JOURNAL OF BACTERIOLOGY, Aug. 2004, p. 5551–5554 Vol. 186, No. 16
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.16.5551–5554.2004

Excision of the *Shigella* Resistance Locus Pathogenicity Island in *Shigella flexneri* Is Stimulated by a Member of a New Subgroup of Recombination Directionality Factors

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Received 17 March 2004/Accepted 7 May 2004

Pathogenicity islands are capable of excision and insertion within bacterial chromosomes. We describe a protein, Rox, that stimulates excision of the *Shigella* resistance locus pathogenicity island in *Shigella flexneri*. Sequence analysis suggests that Rox belongs to a new subfamily of recombination directionality factors, which includes proteins from P4, enterohemorrhagic *Escherichia coli*, and *Yersinia pestis*.

A variety of mobile genetic elements, including insertion sequences, transposons, plasmids, bacteriophages, and pathogenicity islands (PAIs), mediate the lateral acquisition of foreign DNA in bacteria. PAIs are large regions of the chromosome that are genetically and functionally distinct units (7). These elements are often incorporated into bacterial chromosomes by site-specific integration at tRNA genes, and some are capable of excision. PAIs often have G+C contents that vary from those of their host bacteria, suggesting that they are acquired by horizontal transfer. Thus far, PAIs have been identified predominantly in gram-negative enteric and uropathogenic organisms, such as *Salmonella* spp. and *Vibrio cholerae*, and in a variety of other pathogens, e.g., *Escherichia coli*, *Helicobacter pylori*, *Yersinia enterocolitica*, and *Shigella flexneri* (9). *S. flexneri* carries five distinct PAIs: *Shigella* island 2 (SHI-2), SHI-3, the *she* PAI, the *Shigella* resistance locus (SRL) PAI, and the *mxi-spa* gene cluster on the large virulence plasmid (1, 13, 15, 17, 28, 29).

The SRL PAI is a 67-kb island first identified in *S. flexneri* 2a YSH6000 (13). The element was discovered due to its carriage of a locus mediating resistance to ampicillin, streptomycin, chloramphenicol, and tetracycline, the SRL. The SRL PAI also encodes a functional ferric citrate transport system, a number of insertion sequences, and a large number of phage-like genes. Turner et al. have shown that the SRL PAI can undergo precise insertion and deletion in an integrase-dependent manner (26, 27).

The importance of genetic acquisition and loss was highlighted by the recent sequencing of the *S. flexneri* 2a genome, which showed that the major differences between *S. flexneri* and *E. coli* arose primarily through the loss or acquisition of DNA, including PAIs and bacteriophages (8). However, very little is known about the movement of PAIs into and out of bacterial genomes. Several PAIs, including the SRL and *she* PAIs, are capable of spontaneous deletion from the chromosomes in which they normally reside (18, 27). Like many PAIs, the SRL PAI encodes an integrase that is homologous to members of the P4 family of integrases (13). Integrases mediate PAI integration into the chromosome (19, 26) and are also essential for PAI excision (11, 20, 27). However, little is known about how the excision of PAIs is regulated.

The regulation of integrase-mediated excision of mobile genetic elements, in the cases where it is understood, follows two distinct mechanisms. In the majority of cases, excision is stimulated by a class of proteins collectively known as excisionases or recombination directionality factors (RDFs) (12). RDFs have been identified mostly in phage systems but have also been identified in transposons (22) and plasmids (14). Typical members of this family include the bacteriophage lambda excisionase (Xis) and bacteriophage P2 Cox proteins. RDFs are usually small DNA-binding proteins encoded by genes in close proximity to the integrase genes carried on mobile elements. In the best-studied case, that of lambda Xis, the protein regulates excision by binding directly to DNA in the vicinity of the recombination site and to integrase to form a complex that stimulates excision and inhibits integration (23). The excision of CP4-57, a genomic island of *E. coli* K-12, is stimulated by an alternative mechanism that relies instead on the transcriptional activation of the CP4-57 integrase gene by a protein designated AlpA (25). The resulting increase in integrase expression is responsible for the stimulation of excision. In the present study, we tested whether excision of the SRL PAI was also regulated and investigated the mechanism by which this regulation occurs.

