified and concentrated to 1 μg/μl by ethanol precipitation.

Recombineering in *P. syringae* pv. tomato DC3000. Electrocompetent *P. syringae* pv. tomato DC3000 (Table 1) was prepared using the method described by Choi et al. (5). Briefly, overnight *P. syringae* cultures inoculated from a single colony were diluted to an optical density at 600 nm (OD_{600}) of 0.4 in KB medium (18) and grown to an OD_{600} of 0.8 to 1.0. Cells were harvested by centrifugation at 20°C, washed twice with equal volumes of room temperature 300 mM sucrose, and finally resuspended at 1/60 of the original culture volume in 300 mM sucrose. In each experiment, oligonucleotide or PCR product was added to 100 μl of electrocompetent cells and transformed by electroporation at 2.5 kV, 25 μF, and 200 Ω in a 0.2-cm cuvette using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA). KB medium (5.0 ml) was then added, and cells were incubated with shaking at 30°C overnight to allow segregation of the wild-type *rpsL* and recombinant *rpsL*(*K43R*) alleles. To determine the frequency of recombination, dilutions of transformation outgrowth cultures were spread on selective (with 100 μg/ml streptomycin) or nonselective KB agar plates, and the numbers of streptomycin-resistant transformants were standardized to 10^8 viable cells. Recombination frequencies are averages from at least three independent experiments, and error bars indicate standard deviations.

For routine recombineering as described below in the section entitled "Recombineering in practice," we used frozen competent cells. These cells were prepared as described above, except that after being washed twice with 300 mM sucrose, the cells were washed two additional times in 10% glycerol prior to the final resuspension at 1/60 the original culture volume in 10% glycerol and then frozen in 100-μl aliquots at −80°C. To prepare the cells for use, they were thawed on ice for 10 min and then incubated at room temperature for 10 min prior to being used for electroporation.

Nucleotide sequence accession numbers. The sequences of pUCP24/*recT*, pUCP24/*recTE*, and pUCP24/61 were submitted to GenBank under accession numbers HM368667, HM368666, and HM368668, respectively.

RESULTS

Identification of RecT and RecE orthologs in *P. syringae*. The goal of this study was to identify a recombinase that functions in *P. syringae* pv. tomato DC3000 and could be developed as a tool for recombineering. Preliminary data in our laboratory suggested that the lambda Red genes were not capable of facilitating recombination in *P. syringae*, which is not surprising, because organism-specific biases have been observed for other phage-encoded recombinases (9). To find a suitable recombinase, we searched for RecT-like proteins encoded by pseudomonad phages and genomes with the expectation that they would be more likely to function in *P. syringae* than similar proteins associated with more distantly related

