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The SXT/R391 Family of Integrative Conjugative Elements Is Composed of Two Exclusion Groups▽

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Conjugative elements often encode entry exclusion systems that convert host cells into poor recipients for identical or similar elements. The diversity of exclusion systems within families of conjugative elements has received little attention. We report here the most comprehensive study to date of the diversity of exclusion determinants within a single family of conjugative elements. Unexpectedly, our analyses indicate that there are only two exclusion groups among the diverse members of the SXT/R391 family of integrative conjugative elements.

Integrative and conjugative elements (ICEs) are a diverse group of mobile genetic elements with the capacity to incorporate and disseminate genes encoding a variety of properties, including antibiotic resistance, into many bacterial hosts (6). ICEs are self-transmissible, and they transfer from cell to cell via conjugation, similar to conjugative plasmids. However, unlike conjugative plasmids, ICEs do not replicate autonomously; instead, they integrate into the host chromosome. SXT and R391 are closely related ICEs derived from clinical isolates of *Vibrio cholerae* and *Providencia rettgeri*, respectively (9, 23). SXT and R391 were the first recognized members of the SXT/R391 family of ICEs, which now includes ~25 members (5). These ICEs were grouped as a family since they encode nearly identical integrases that mediate the integration of the elements into the host chromosome at the *prfC* locus (8, 14). In addition, the members of the SXT/R391 family of ICEs appear to carry a conserved set of genes involved in DNA transfer (8).

The conjugal transfer genes in SXT and R391 are distantly related to those found in the F plasmid (4). Similar to F (3) and other conjugative plasmids (11), SXT and R391 carry genes for an entry exclusion system mediated by two inner membrane proteins, one (TraG) in the donor and the other (Eex) in the recipient (16). Entry exclusion systems function to specifically inhibit redundant conjugative transfers between cells that carry the same element (1, 10, 11, 21). We recently showed that even though SXT and R391 have nearly identical conjugative transfer genes, these ICEs do not exclude each other (16). Thus, cells harboring SXT inhibit the acquisition of SXT but not R391 and vice versa. We found that element-specific exclusion activity is mediated by Eex variants EexS and EexR and TraG variants $TraG_S$ and $TraG_R$ (encoded within SXT and R391, respectively). For example, exclusion was observed when EexS was produced in the recipient and $TraG_S$ in the donor; however, EexS did not exclude ICE transfer from a donor producing $TraG_R$. The specificity of the Eex variants is dictated by residues found in their respective carboxyl termini. While the first 86 amino-terminal residues of EexS and EexR are 87% identical, the remaining 56 carboxyl-terminal residues are only 41% identical. Interestingly, a stretch of only 3 amino acids (amino acids 606, 607, and 608) determines TraG's exclusion specificity. At these positions, $TraG_S$ has the amino acid sequence P-G-E, whereas $TraG_R$ has the sequence T-D-D. Here we evaluated the diversity and activity of Eex and TraG proteins from the SXT/R391 family of ICEs derived from several gamma-proteobacteria isolated from four continents.

Eex sequences from diverse ICEs segregate into two groups. We sequenced *eex* genes from 19 SXT/R391 family ICEs to explore the diversity of exclusion proteins found in this group of conjugative elements. These ICEs were obtained from five different genera of gram-negative organisms isolated from either clinical or environmental specimens from diverse locations (Table 1). PCR primers for the amplification of these *eex* genes were complementary to the sequences that flank *eexS* in the ICE SXT^{MO10}. We also obtained the sequences of *eex* genes from the SXT/R391-related ICEs pMERPH (17) and ICE*Spu*PO1 (19) from the NCBI database. Unexpectedly, the predicted Eex proteins segregated unambiguously into two groups (Fig. 1) and were almost identical (>93%) to either EexS or EexR. Like the residues that distinguish EexS and EexR, the majority of amino acids that distinguished EexS-like proteins from EexR-like proteins were found within the carboxyl-terminal 56 amino acids. Since this region determines the exclusion specificities of EexS and EexR, and since the corresponding regions of the newly sequenced genes appear to encode proteins essentially identical to either EexS or EexR, it appears that the SXT/R391 family of ICEs is divided into two exclusion groups: the S and R exclusion groups.