Sequence analysis of AlpA homologues. Analysis of the SRL PAI sequence revealed two open reading frames (ORFs) with corresponding sequence similarity to proteins that regulate the excision of other mobile elements, namely, ORF3, which was subsequently termed Rox (regulator of excision), and ORF41 (13). Rox shows 66% sequence similarity to AlpA from CP4-57, while ORF41 is more closely related (53% similarity) to Vis, another AlpA homologue from the satellite phage P4. Vis is a multifunctional protein that represses transcription from the P_{LL} late promoter of P4 and activates transcription of P_{sid} (16). In addition, Vis represses transcription of the P4 integrase gene and is also an excisionase that promotes integrase-dependent P4 excision (D. Ghisotti, personal communication).

The recent sequencing of the enterohemorrhagic *E. coli*

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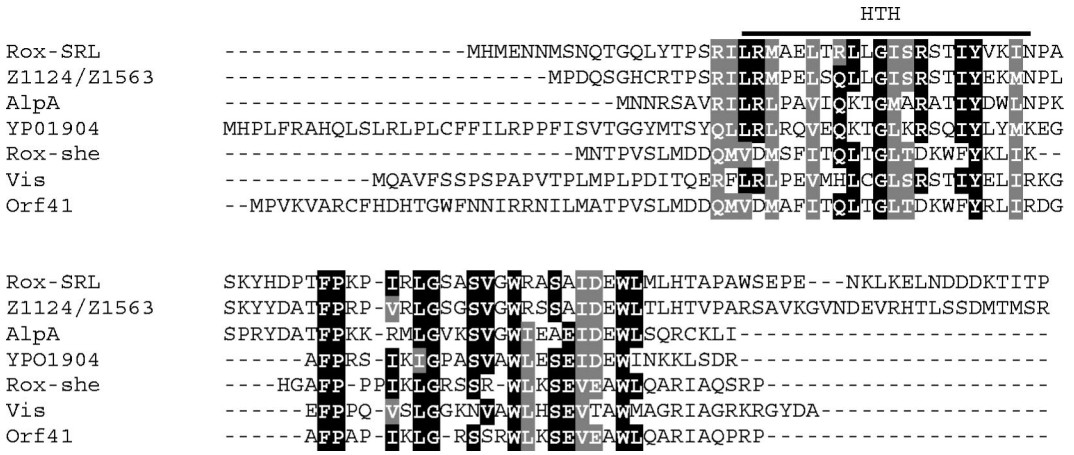

FIG. 1. Multiple alignment of Rox homologues. Amino acids that are identical or similar in at least two-thirds of the sequences are rendered as white characters on a black or grey background, respectively. Z1124 and Z1563 are encoded by EHEC EDL933 O islands 43 and 48, respectively. YPO1904, Rox_{she}, Vis, and AlpA are proteins encoded by the HPIs of *Y. pestis*, the *S. flexneri she* PAI, bacteriophage P4, and cryptic prophage CP4-57, respectively. The proposed helix-turn-helix (HTH) motif is indicated by a bar. Alignment was performed by using Clustal W (24).

(EHEC) strain O157:H7 EDL933 has revealed AlpA homologues, Z1124 and Z1563, on duplicate O islands 43 and 48, which have an integrase nearly identical with that encoded by the SRL PAI. Unlike *alpA*, the EHEC homologues show significant nucleotide identity with *rox*. EHEC EDL933 O islands 43 and 48 encode the proteins required for tellurite resistance and urease biosynthesis and share no similarity with the SRL PAI aside from the presence of a number of putative prophage ORFs (13). Database probing also revealed Rox homologues on other previously described elements, including the *she* PAI in *S. flexneri* and the high pathogenicity island (HPI) of *Yersinia pestis*. An alignment of these seven homologues shows two areas of conservation (Fig. 1). The first region corresponds to a predicted DNA-binding, helix-turn-helix motif in all homologues except Rox_{she} and ORF41, with Dodd and Egan scores (5) for Rox_{SRL}, AlpA, Vis, EHEC Z1124 or Z1563, and YP01904 of 6.35, 3.98, 5.25, 4.94, and 3.45, respectively, where a value of $\geq$2.5 is indicative of a likely helix-turn-helix motif. Database and motif probing has provided no clues as to the potential role of the second conserved region. Mutational analysis may provide clues as to the potential function of this region.