TABLE 2. Oligonucleotides used

Identifier	Description[a]	Sequence[b]
oSWC1255	*rpsL*(*K43R*) GAAA to ACGG; lagging strand	5′GGCGCACACGGCAGACTTTACGCAATGCCGAGTTAGGTTTCCGAGGCGTAGTGGTATACACACGAGTGCACACACCGCGACGTT
oSWC1978	*rpsL*(*K43R*) GAAA to ACGG; lagging strand	5′GGTTTCCGAGGCGT
oSWC1518	*rpsL*(*K43R*) GAAA to ACGG; lagging strand	5′AGTTAGGTTTCCGAGGCGTAGTGG
oSWC1251	*rpsL*(*K43R*) GAAA to ACGG; lagging strand	5′CGCAATGCCGAGTTAGGTTTCCGAGGCGTAGTGGTATACACACG
oSWC1253	*rpsL*(*K43R*) GAAA to ACGG; lagging strand	5′GCAGACTTTACGCAATGCCGAGTTAGGTTTCCGAGGCGTAGTGGTATACACACGAGTGCACACA
oSWC1257	*rpsL*(*K43R*) GAAA to ACGG; lagging strand	5′AACCTCGAAACCGTTGGTCAGGCGCACACGGCAGACTTTACGCAATGCCGAGTTAGGTTTCCGAGGCGTAGTGGTATACACACGAGTGCACACACCGCGACGTTGCGGGCAGTTCTGCAGCGCA
oSWC1513	Amplified 186-bp fragment of *rpsL*(*K43R*) GAAA to ACGG	5′CGTAAGCGTATCGTCGAGAAATCC
oSWC1514	Amplified 186-bp fragment of *rpsL*(*K43R*) GAAA to ACGG	5′GTTGTGACCTTCACCACCGATGTA
oSWC1694	Cloning primer; 5′ end of $recT_{Psy}$	5′CACCATGTCCGCAAGAAACGTTGCGTCC
oSWC1695	Cloning primer; 3′ end of $recT_{Psy}$	5′TCATGCGGTTTCTCCGGCGATAGG
oSWC2526	Cloning primer; 3′ end of $recE_{Psy}$	5′TCATGCTGCCACCTGCGTGATGG
oSWC2545	PCR-amplified 500-bp region flanking the Tn*5* insertion in PSPTO_0362	5′CAGCCATCAGTCCAAAGCCGTGGT
oSWC2546	PCR-amplified 500-bp region flanking the Tn*5* insertion in PSPTO_0362	5′AAACACTGCAAGCCCGTGGCAAG
oSWC2547	PCR-amplified Tn*5* insertion in PSPTO_0362; 80-bp flank	5′CCACCAGCTCCAAATACGGCACCT
oSWC2548	PCR-amplified Tn*5* insertion in PSPTO_0362; 83-bp flank	5′AGCCACCTCTACTTTGAGCGACCGC
oSWC2670	PCR primer for PSPTO_1206 deletion, upstream fragment, fwd	5′TGTCTGTTTTGCGAGATGTGTCAG
oSWC2671	PCR primer for PSPTO_1206 deletion, upstream fragment, rev (*tet*)	5′GATAAGCTGTCAAACATGAGAATTGGCGGGACGGGGCATGACAGATCCTTG
oSWC2672	PCR primer for PSPTO_1206 deletion, downstream fragment, fwd (*tet*)	5′CTAACGGATTCACCACTCCAAGAAACGTATCGGTGGTGAGACAGGCTGAAAAG
oSWC2673	PCR primer for PSPTO_1206 deletion, downstream fragment, rev	5′ACACGGTCATCGCACGCGGCGAGAC

[a] fwd, forward; rev, reverse.
[b] Underlining shows the position of sequence changes.

organisms. Pseudomonad-associated RecT orthologs were identified by searching the NCBI NR database using PSI-BLAST for proteins similar to known phage-encoded recombinases, as described by Iyer et al. (16). Coding sequences for proteins meeting our criteria were found in several pseudomonads (Table 3). However, we immediately focused our attention on Psyr_2820 from *P. syringae* pv. syringae B728a, which is annotated as encoding a RecT protein and matches the RecT protein motif in the Conserved Domain Database (16, 25, 26). To distinguish this gene and its product from similar proteins, we refer to Psyr_2820 using a Psy subscript (e.g., $RecT_{Psy}$). Sequence analysis of genes in the vicinity of $recT_{Psy}$ in the *P. syringae* B728a genome indicated that the downstream gene (Psyr_2821) potentially encodes a protein with similarity to RecE and lambda exonuclease. Based on the sequence similarity of the protein and the genomic context of the coding sequence, we refer to this gene as $recE_{Psy}$ and to its product as $RecE_{Psy}$. Comparative genomic analysis of closely related *P. syringae* strains suggests that the $recTE_{Psy}$ genes are located in a 72-kb region that has been horizontally acquired in *P. syringae* pv. syringae B728a. This region contains 86 annotated open reading frames (ORFs), the majority (58/86) coding for hypothetical proteins; however, 13 are annotated as encoding proteins related to phage. Therefore, we suspect that this region corresponds to a prophage or a remnant of a prophage.

$RecT_{Psy}$ facilitates oligonucleotide recombination. Several phage-encoded recombinase proteins have previously been reported to catalyze the recombination of ssDNA oligonucleotide substrates with genomic target loci (9). These recombination proteins are sufficient for this reaction because oligonucleotides are analogous to their natural substrates, which are 3′ single-stranded sequences generated by the genetically linked exonuclease. Supplying oligonucleotide substrates for recombination avoids the requirement for initial processing by the exonuclease, allowing the activity of RecT to be tested independently of RecE.

