

Analysis of the Role of *recA* in Phenotypic Switching of *Pseudomonas tolaasii*

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Switching between the pathogenic smooth (1116S) and nonpathogenic rough (1116R) forms of *Pseudomonas tolaasii* occurs due to the reversible duplication of a 661-bp element within the *pheN* locus. Disruption of the chromosomal *recA* locus of 1116S and 1116R produced strains 1116S*recA* and 1116R*recA*, respectively, which showed typical loss of UV resistance. Switching from the smooth to the rough form was virtually eliminated in the 1116S*recA* strain, whereas the extent of switching from the rough to the smooth form was almost identical in 1116R and 1116R*recA*. It is concluded that phenotypic switching from 1116S to 1116R is *recA* dependent whereas that from 1116R to 1116S is *recA* independent.

Pseudomonas tolaasii is the causal agent of the economically important brown blotch disease of the cultivated mushroom *Agaricus bisporus* (Lange) Imbach (19). Colonies of the wild-type strain of *P. tolaasii* (designated 1116S) are opaque, mucoid, pathogenic, and nonfluorescent and produce tolaasin (6). In contrast, colonies of the stable phenotypic variant form of 1116S (designated 1116R) are translucent, nonmucoid, nonpathogenic, and fluorescent and do not produce tolaasin (6). Phenotypic switching in *P. tolaasii* from 1116S to 1116R is due to a reversible 661-bp duplication in the putative kinase domain of the regulatory locus *pheN* (8). This DNA duplication causes a frameshift mutation in the predicted *pheN* open reading frame (ORF), resulting in a truncated *pheN'* ORF encoding a 77-kDa protein, which lacks part of the PheN sensor domain (8). Spontaneous switching from 1116R to 1116S occurs by precise deletion of the 661-bp element, restoring full functionality of PheN (8). This reversible mutation within *pheN* therefore represents one mechanism whereby *P. tolaasii* can switch its phenotype between pathogenic and nonpathogenic forms.

Studies with *Escherichia coli* and other bacteria have shown that RecA-dependent DNA recombination is the main mechanism of general homologous recombination (4). RecA-dependent recombination has been demonstrated in antigenic variation in *Borrelia hermsii* (17), in differential expression of surface layer proteins in *Campylobacter fetus* (3), in the instability of capsule production in *Haemophilus influenzae* type b (12), in switching of type IV pilin in *Neisseria gonorrhoeae* (15), in amplification of toxin genes of *Vibrio cholerae* (5), and in virulence determination in *Yersinia pestis* (10). In this paper we show that RecA plays a functional role in DNA rearrangement associated with phenotypic switching in *P. tolaasii*.

Putative cosmid clones containing the *P. tolaasii recA* gene were isolated by functional complementation of the *recA*-deficient *E. coli* host strain HB101 after en masse mobilization of a *P. tolaasii* genomic library and selection at 25°C on Pseudo-

monas agar F (PAF) (21) plates containing 0.02% methyl methanesulfonate (2). Complementation of *E. coli* DH5 α (9) showed that pAPRL1 (Fig. 1) was able to restore RecA function as assessed by methyl methanesulfonate sensitivity. Subcloning of pAPRL1 in pBS (SK⁺) and subsequent DNA sequence analysis of subclones pAPR1 and pAPR2 revealed the presence of two ORFs of 1,059 and 468 nucleotides (EMBL accession number, *recAX*, AJ249265), coding for two proteins of 353 and 155 residues with predicted molecular masses of 37.6 and 17.9 kDa, which were designated *recA* and *recX*, respectively. This region had >90% homology to the *P. fluorescens recA-recX* region (1, 2).