Recently, an *xis* gene was identified on the SXT element of *V. cholerae* and found to have a role in SXT excision (4). SXT Xis did not belong to any of the RDF subgroups previously described by Lewis and Hatfull (12). We compared a member of each previously described subgroup of RDFs, including SXT Xis, with Rox, ORF41, and the homologues described above. Clustal W analysis suggested that these homologues are most closely related to the SLP1 subgroup, which is typified by an RDF encoded by the SLP1 plasmid from *Streptomyces coelicolor* (12), but that they form a distinct and thus a new subgroup (Fig. 2). Of the new subgroup, Rox_{she}, YPO1904, and Vis have been shown experimentally to have a role in excision (10, 20; D. Ghisotti, personal communication).

Effect of Rox and ORF41 on SRL PAI excision. In order to determine if Rox or ORF41 had a role in the excision of the SRL PAI, assays were performed to test SRL PAI excision when these genes were overexpressed. *rox* and *orf41* were amplified by PCR from *S. flexneri* YSH6000 genomic DNA and cloned directionally into the EcoRI and BamHI sites of pPBA1100 (pUC18 carrying *kan* from pMK3), giving rise to plasmids pAL34 and pAL31, respectively. Accurate amplification of PCR products was confirmed by sequencing the inserts of the plasmids obtained. These plasmids, along with pPBA1100, were transformed separately into *S. flexneri* 2a strain YSH6000, resulting in strains AL66(pAL34), AL67(pAL31), and AL75(pPBA1100). Each strain was grown overnight with aeration in 2.5 ml of 2YT medium supplemented with 50 μg of kanamycin/ml and 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Extraction of genomic DNA was performed as described previously (2). Excision of the SRL PAI was assayed by semiquantitative PCR with inward-facing primers flanking the SRL PAI (BAP679 [5′-GTG CTGCTTTCGGTGTGC-3′] and BAP1157 [5′-GCCAGCAT TTCAACAGGAGG]) (Fig. 3). The PCR amplification of *recA* (BAP1643 [5′-CTACGCACGTAAACTGGGCG-3′] and BAP1644 [5′-ACCGGTAGTGGTTTCCGGG-3′]) served as controls for template concentration. Although *recA* controls indicated that the concentration of chromosomal DNA was 10-fold greater in the AL66 template than in the wild-type and AL67 templates (data not shown), the level of SRL PAI excision was 10^4-fold greater in the AL66 template, where Rox was overexpressed, than in the wild-type template (Fig. 3). In contrast, overexpression of *orf41* had no effect on SRL PAI excision. The PCR product obtained from strain AL66 was sequenced and shown to be the reconstitution of *serX* with a single copy of the 14-bp direct repeat that flanks the SRL PAI, as was previously observed upon deletion of the SRL PAI (27). Therefore, Rox, but not ORF41, stimulates SRL PAI excision.

In order to determine if the effect of Rox on PAI excision was dependent on the SRL PAI integrase, a PCR excision assay was performed on an *int* mutant, AL11 (27). AL11 was transformed with pUC19-Tp and pAL85 (pUC19-Tp carrying *rox*), giving rise to strains AL296 and AL295, respectively. The excision assay was performed as described previously, with strains AL325 (wild-type *int* with pAL85), YSH6000 (parent), and SBA1363 (SRL PAI$^-$) as controls. The results showed that