S exclusion group. The S group is composed of eight ICEs that were derived from bacteria isolated in distinct locations, ranging from an environmental isolate from Sri Lanka to a fish pathogen from Spain (Table 1). There are six different EexS-like sequences in our ICE collection. EexS was found in three *V. cholerae* O139 clinical isolates, those carrying ICEs SXT^{MO10}, ICE*Vch*Ban7, and ICE*Vch*Ind4, from the Indian subcontinent (Table 1). However, these ICEs do not carry the same antibiotic resistance genes, indicating that *eexS* is found

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TABLE 1. SXT/R391 ICE family members analyzed in this study

ICE name[a]	*eex* allele[b]	TraG exclusion amino acids[c]	Drug(s) to which resistance is conferred[d]	Isolation			Reference
				Host	Location[e]	Year	
SXT group							
SXT^{MO10}	*eexS*	P-G-E	SXT, Cm, Sm	*Vibrio cholerae* O139	**India**	1992	23
ICE*Vch*Ban7	*eexS*	P-G-E	None	*Vibrio cholerae* O139	**Bangladesh**	1998	This study
ICE*Vch*Ind4	*eexS*	P-G-E	Sul, Cm	*Vibrio cholerae* O139	**India**	1997	This study
ICE*Vf*Ind1	*eexS1*	P-G-E	SXT	*Vibrio fluvialis*	**India**	2002	2
R997	*eexS2*	P-G-E	Amp, Sul, Sm	*Proteus mirabilis*	**India**	1979	16a
ICE*Pda*Spa1	*eexS3*	P-G-E	Tet	*Photobacterium damselae*	**Spain**	2001	15
ICE*Spu*PO1	*eexS4*	P-G-E	NA	*Shewanella* sp. strain W3-18-1	Pacific Ocean marine sediment	NA	1
ICE*Vch*SL1	*eexS5*	P-G-E	SXT, Cm, Sm	*Vibrio cholerae* O139	Sri Lanka	1994	21a
R391 group							
R391	*eexR*	T-D-D	Kan, Mer	*Providencia rettgeri*	**South Africa**	1967	9
ICE*Vch*Ban1	*eexR1*	T-G-D	SXT, Cm	*Vibrio cholerae* O1	**Bangladesh**	1998	12
ICE*Vch*Ban2	*eexR1*	T-G-D	SXT	*Vibrio cholerae* O1	**Bangladesh**	2005	This study
ICE*Vch*Ban3	*eexR1*	T-G-D	SXT, Cm	*Vibrio cholerae* O1	**Bangladesh**	2005	This study
ICE*Vch*Ban4	*eexR1*	T-G-D	SXT	*Vibrio cholerae* O1	**Bangladesh**	1998	This study
ICE*Vch*Ban5	*eexR1*	T-G-D	SXT	*Vibrio cholerae* O1	**Bangladesh**	1998	12
ICE*Vch*Ban6	*eexR1*	T-G-D	SXT	*Vibrio cholerae* O1	**Bangladesh**	1998	This study
ICE*Vch*Ind1	*eexR1*	T-G-D	SXT	*Vibrio cholerae* O1	**India**	1994	This study
ICE*Vch*Ind2	*eexR1*	T-G-D	SXT	*Vibrio cholerae* O1	**India**	1994	This study
ICE*Vch*Ind3	*eexR1*	T-G-D	SXT	*Vibrio cholerae* O1	**India**	1994	This study
ICE*Pal*Ban1	*eexR2*	T-G-D	SXT	*Providencia alcalifaciens*	**Bangladesh**	1999	12
ICE*Vch*Mex1	*eexR3*	T-G-D	None	*Vibrio cholerae* non-O1	Mexico	2001	7
pMERPH	*eexR4*	T-G-D	Mer	*Shewanella putrefaciens*	United Kingdom	1990	20