A 1.4-kb *BsrBI-HindIII* fragment from pAPR1 containing the *P. tolaasii recA* gene was ligated into *SmaI-HindIII*-digested pBS (SK⁺) to generate pHSR1. The 1.3-kb *EcoRI* fragment containing the kanamycin cassette from pUC4K (Promega) was ligated into the *EcoRI* site within the *recA* gene in pHSR1 to yield plasmid pHSR2. The 2.7-kb *XbaI-ApaI* fragment containing the *recA* knockout construct was ligated into *XbaI-ApaI*-digested pKNG101 (13) to give the suicide plasmid construct pHSR3, which was transformed into *E. coli* CC118 λ *pir* (11). Marker exchange mutagenesis following triparental mating, between *P. tolaasii* 1116S or 1116R together with the *E. coli* CC118 λ *pir* strain containing pHSR3 and *E. coli* containing the *tra*⁺ helper plasmid pNJ5000 (7), was as described previously (13, 14). Putative *recA*-disrupted strains, which were kanamycin resistant, streptomycin sensitive, and able to grow in the presence of 10% (wt/vol) sucrose, designated 1116S*recA* and 1116R*recA*, were selected. The site of *recA* disruption was confirmed by Southern hybridization of genomic DNA of 1116S*recA* and 1116R*recA* with an appropriate 1.1-kb *recA* probe (produced by PCR with primers PRECA1 [5'-AATGG ACGACAACAA-3', *recA* residues 413 to 427, accession number AJ249265] and PRECA2 [5'-GAACAGCGACGAGTG-3', *recA* residues 1514 to 1500, accession number AJ249265] from 25 ng of pAPR1 [Fig. 1]) under optimum PCR conditions. The observed change in band sizes in 1116S*recA* and 1116R*recA* compared to 1116S and 1116R was consistent with disruption of chromosomal *recA* with the kanamycin resistance cassette at the *EcoRI* site (data not shown). The functional disruption of the *recA* transcript was further confirmed by Northern blotting using total cellular RNA and a *recA-recX*-specific probe. No *recA* or *recX* transcripts were detected in the *recA*-disrupted strains (1116S*recA* and 1116R*recA*) of *P. tolaasii*, whereas a positive signal was observed in Northern blots

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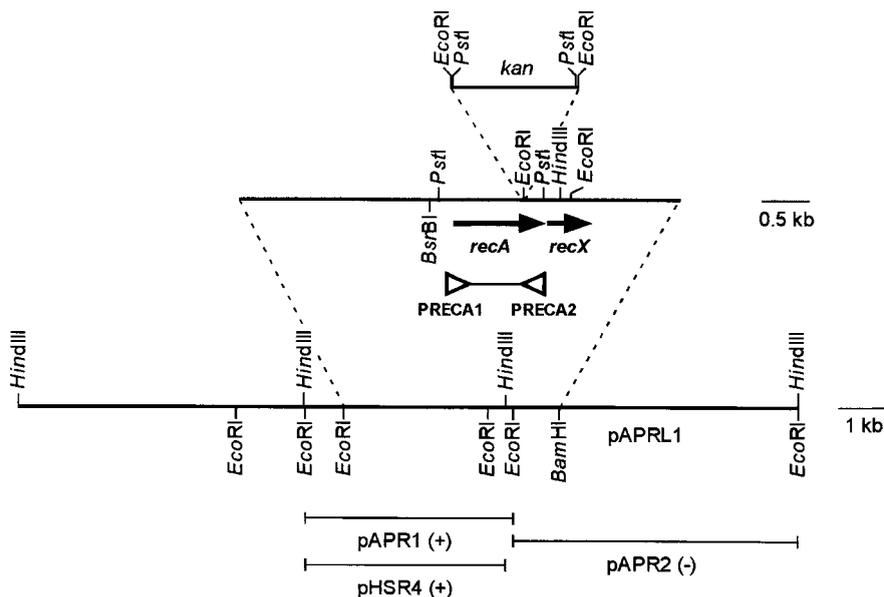


FIG. 1. Restriction map of pAPRL1 showing the positions of *recA* and *recX* and the ability or inability of clones pAPR1 and pAPR2 to restore methyl methanesulfonate resistance (indicated by + and -, respectively) to *E. coli* HB101 and of pHSR4 to restore UV resistance (indicated by +) to *P. tolaasii* 1116*SrecA*. The primers used for *recA* PCR PRECA1 and PRECA2 are indicated as open triangles. Cloning sites, the kanamycin resistance cassette *kan* used, and the site of disruption in *recA* are also shown.

of wild-type *P. tolaasii* (total RNA extracted 6 h after induction with 2 µg of ofloxacin per ml [data not shown]).

Both 1116*SrecA* and 1116*RrecA* were killed by lower doses of UV than were 1116*S* and 1116*R*, respectively (Fig. 2), and UV resistance was at least 75% restored at a UV dose of 400 µJ/cm² by complementation with pHSR4 (a pLAFR3 clone containing a 4.3-kb *Hind*III fragment of pAPRL1 with a functional copy of *recA* [Fig. 1]) in both *recA*-disrupted strains and with pAPRL1 (containing a functional copy of *recA/X* [Fig. 1]) in 1116*SrecA*.