The function of $RecT_{Psy}$ was evaluated in terms of its ability to mediate recombination of an ssDNA oligonucleotide with the *P. syringae* pv. tomato DC3000 genome, which is not predicted to encode any proteins with similarity to known phage-encoded recombinase proteins (34). To assess the effect of $RecT_{Psy}$ on recombination, *P. syringae* cells were transformed with oligonucleotides that are homologous to a specific genomic locus and contain nucleotide changes that confer a selectable phenotype upon recombination. Recombinants were identified by conversion of the wild-type *rpsL* gene to the

TABLE 3. *Pseudomonas* proteins with similarity to lambda Red Beta and RecT from the Rac prophage of *E. coli*[a]

GenBank accession no.	Source	Description	Beta[b]		RecT[c]		Exo gene[d]
			% identity	E value	% identity	E value	
YP_235897.1	*P. syringae* pv. syringae B728a	Psyr_2820; RecT protein	14	2×10^{-18}	10	2×10^{-24}	Psyr_2821
ACD38827.1	*P. aeruginosa*	PACL_0579; hypothetical protein	12	4×10^{-22}	11	9×10^{-24}	—
ACD38903.1	*P. aeruginosa*	PACL_0645; hypothetical protein	12	4×10^{-22}	11	9×10^{-24}	—
ABR85240.1	*P. aeruginosa* PA7	PSPA7_2380; hypothetical protein	13	2×10^{-20}	10	3×10^{-24}	—
EAZ57804.1	*P. aeruginosa* 2192	PA2G_01010; hypothetical protein	14	8×10^{-11}	7	2×10^{-22}	—
ABY97628.1	*Pseudomonas putida* GB-1	PputGB1_1725; hypothetical protein	12	4×10^{-21}	10	9×10^{-22}	—
YP_002872416.1	*P. fluorescens* SBW25	PFLU2837; hypothetical protein	14	6×10^{-19}	11	2×10^{-13}	PFLU2836
NP_758659.1	*Pseudomonas resinovorans*	pCAR1_p118; RecT protein	12	4×10^{-15}	31	7×10^{-35}	—

[a] Each potential recombinase satisfies the following criteria: each protein has an Expect (E) value less than 0.01 based on PSI-BLAST-computed alignments with the query sequence (Beta or RecT) and is found in a pseudomonad genome or phage. Descriptions are of *Pseudomonas* genes identified using an iterative PSI-BLAST search.
[b] PSI-BLAST results of searches initiated with lambda Red Beta (NP_040617.1). Convergence was achieved in 8 iterations.
[c] PSI-BLAST results of searches initiated with RecT (NP_415865.1) from *E. coli* DH10B. Convergence was achieved in 14 iterations.
[d] Adjacent genes encoding a putative exonuclease. The dashes indicate that a gene encoding an exonuclease was not found.

streptomycin-resistant *rpsL*(*K43R*) allele, and the frequency of recombination was calculated by determining the number of colonies that grew on streptomycin-containing selective medium. Using the streptomycin resistance assay, the average recombination frequency of *P. syringae* cells transformed with 5 μg of an 84-nucleotide (nt) oligonucleotide containing a four-base change (oSWC1255) was 2.4×10^4 recombinants/10^8 viable cells in the presence of a plasmid (pUCP24/*recT*) designed to constitutively express $recT_{Psy}$, compared to 975 recombinants/10^8 viable cells for a *P. syringae* strain containing the empty vector control (pUCP24/61), a 25-fold difference in recombination frequency (Fig. 1). No colonies were observed in control transformations in which oligonucleotides were not added, indicating that $RecT_{Psy}$-mediated recombination in *P. syringae* is at least 5 orders of magnitude higher than the background rate for spontaneous streptomycin resistance,