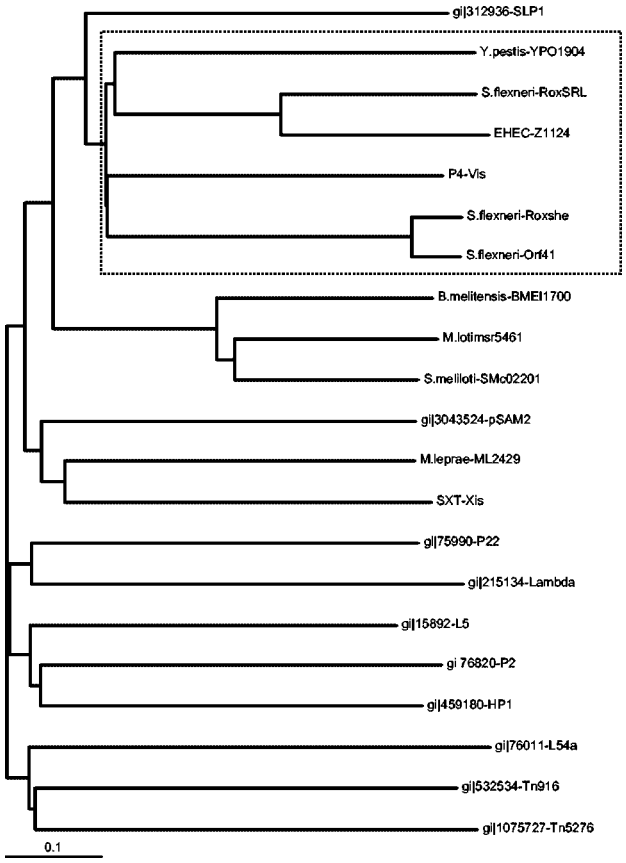

FIG. 2. Phylogenetic tree representing subgroups of RDFs. Members of each subgroup were chosen based on the Lewis and Hatfull definition of RDF subgroups (12). The proposed new subgroup is boxed. Mapping was performed by using Clustal W (24). B.melitensis, *Brucella melitensis*; M.loti, *Mesorhizobium loti*; S.melioti, *Sinorhizobium meliloti*; M.leprae, *Mycobacterium leprae*.

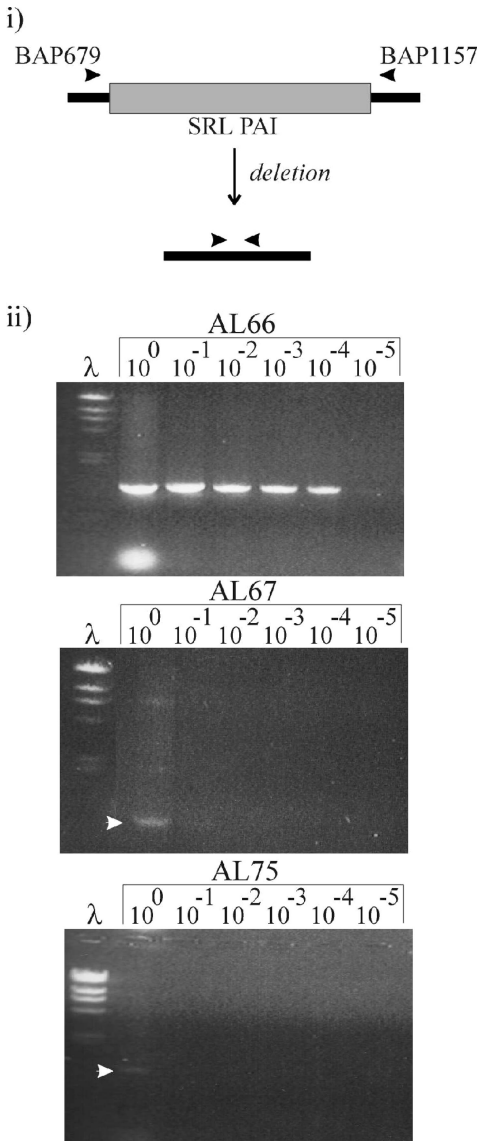

FIG. 3. The effect of Rox_{SRL} and ORF41 on SRL PAI excision. (i) The shaded box denotes the SRL PAI, and the lines denote YSH6000-flanking chromosomal DNA. The primers used to determine if the PAI has excised are shown by arrowheads. (ii) Lane λ, lambda HindIII markers. Other lanes contain 10-fold serial dilutions of genomic DNA. AL66, AL67, and AL75 are *S. flexneri* 2a strains overexpressing *rox*, overexpressing *orf41*, and carrying an empty plasmid, respectively. The SRL PAI excisions in AL67 and the plasmid control can be seen as PCR products and are indicated by the arrowheads.