The effect of disruption of the chromosomal *recA* locus on phenotypic switching was determined for both 1116*S* and 1116*R*. The extent of switching from 1116*S* to 1116*R* was determined in a shake culture, inoculated with a single 2-day colony of 1116*S*, after 7 days by measuring the percentage of S and R forms in the resulting population. Since the culture reaches stationary phase after 2 to 3 days, it is not appropriate in this assay to relate the extent of switching to the number of generations of the test organism. Under these conditions, 0.2 to 0.3% of the 1116*S* population were present as the 1116*R* phenotype, whereas in 1116*SrecA*, this was reduced to less than 0.01% and was restored to 0.14% when complemented with pHSR4 (Fig. 3A). Colony PCR analysis (data not shown) of five randomly chosen R forms arising from 1116*SrecA*, with PHEN1 (5'-GGGCTATTTAC25692-3', *pheN* residues 339 to 357, accession number U25692 [6]) and PHEN2 (5'-GCGGATTTCTGGCTCAT-3', *pheN* residues 1132 to 1114, accession number U25692 [6]) primers which flank the 661-bp duplication site in *pheN* (8), established that in each case no duplication had occurred in the *pheN* locus as seen in the 1116*S*-to-1116*R* conversion (data not shown). In contrast, the expected 661-bp duplication was observed by PCR analysis of five R forms arising from 1116*SrecA*/pHSR4 using the same primers (data not shown). When 1116*SrecA* was complemented with pAPRL1 containing the whole *recA/X* locus, the extent of switching was restored to 0.13%, which was not significantly different (at the 99% confidence level) from that in 1116*SrecA*/pHSR4 (Fig. 3A). PCR analysis (as described above) of five

randomly chosen colonies of R forms arising from 1116*SrecA*/pAPRL1 confirmed that in all cases the expected 661-bp duplication had occurred in the *pheN* locus (data not shown). Finally, introduction of pLAFR3 into 1116*SrecA* had no effect

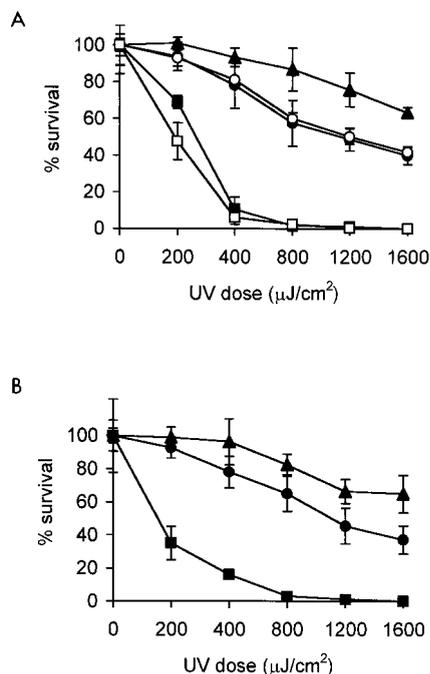


FIG. 2. UV kill curves for wild-type, *recA*-disrupted, and complemented strains of *P. tolaasii* 1116*S* (A) and 1116*R* (B). ▲, wild type; ■, *recA* disrupted strain; □, *recA* disrupted strain complemented with pLAFR3; ●, *recA* disrupted strain complemented with pHSR4; ○, *recA* disrupted strain complemented with pAPRL1. Cells were plated on Pseudomonas F plates, exposed to UV light in a UV Stratalinker (Stratagene), and incubated at 25°C in the dark for 2 days. Error bars indicate the standard deviation from triplicate samples.