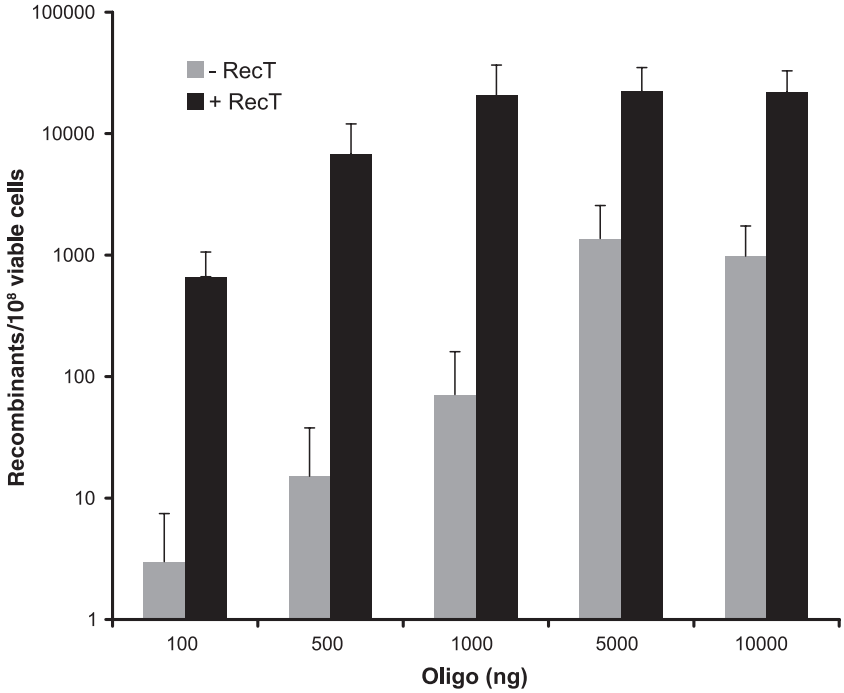

FIG. 1. Oligonucleotide concentration affects recombination frequency. The influences of oligonucleotide concentration on recombination frequency were compared in *P. syringae* cells containing the $RecT_{Psy}$ expression vector (black bars) or the empty vector control (gray bars). For each concentration, cells were transformed in equal volumes with the indicated amount of oligonucleotide containing changes coding for streptomycin resistance. Results here and in succeeding figures are the averages from three independent experiments, with standard deviations indicated by error bars.

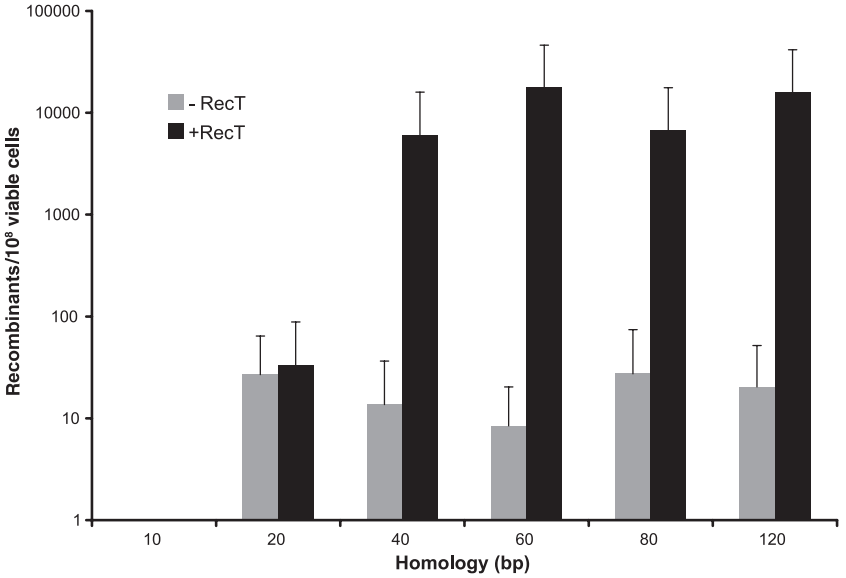

FIG. 2. Effect of oligonucleotide length on $RecT_{Psy}$-mediated recombination. *P. syringae* cells containing either the $RecT_{Psy}$ expression vector or the empty vector control were transformed with 1 μg of oligonucleotide. Each oligonucleotide was designed with two equal-length regions of homology (combined lengths are indicated on the *x* axis) flanking the 4-base change coding for streptomycin resistance. For example, the 40-bp homology oligonucleotide is composed of two 20-nt segments of the *rpsL* sequence flanking the 4-base change. The oligonucleotides used in this experiment are oSWC1978 (10 nt), oSWC1518 (20 nt), oSWC1251 (40 nt), oSWC1253 (60 nt), oSWC1255 (80 nt), and oSWC1257 (120 nt). See Table 2 for the complete sequence of each oligonucleotide.

which was below (0.17 streptomycin-resistant cells/10^8 viable cells) the limit of detection for this assay. Lambda Beta was also tested using our expression system, but we did not see any evidence of Beta-mediated recombination in *P. syringae* (data not shown).