[a] Sequence previously published in NCBI. Accession numbers: SXT^{MO10}, AY055428; ICE*Spu*PO1, NZ_AALN01000002; R391, AY090559; pMERPH, Z49196. New sequences have the following accession numbers *traG* sequences: middle_traG_region_ICEVchSL1 EF434278, middle_traG_region_ICEVchBan1 EF434279, middle_traG_region_ICEVchBan2 EF434280, middle_traG_region_ICEVchBan4 EF434281, middle_traG_region_ICEVchBan5 EF434282, middle_traG_region_ICEVchBan6 EF434283, middle_traG_region_ICEVchInd2 EF434284, middle_traG_region_ICEVchInd3 EF434285, middle_traG_region_ICEVchMex1 EF434286, middle_traG_region_ICEPdaSpa1 EF434287, middle_traG_region_ICEBan2 EF434288, middle_traG_region_ICEVchBan1 EF434289, middle_traG_region_ICEPalBan1 EF434290, middle_traG_region_pMERPH EF434291, middle_traG_region_R997 EF434292, middle_traG_region_ICEVflInd1 EF434293, middle_traG_region_ICEVchBan7 EF434294, and middle_traG_region_ICEInd4 EF434295. eex sequences: eexS ICEVchBan7 EF434296, eexS ICEVchInd4 EF434297, eexS1 ICEVflInd1 EF434298, eexS2 R997 EF434299, eexS3 ICEPdaSpa1 EF434300, eexS5 ICEVchSL1 EF434301, eexR1 ICEVchBan1 EF434302, eexR1 ICEVchBan2 EF434303, eexR1 ICEVchBan3 EF434304, eexR1 ICEVchBan4 EF434305, eexR1 ICEVchBan5 EF434306, eexR1 ICEVchBan6 EF434307, eexR1 ICEVchInd1 EF434308, eexR1 ICEVchInd2 EF434309, eexR1 ICEVchInd3 EF434310, eexR2 ICEPalBan1 EF434311, and eexR3 ICEVchMex1 EF434312.

[b] The indicated region was PCR amplified and sequenced using primers E1 (5′-TTGCGGGAGATTATGCTC-3′) and E2 (5′-TGACCATCAATGAAGGTTG-3′).

[c] Amino acids at positions 606-607-608. *traG* was PCR amplified and sequenced using primers T1 (5′-CATCTAGCGCCGTTGTTAATCAGGT-3′) and T2 (5′-ATCGCGATACTCAGCACGTCGTGAA-3′).

[d] Abbreviations: SXT, trimethoprim-sulfamethoxazole; Cm, chloramphenicol; Sm, streptomycin; Sul, sulfamethoxazole; Amp, ampicillin; Tet, tetracycline; Kan, kanamycin; Mer, mercury; NA, not available.

[e] Origins of clinical isolates are highlighted in bold, while those of environmental isolates are not highlighted.

in ICEs that are not identical (Table 1). On the other hand, ICE*Vch*SL1, an ICE derived from an environmental *V. cholerae* O139 isolate, carries *eexS5*, demonstrating that not all *V. cholerae* O139 strains contain *eexS*. Overall, the predicted differences between the six EexS-related sequences are slight, and most of them are located within the amino termini and thus are not expected to influence exclusion specificity (Fig. 1).

R exclusion group. The R group is composed of 13 ICEs derived from four continents (Table 1). Overall, this group consists of only five distinct Eex sequences, as nine of the ICEs appear to encode the same protein, EexR1 (Table 1). The ICEs encoding EexR1 were all derived from *V. cholerae* O1 clinical isolates from India or Bangladesh (Table 1). Although our sample size was fairly small, it is interesting that the exclusion proteins encoded by SXT/R391 family ICEs in *V. cholerae* O1 and *V. cholerae* O139 belong to the R and S exclusion groups, respectively. These findings suggest that more than one SXT/R391 family ICE may have been acquired by pathogenic *V. cholerae* strains relatively recently, as was also suggested by Hochhut et al. (12) based on differences between the antibiotic resistance genes in ICEs from *V. cholerae* O1 and O139 clinical isolates. As that among the EexS-related sequences, the variation among the five R exclusion group protein sequences is minor (Fig. 1). The predicted carboxyl-terminal 3 amino acids of two of the five EexR group sequences, EexR and EexR4, differ from the amino acids found at the C termini of all the other Eex sequences (Fig. 1). R391 and pMERPH, the ICEs that contain *eexR* and *eexR4*, respectively, both contain an insertion of genes conferring resistance to mercury immediately downstream of the *eexR* and *eexR4* genes (4, 17). It is possible that the acquisition of mercury resistance genes at this locus was accompanied by the alteration of the 3′ end of *eexR* in both R391 and pMERPH.