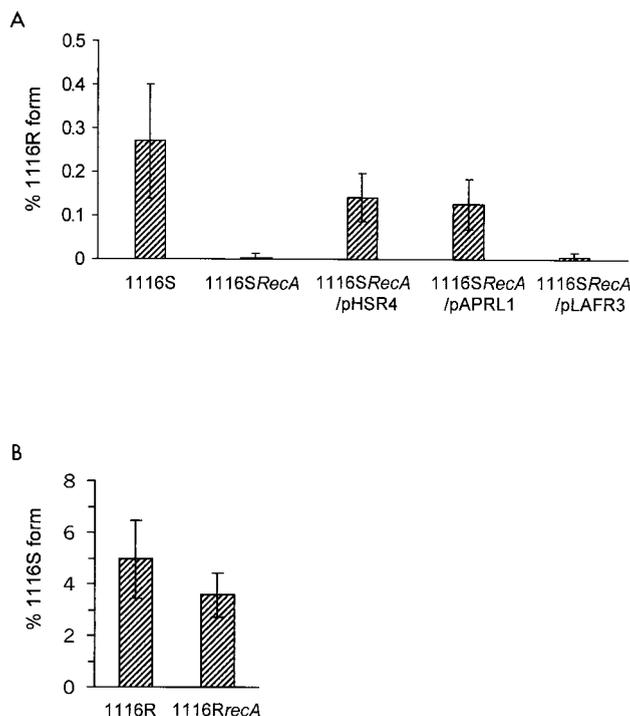


FIG. 3. (A) Extent of phenotypic switching from *P. tolaasii* 1116S, 1116S*recA*, 1116S*recA*/pHSR4, 1116S*recA*/pAPRL1, and 1116S*recA*/pLAFR3 to the corresponding R form. Serial dilutions of a shake culture (7 days at 25°C) in *Pseudomonas* F broth with appropriate antibodies were plated on PAF agar plates and incubated at 25°C for 2 days, and colonies were scored for phenotype. The extent of switching was calculated as the percentage of R forms in the total number of colonies. Error bars are from 10 replicate determinations in two independent experiments. The mean values for 1116S*recA* and 1116S*recA*/pLAFR3 are significantly different at the 99% confidence level (using the χ^2 test) from the mean values for 1116S, 1116S*recA*/pHSR4, and 1116S*recA*/pAPRL1. (B) Extent of phenotypic switching from *P. tolaasii* 1116R and 1116R*recA* to the S form in 7-day-old colonies. Serial dilutions of an 18-h culture grown at 25°C were plated out on PAF agar plates with appropriate antibiotics to give 10 to 15 colonies per plate. After incubation for 7 days at 25°C, three individual colonies were randomly selected, removed, and resuspended in 5 ml of sterile deionized water, and serial dilutions were plated out on PAF agar. The resulting colonies were scored for phenotype after a 2-day incubation at 25°C, and the extent of switching was calculated as the percentage of S forms in the total number of colonies. Error bars are from 10 replicate determinations in two independent experiments. The mean values are not significantly different at the 99% confidence level (using the χ^2 test).

on the extent of switching observed (Fig. 3A). Although both *recA* and *recX* functions are disrupted in 1116S*recA* and these functions should both be restored in 1116S*recA*/pAPRL1, the ability of pHSR4, which contains only the *recA* gene (Fig. 1), to restore phenotypic switching establishes that conversion of 1116S to 1116R forms is *recA* dependent. However in a *recA*-deficient background, phenotypic switching occurs to a reduced extent and is due to a mechanism other than the duplication event observed in 1116S (8).

The extent of switching from 1116R to 1116S was determined by analyzing the percentage of S and R forms in 7-day-old colonies of 1116R on PAF. In this assay the S form appears from day 4 onward as microcolonies within the 1116R colony, and it is not possible to determine the number of generations of the 1116R and 1116S forms. Under these conditions, the extent of switching from the wild-type 1116R to the 1116S form was 4.8%, and there was no significant reduction in the extent of switching in 1116R*recA* (Fig. 3B). These data establish that *recA* is not required for the 1116R-to-1116S reversion. Analysis of five independently isolated S forms from 1116R*recA* using the PHEN1 and PHEN2 primers described above confirmed

the expected precise deletion of the 661-bp fragment in *pheN* in all colonies examined, as seen in 1116S forms arising from 1116R (data not shown). It is therefore concluded that under the assay conditions used, transition of the pathogenic form (1116S) to the nonpathogenic form (1116R) is dependent on *RecA* function but that reversion from 1116R to 1116S is independent of *RecA* function.

Disruption of *recA* in 1116S and 1116R also abolished expression of the downstream ORF *recX* (data not shown). Plasmids pAPRL1 and pHSR4 restored UV resistance and the extent of switching to the 1116R form in 1116S*recA* (Fig. 2) to the same extent. This argues against a direct role of *recX* in *pheN* duplication-related phenotypic switching from 1116S to 1116R forms in *P. tolaasii*. Although the precise role of *recX* in *recA*-mediated recombination is still under investigation, the ability of *recX* to reduce the toxicity of *recA* overexpression has been demonstrated in *P. aeruginosa* (18), *Streptococcus lividans* (20), and *Mycobacterium smegmatis* (16).

The biological significance of the phenotypic switch in *P. tolaasii* remains to be established. The higher extent of reversion from the avirulent 1116R form to the virulent 1116S form may be important in the epidemiology of brown blotch disease. The failure to find the origin of primary inoculum in mushroom farms may be due to the inadequacy of current diagnostic tests, which are able to detect only the 1116S form (21). Thus, the 1116R form may constitute the primary inoculum, which could revert to the virulent form either spontaneously or as a result of receiving environmental cues.

Himanshu Sinha and Arnab Pain contributed equally to the work.

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