Effect of oligonucleotide concentration on $RecT_{Psy}$-mediated recombination. The amount of oligonucleotide used in the transformations was varied in order to assess whether oligonucleotide concentration influences the frequency of $RecT_{Psy}$-mediated recombination events. In this experiment, *P. syringae* cells containing either the $RecT_{Psy}$ expression vector or the empty vector control were transformed in a constant volume containing 0.1 μg, 0.5 μg, 1 μg, 5 μg, or 10 μg of the *rpsL*(*K43R*)-carrying oligonucleotide (oSWC1255), and the recombination frequency was determined (Fig. 1). Over the range of concentrations tested, maximal levels of $RecT_{Psy}$-mediated recombination were attained using 1 μg of oligonucleotide. In contrast, the saturation threshold for RecT-independent recombination events was 5 μg, as has been reported elsewhere (34).

Comparing the recombination frequencies of $RecT_{Psy}$-mediated and recombinase-independent events at each concentration of oligonucleotide also helps to illustrate the role of $RecT_{Psy}$. The data show that the influence of $RecT_{Psy}$ is greater at lower concentrations. For example, we observed a 450-fold difference in recombination frequency between the $RecT_{Psy}$ expression and empty vector control strains at 0.5 μg (Fig. 1). On the other hand, the relative difference between the $RecT_{Psy}$ and control strains was only 25-fold at 5 μg of oligonucleotide, showing that under these conditions, the relative influence of RecT diminishes as the concentration of the oligonucleotide increases. These data suggest that in the absence of RecT, the recombination frequency is more dependent on oligonucleotide concentration, possibly because efficient recombination is more strongly affected by the actions of inhibitors, such as nucleases that degrade the oligonucleotide and prevent it from being able to participate in recombination (10, 34). With RecT present, oligonucleotides are capable of efficient recombination at lower concentrations. These results are consistent with the role that has been proposed for other ssDNA-annealing recombinases, which is to protect the ssDNA and promote annealing to the target DNA by increasing the rate of invasion or stabilizing the interactions (15, 17, 32).

$RecT_{Psy}$-mediated recombination requires oligonucleotides of sufficient length. The effect of oligonucleotide length on $RecT_{Psy}$-mediated recombination was investigated by transforming *P. syringae* cells with oligonucleotides of different lengths in the presence of the $RecT_{Psy}$ expression vector or the empty vector control. Each oligonucleotide contained the same 4-base change mutating the *rpsL* gene to streptomycin resistance. The results of this experiment can be grouped into three categories (Fig. 2). In the first category, which applies to the 10-nt oligonucleotides, there was no evidence of recombination in either the presence or absence of the recombinase. In the second category were the 20-nt oligonucleotides, with which the rates of recombination were the same in the presence or absence of $RecT_{Psy}$. This result suggests that a 20-mer is long enough for RecT-independent recombination but too short for RecT-dependent recombination. In the final category were oligonucleotides ranging from 40 nt to 120 nt. For these lengths, recombination rates were increased in the presence of RecT but not in its absence. These results suggest that there is a length threshold between 20 nt and 40 nt that must be exceeded for RecT to mediate recombination. Lambda Beta also displays a dramatic length dependence that has been attributed to the requirement for the substrate oligonucleotides