both Rox and Int have critical roles in SRL PAI excision, as excision was not observed in AL295, the *int* mutant strain that overexpressed Rox. In contrast, SRL PAI excision was clearly visible when *rox* was overexpressed in the presence of an intact *int* gene (AL325) (Fig. 4).

Does Rox affect *int* transcription? A potential binding site (YYRTTCGRNRY) for the bacteriophage P4 Vis protein was proposed by Polo et al. (16). This binding site is present upstream of *slpA*, the gene encoding the CP4-57 integrase, and the SRL PAI *int* gene. The similarity to AlpA and the presence of a potential binding site upstream of *int* suggested that Rox may be required for the regulation of *int* transcription. In order to test this hypothesis, the *int* gene of *S. flexneri* 2a strain SBA1366, an antibiotic-sensitive derivative of YSH6000, was disrupted by a Campbell insertion with pJP5603 as previously described (27), giving rise to strain AL467. It was necessary to inactivate *int* to avoid potential complications associated with SRL PAI excision, leading to the loss of the PAI and therefore the *int* gene. AL467 was transformed with pBAD30 (6) and pAL216 (pBAD carrying *rox*), giving rise to strains AL468 and AL469, respectively. The use of pBAD allowed the tight regulation of Rox expression. RNA was extracted as described previously (21) with the following modifications: cells were grown for 1 h in a solution containing Luria broth, 50 μg of kanamycin/ml, and 100 μg of ampicillin/ml and then induced with 1 mM arabinose for an additional 2 h before RNA extraction. Real-time reverse transcriptase PCR was performed as described by Boyce et al. (3). Assays of *gyrB* transcription served as controls for RNA concentration. Levels of transcription of *rox* were also compared between strains, and as expected, the level of *rox* transcription was significantly higher (27-fold) in AL469 than in AL468. However, there was no statistically significant change (P value = 0.385; two-tailed t test) in *int* transcription when *rox* was overexpressed following

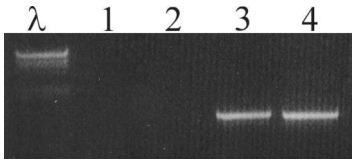

FIG. 4. The effect of Int and Rox_{SRL} on SRL PAI excision. Lane λ, lambda HindIII markers. The various strains used as templates for PCR were as follows: AL296 (*int* mutant, pUC19-Tp) (lane 1), AL295 (*int* mutant, pUC19-Tp/*rox*) (lane 2), SBA1363 (SRL PAI^-) (lane 3), AL325 (wild-type *int*, pUC19-Tp/*rox*) (lane 4).

arabinose induction. Therefore, although Rox stimulates integrase-dependent SRL PAI excision, it is not an activator of *int* transcription. Multiple attempts to purify Rox for further characterization and DNA-binding experiments were prevented by the exceptional instability of the protein.

Little is known about the mechanisms of PAI mobility. However, Turner et al. (27) showed that the SRL PAI *int* gene is required for excision of the SRL PAI, and it has been demonstrated that HPI *int* and SRL PAI *int* modules are capable of site-specific integration (19, 26). Together, these results support the significance of *int* genes in PAI mobility. This study investigated the role of Rox in the regulation of SRL PAI excision. Our findings suggest that although Rox stimulates the excision of the SRL PAI, regulation of excision does not follow the model proposed for CP4-57 (10). Since Rox does not act like AlpA, the transcriptional activator of CP4-57, we propose based on its homology to Vis and its presently proven ability to potently stimulate excision of the SRL PAI that it probably has direct excisionase activity. On the basis of sequence comparisons, Rox_{SRL} appears to be a member of a new subgroup of RDFs that includes Rox_{she}, Vis, YP01904, and Z1124 or Z1563.

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