For each ICE, the corresponding TraG and Eex proteins belong to the same exclusion group. We sequenced and analyzed the *traG* exclusion specificity regions in our set of 21 ICEs. As the predicted Eex amino acid sequences, the predicted amino acid sequences of the TraG exclusion regions segregated into two exclusion groups (Table 1). All of the ICEs that encoded an S exclusion group protein also encoded the $TraG_S$ exclusion determinant residues P-G-E (Table 1). In contrast, all of the newly analyzed ICEs that encoded an R

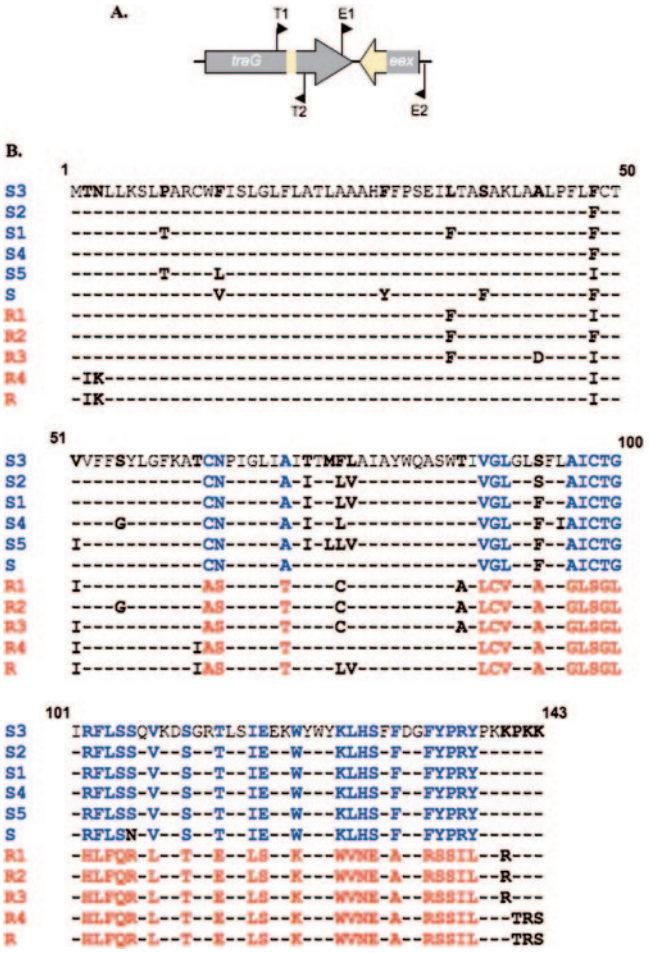

FIG. 1. ClustalW alignment of the predicted amino acid sequences of Eex proteins from SXT/R391 family ICEs. (A) Map of the *traG* and *eex* regions depicting the regions encoding exclusion specificity (yellow) and the primers used in this study. (B) The Eex sequences of the S and R groups are presented. Amino acids that are conserved in all sequences are shown with dashed lines; amino acids that distinguish EexS and related proteins from EexR and related proteins are shown in blue and red, respectively; amino acids that are divergent in some members of the EexS or EexR groups are shown in boldface black.

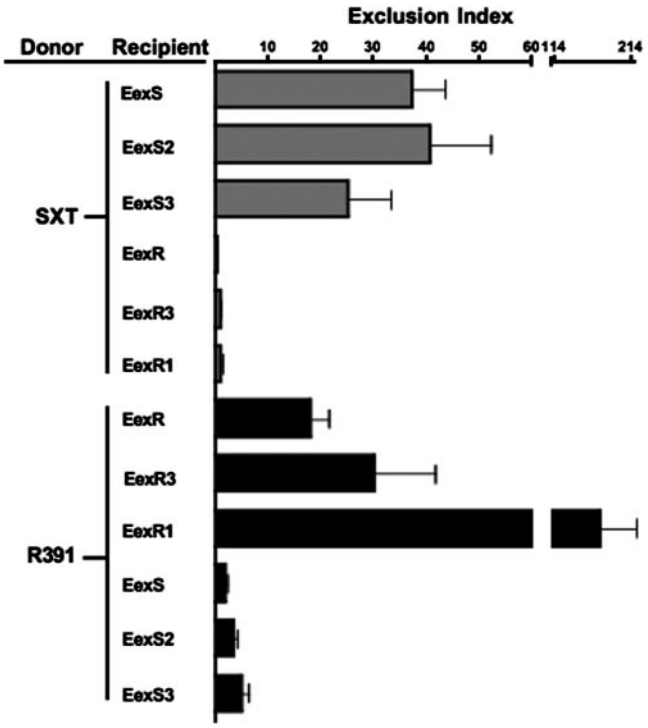

FIG. 2. Exclusion specificity and activity of SXT and R391 exclusion group proteins. In all cases, the recipient strain was a derivative of *Escherichia coli* CAG18439 (22) expressing the indicated Eex protein from pBAD-Topo. The donor strains were derivatives of MG1655 (13) harboring either SXT or R391. The exclusion index is the ratio of an element's frequency of transfer to an ICE-free recipient to the frequency of transfer to the indicated recipient. Each bar represents the mean of results from three experiments, with error bars indicating the standard deviations. Mating assays and calculations of transfer frequencies and exclusion indices were done as previously described (16).