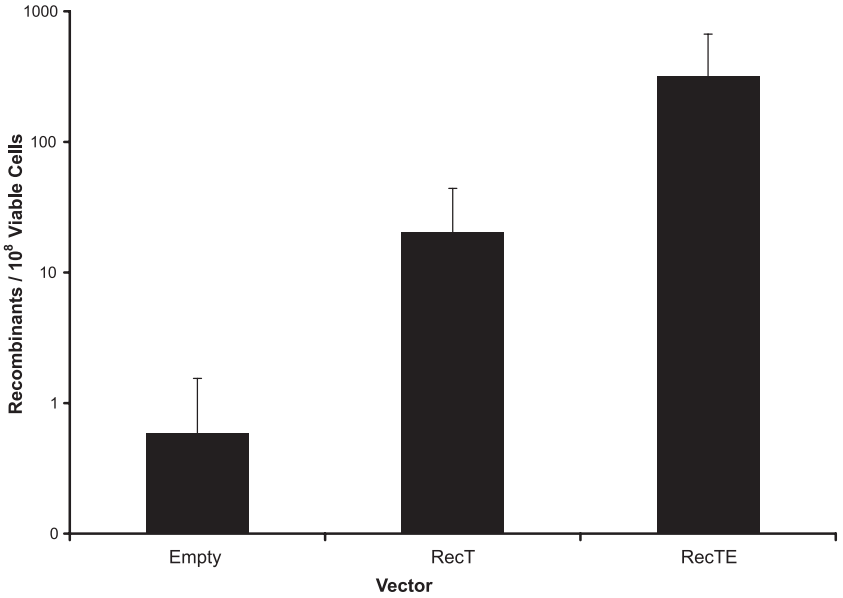

FIG. 3. Frequency of $RecT_{Psy}$- and $RecTE_{Psy}$-mediated recombination. The recombination frequency was assessed in *P. syringae* cells containing expression plasmids for $RecT_{Psy}$ and $RecTE_{Psy}$ or the empty vector control. These strains were used to compare the recombination frequencies of cells transformed with 1 μg of a 186-bp PCR product containing the *rpsL*(*K43R*) change.

to be long enough for Beta to bind (31), and therefore efficient Beta-mediated recombination requires substrate DNA to be at least 30 to 40 bases long (11, 31).

$RecT_{Psy}$ and $RecE_{Psy}$ are required for efficient recombination of double-stranded substrates. The gene encoding the $RecT_{Psy}$ recombinase is located in the *P. syringae* pv. syringae B728a genome adjacent to an ORF that is expected to encode a protein with similarity to exonucleases found in other phage recombination systems (Table 3). These proteins assist recombination by digesting DNA in the 5′-to-3′ direction, creating 3′ ssDNA overhangs that are acted upon by the recombinase protein to promote strand invasion and annealing at the homologous target location (8, 37, 43). Based on this paradigm, if $RecE_{Psy}$ is functional, then it should be able to assist $RecT_{Psy}$ in the recombination of a dsDNA substrate. We tested this by determining the recombination frequency of a 186-bp PCR product encoding the *rpsL*(*K43R*) change in cells containing the vector designed to express either $RecTE_{Psy}$, $RecT_{Psy}$, or the empty control. The results show that including both the $recE_{Psy}$ and $recT_{Psy}$ genes increased the recombination frequency 17-fold relative to the recombination frequency of $recT_{Psy}$ alone (Fig. 3). The low level of recombination (20 recombinants/10^8 viable cells) in the absence of $RecE_{Psy}$ may be due to the presence of ssDNA in the PCR product as a result of incomplete DNA synthesis. We also assessed the ability of $RecTE_{Psy}$ to facilitate ssDNA oligonucleotide recombination and found that coexpression of $RecT_{Psy}$ with $RecE_{Psy}$ did not further increase the frequency of oligonucleotide recombination in comparison to expression of $RecT_{Psy}$ alone (data not shown).

Recombineering in practice. We have begun to take advantage of the $RecTE_{Psy}$ system to modify the *P. syringae* pv. tomato DC3000 genome for experimental purposes that are not directly related to understanding the function of the $RecTE_{Psy}$ proteins *per se*. Many strategies that employ $RecTE_{Psy}$-mediated recombination for genetic engineering can be imagined (28), and we have successfully used $RecTE_{Psy}$ to facilitate two types of structurally related genomic disruptions (Fig. 4).

In the first example, we used $RecTE_{Psy}$ to move a marked mutation from a parental strain into alternative strain backgrounds, as is typically done using a transducing phage in other model systems. In this experiment, PCR products were generated from *P. syringae* genomic DNA using primers that flank a mini-Tn*5* insertion encoding kanamycin resistance in gene PSPTO_0362 (Fig. 4A). These primers amplified the transposon (1.2 kb) in addition to 80 and 83 bp of the flanking sequences, which provide homology to direct the insertion to the genomic sequence of the wild-type target allele in the recipient strain. The PCR product (1.5 μg) was used to transform *P. syringae* cells containing the $RecTE_{Psy}$ expression vector or empty vector control, and recombinants were selected for growth on a medium containing kanamycin. An average of 11.3 recombinants per 10^8 viable cells were obtained in cells expressing $RecTE_{Psy}$, and no recombinants were obtained in cells of the control strains. A subset of the individual kanamycin-resistant isolates was analyzed using PCR. All of the clones analyzed (32/32) were confirmed to contain the insert in the correct chromosomal location.

We have also adapted a PCR-based strategy for generating *de novo* deletions that could be applied to any nonessential genomic region. The substrate used to direct the deletion was produced using long flanking homology (LFH) PCR (23, 27, 39), where the product has the structure of the desired mutation and flanking sequences (Fig. 4B). For example, we deleted a gene coding for a TonB-dependent siderophore receptor (PSPTO_1206). This gene was deleted from the genome by generating a PCR product with 744 bp and 698 bp of homology

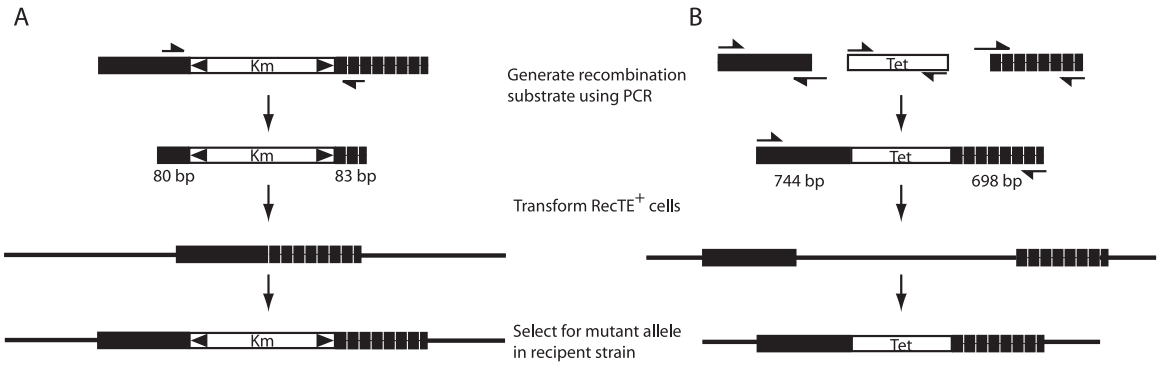

FIG. 4. $RecTE_{Psy}$-mediated recombineering strategies. PCR products with sequences encoding antibiotic resistance and flanking homologies to genomic loci were used to introduce site-directed mutations in *P. syringae* DC3000. (A) The PCR product was amplified directly from genomic DNA using primers flanking a Tn*5* insertion encoding kanamycin resistance. (B) The PCR product was generated using an LFH PCR technique in which flanks were amplified with primers that introduce sequences homologous to the 5′ and 3′ ends of a tetracycline resistance-encoding cassette. In a second round of PCR, the three fragments join to make one large molecule by the annealing of overlaps, followed by extension.

flanking a 1,323-bp region of pBR322 containing a tetracycline resistance gene. This product (1.0 μg) was transformed into *P. syringae* cells containing the $RecTE_{Psy}$ expression vector or empty vector control, and recombinants were selected for growth on tetracycline-containing culture medium. In this experiment, we observed 45 recombinants per 10^8 viable cells in cells containing the $RecTE_{Psy}$ expression vector, compared to 0 recombinants in the empty vector control strain. PCR was used to confirm that all of the tetracycline-resistant *P. syringae* cells tested (11/11) contained the mutant allele in the correct genomic location, which was further verified by sequence analysis.

DISCUSSION

We have identified and characterized key components of a new recombineering system derived from a pseudomonad, belonging to a group that includes many pathogens and environmentally important organisms. The genes that encode these functions were identified in the plant pathogen *P. syringae* pv. syringae B728a based on similarity to genes used for recombineering in other bacteria. We have designated these genes *recT*$_{Psy}$ and *recE*$_{Psy}$ and their products $RecT_{Psy}$ and $RecE_{Psy}$ based on evidence that the genes encode the functional orthologs of lambda Exo/Beta and Rac RecET.

The strategy described here could be used to identify new phage-encoded recombinases in other bacteria that currently lack recombineering tools. The utility of individual recombinases appears to be restricted to a limited range of species (9), possibly due to a requirement for specific adaptations to facilitate interaction with host-encoded functions. Based on this idea and on our failure to observe lambda Red-mediated recombination in *P. syringae*, we formulated the hypothesis that recombinases found in or associated with particular species would be more likely to function in close relatives than in more distantly related species. A similar approach was used to identify a phage-encoded recombinase and exonuclease that have been used for recombineering in *Mycobacteria* (37). Our analysis focused on identifying a recombineering system for use in *P. syringae* pv. tomato DC3000, but we also identified recombinase candidates in other *Pseudomonas* species (Table 3). The canonical paired arrangement of the genes encoding the recombinase and exonuclease in *P. fluorescens* SBW25 suggests that these genes may also be useful for recombineering. Additionally, it will be interesting to determine to what extent $RecT_{Psy}$ and $RecE_{Psy}$ will function in other pseudomonads.