exclusion group protein encoded T-G-D at amino acids 606 to 608 of TraG. As the G residue at position 607 in the TraG exclusion determinant region is conserved between the two exclusion groups, this result suggests that only two TraG residues, those at positions 606 and 608, confer TraG exclusion specificity; the D residue at position 607 in $TraG_R$ is unique and therefore unlikely to play a role in exclusion specificity. Thus, for all the ICEs examined, the corresponding Eex and TraG sequences were from the same exclusion group. This association likely reflects the functional interaction between these proteins that mediates exclusion.

Exclusion activity of EexS and EexR exclusion group proteins. Since the EexS and EexR exclusion groups were defined by differences in their carboxyl termini, the part of the Eex protein that determines exclusion specificity, we hypothesized that S group proteins would exclude SXT transfer and not R391 transfer and that R group proteins would exclude R391 transfer and not SXT transfer. To test this hypothesis, we cloned *eexS*, *eexS2*, *eexS3*, *eexR*, *eexR1*, and *eexR3* into an expression vector and then assessed whether the expression of these exclusion genes in a recipient cell excluded the transfer of either SXT or R391. We measured exclusion activity and specificity by calculating an exclusion index towards SXT (EI_S) or R391 (EI_R) transfer. This index is the ratio of an element's frequency of transfer to an ICE-free recipient to the frequency of transfer to the indicated recipient. We found that all Eex variants excluded ICE transfer as predicted. Thus, when expressed in a recipient, the three S exclusion group proteins excluded SXT transfer (EI_S ranged from 26 to 41) but did not exclude R391 transfer (EI_R ranged from 0.5 to 1) (Fig. 2). Similarly, R391 transfer was excluded by the three R exclusion group proteins (EI_R ranged from 18 to 174), but SXT transfer was excluded only to a small extent (EI_S ranged from 2 to 4) (Fig. 2). These results corroborate the previous report that the carboxyl termini of Eex proteins determine the specificities of the proteins. However, the exclusion potencies of the three EexR group proteins varied to a surprising extent, especially considering that the sequence of the highly active EexR1 protein (EI_R of 174) differs by only a single amino acid (at residue 43) from that of EexR3 (EI_R of 30). The basis for this variability requires further studies.

Conclusions. We report here the most comprehensive study to date of the diversity of exclusion determinants within a single family of conjugative elements. We analyzed the predicted amino acid sequences of 19 novel Eex and TraG proteins from the SXT/R391 family of ICEs derived from several bacterial species isolated from diverse parts of the world over the past four decades. All of the Eex proteins shared well-conserved amino termini. The distinguishing feature of these proteins was their carboxyl-terminal sequences, which determine exclusion specificity. All of the new Eex sequences contained carboxyl-terminal sequences that were nearly identical to that in either EexS or EexR. Remarkably, every ICE that encoded an S group Eex also encoded an S group TraG; this linkage was also observed in the ICEs of the R exclusion group. Thus, our sequence analyses strongly suggested that there are only two (S and R) exclusion groups in the SXT/R391 family of ICEs. Functional studies confirmed this prediction; the new EexS-like and EexR-like variants specifically excluded SXT and R391, respectively.

It is not clear why there are only two exclusion groups in the SXT/R391 family of ICEs. Considering that the *traG* and *eex* genes are linked, a potential scenario to explain the emergence of the S and R groups is a recombination event between the region encompassing exclusion specificities in both genes (Fig. 1) and a similar region from another conjugative element. Even though ICEs appear to have fairly plastic genomes (7, 8), their exclusion genes are conserved. Although entry exclusion in plasmids has been studied for many years (1, 10, 11, 21), there is relatively scant knowledge of the extent of the diversity of exclusion determinants within plasmid families. Further definition of the diversity of exclusion genes in plasmid families will provide useful tools for analyses of the bases of exclusion in plasmids and ultimately further our understanding of the conjugative process.

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