The $RecT_{Psy}$ protein identified in *P. syringae* is likely a member of the RecT/Redβ superfamily. Proteins in this superfamily have limited sequence identity (Table 3) but share a characteristic sequence conservation pattern that can be detected by the PSI-BLAST algorithm (16). As a superfamily, these proteins are distinct from other proteins capable of similar recombination reactions, such as members of the Rad52 and ERF superfamilies (16). Because of the limited sequence conservation among the RecT/Redβ superfamily, detecting members in sequence databases often requires sophisticated sequence profile matching software such as PSI-BLAST. Additional information for identifying candidates for recombineering can be gleaned from examination of the genomic context. Finding the gene coding for a recombinase in the genetic neighborhood of an exonuclease gene provides evidence supporting orthology and is critical for a recombinase to have full utility. Existing data suggest that hybrid systems consisting of recombinases and exonucleases from different organisms do not support maximal activity (9, 30), implying a potential physical association or some other type of optimization between the proteins for coordinating recombination. Furthermore, Iyer et al. have proposed that the entire class of ssDNA-annealing proteins, including the RecT/Redβ, ERF, and Rad52 families, are of phage origin (16). Therefore, evaluating whether a given recombinase is situated in the genome as part of a prophage is useful to further support membership in this class of proteins.

The factors that determine the host range of phage-encoded recombinases are not known, so identifying candidates that facilitate recombination requires empirical testing. Functional analyses were accomplished using simple assays for recombination frequency, in which recombinants were counted and standardized to a fixed number of viable cells from each transformation. Initial experiments focused on characterizing $RecT_{Psy}$ alone by testing its ability to promote recombination of ssDNA oligonucleotide substrates, because this reaction requires only the action of the recombinase protein, as has

been demonstrated previously for other proteins in the same class (9, 11, 36). Our results show that $RecT_{Psy}$ promotes oligonucleotide recombination and supports the conclusion that $RecT_{Psy}$ is functionally equivalent to *E. coli* RecT, which has been demonstrated to facilitate strand invasion, annealing, and strand transfer *in vitro* (15, 32). The ability of $RecT_{Psy}$ and $RecE_{Psy}$ to facilitate dsDNA recombination was also tested. This experiment demonstrated that both $RecT_{Psy}$ and $RecE_{Psy}$ were required for efficient recombination of a dsDNA substrate, which is consistent with the proposed role of RecE as a $5' \rightarrow 3'$ DNA exonuclease (6, 20, 42). Together, these experiments demonstrate that we have identified new RecT and RecE proteins that are capable of promoting recombineering-type recombination in a pseudomonad.

Recombineering dramatically changes what is possible for scientists engaged in genetic analysis and engineering. Because of the technical differences between recombineering and traditional approaches (i.e., recombineering does not use restriction enzymes), constructions can be accomplished directly in the genome of the target organism. This offers a new way to approach experiments, and we have already begun to employ this system for routine genetic manipulation of *P. syringae* pv. tomato DC3000 (Fig. 4). These methods are robust; we have succeeded in obtaining the correct mutant in all of our attempts using $RecTE_{Psy}$, and under the conditions tested, no recombinants arose in the absence of the $RecTE_{Psy}$ expression plasmid. For example, we have successfully moved eight separate mutant alleles between strains for the purpose of isolating mutations as well as to achieve optimal genetic backgrounds for experimental purposes. These recombinants were generated using short homologies (~80 nt), which is typical for phage-mediated recombination systems. In this example, recombineering was used as an alternative to phage-mediated transduction. Currently, a characterized phage capable of generalized transduction is not available for *P. syringae*, and the absence of this tool has imposed practical limitations in genetic analysis. We offer that $RecTE_{Psy}$ might be used as an approach to circumvent this problem in lieu of real alternatives. Also, we have successfully used the $RecTE_{Psy}$ system to generate *de novo* gene deletions using LFH PCR. This approach can be used, at least in theory, to disrupt any nonessential locus, and demonstrating the integration of an antibiotic resistance cassette establishes a compelling measure of this system's utility.

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