

R391: a Conjugative Integrating Mosaic Comprised of Phage, Plasmid, and Transposon Elements

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The conjugative, chromosomally integrating element R391 is the archetype of the IncJ class of mobile genetic elements. Originally found in a South African *Providencia rettgeri* strain, R391 carries antibiotic and mercury resistance traits, as well as genes involved in mutagenic DNA repair. While initially described as a plasmid, R391 has subsequently been shown to be integrated into the bacterial chromosome, employing a phage-like integration mechanism closely related to that of the SXT element from *Vibrio cholerae* O139. Analysis of the complete 89-kb nucleotide sequence of R391 has revealed a mosaic structure consisting of elements originating in bacteriophages and plasmids and of transposable elements. A total of 96 open reading frames were identified; of these, 30 could not be assigned a function. Sequence similarity suggests a relationship of large sections of R391 to sequences from *Salmonella*, in particular those corresponding to the putative conjugative transfer proteins, which are related to the IncHI1 plasmid R27. A composite transposon carrying the kanamycin resistance gene and a novel insertion element were identified. Challenging the previous assumption that IncJ elements are plasmids, no plasmid replicon was identified on R391, suggesting that they cannot replicate autonomously.

Horizontal gene transfer, the intraspecies and interspecies exchange of genetic information, plays an important role in the evolution of bacteria (10, 24, 26, 39, 65). Three major mechanisms, transformation, transduction, and conjugation (9), provide bacterial populations with access to a “horizontal gene pool,” enabling them to rapidly respond to environmental challenges (16, 34).

The most important contributor to horizontal gene transfer is the heterogeneous group of mobile genetic elements that includes plasmids, insertion (IS) elements, transposons, integrons, phages, and genomic islands (10, 17). They are “selfish” elements that promote their own maintenance and distribution and, in addition, can function as vectors for accessory DNA elements. These accessory elements commonly consist of genes that are nonessential for survival but confer a phenotype, which is advantageous under particular environmental conditions. Prominent examples of such traits are antibiotic and heavy metal resistances, degradation of xenobiotic compounds, symbiosis and virulence determinants, resistance to radiation, and increased mutation frequency (15).

A group of related conjugative DNA elements has been identified as carriers of antibiotic resistance genes in members of the γ -*Proteobacteria* from dispersed global locations. R391, the archetype of this group, was originally detected in a South African *Providencia rettgeri* isolate (8); for many years, it was believed to be a plasmid and was assigned to a new incompatibility group, IncJ. Subsequently, other IncJ elements, confer-

ring the same phenotype as that conferred by R391 (19, 20) and possibly of clonal origin, were identified in *Providencia* spp. (R748 and R749) and *Proteus vulgaris* (R705 and R706) from South Africa, *Vibrio cholerae* (pJY1; phenotype, Cm^r Sm^r Su^r) from the Philippines (72), *Proteus vulgaris* (R997; phenotype, Ap^r Sm^r Su^r) from India (32), and *Shewanella putrefaciens* (pMERPH; mercury resistance) from the United Kingdom (48). Previous studies have examined R391's ability to mobilize chromosomal markers (38), its mutagenic DNA repair function (27), and its unusual UV-sensitizing phenotype (46, 47).

More recently, the plasmid nature of these elements was challenged by the discovery of their transfer and stable integration into the host chromosome in recombination-deficient (*recA*) backgrounds, suggesting that they are conjugative transposons (37). Furthermore, Hochhut et al. (21) have since reported a close relationship between R391 and the self-transmissible SXT element from *Vibrio cholerae* O139, which carries multiple antibiotic resistance cassettes (Su^r Tm^r Sm^r). Both code for a nearly identical phage-like integrase, which mediates site-specific integration into the 5' end of the *prfC* gene of the *Escherichia coli* chromosome (23).

In this study, the complete nucleotide sequence of R391 has been determined and analyzed. The main objective of the analysis was to elucidate the nature of R391 and, by inference, of related elements, to identify the genetic systems responsible for both the chromosomal integration of R391 and its conjugative transfer, and to determine whether R391 carries a recognizable autonomous replicon. Additionally, given that the chromosomal association of these elements makes detection difficult, it is possible that such elements are representatives of a much larger and important group of mobile genetic elements, potentially acting as vectors of antibiotic resistances and other

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phenotypes in the γ -*Proteobacteria*. Thus, this study aimed to provide an enhanced understanding of the archetype of this group, to broaden our molecular understanding of its genetic structure, regulatory systems, and evolutionary origins, to identify important accessory functions, and to facilitate the future molecular detection of further related elements.

MATERIALS AND METHODS

Isolation and purification of DNA. An extrachromosomal circular intermediate of R391 can be generated and visualized when it is transferred to an *E. coli* *recA* recipient strain harboring the related element R997 integrated in the chromosome (45). For the purpose of subcloning R391, the circular form was extracted from *E. coli* AB2463 (R997 and R391) with a Qiagen plasmid maxikit purification protocol for plasmids with a very low copy number. To minimize contamination with chromosomal DNA, the preparation was purified further by cesium chloride density gradient centrifugation in accordance with standard protocols (58).

Shotgun cloning and DNA sequencing. Shotgun cloning of R391 was performed with a TOPO shotgun subcloning kit (Invitrogen) in accordance with the manufacturer's protocol. Briefly, DNA was sheared with compressed air by using a nebulizer, generating fragments of about 1 to 2 kb in size. After blunt-end repair and dephosphorylation, the fragments were ligated to the vector pCR4Blunt-TOPO and transformed into *E. coli* TOP10, with subsequent blue/white screening used to detect transformants carrying an insert. Plasmid DNA from clones was isolated with a Qiaprep8 miniprep kit (Qiagen). Library subclones were sequenced by the chain termination method (59) with sequencing kits provided by Applied Biosystems, Amersham, or Cambio. Electrophoresis and analysis were carried out on an ABI Prism 310 genetic analyzer (Applied Biosystems) or a Licor IR4200. The complete nucleotide sequence of R391 has been determined by sequencing 750 shotgun clones, yielding a fourfold coverage in combination with primer walking for the closure of gaps and regions of insufficient coverage. Primers for gap filling were designed with PrimerSelect software (Lasergene) and used either directly for sequencing on shotgun clones or to sequence PCR amplicons. PCR amplification with the extrachromosomal form of R391 as the template DNA was performed according to standard protocols.

Annotation and phylogenetic analysis. Sequences were assembled by using SeqMan software (Lasergene). ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) was employed to search the complete R391 sequence for open reading frames (ORFs), initially with a cutoff value of 50 amino acids, by using the bacterial genetic code. Subsequently, the ORFs were compared to sequences in the public sequence databases of GenBank and EMBL by using the BLAST (1) and FASTA (44) search tools.

Of the original 885 ORFs identified, only those that had a minimum length corresponding to 60 amino acids and were not overlapping other ORFs with significant identity to ORFs in the databases were included in the results. Significant identity was defined as being greater than 20% identity over at least 60% of the ORF or, alternatively, over at least 50 corresponding amino acids. ORF products were further analyzed for the presence of conserved protein domains by using the Web-based tools Cognitor (66), PFAM Search (3), and ProDom (1).

Multiple sequence alignments and bootstrap neighbor-joining phylogenies (12, 56) were generated with ClustalX software (67). Gaps in the alignment were excluded from the calculation of the phylogenetic tree. The G+C plot was created with the program Freak from the EMBOSS package (Human Genome Mapping Project Resource Centre, Cambridge, United Kingdom) with a window size setting of 300 nucleotides (nt) and a step size setting of 10.

Nucleotide sequence accession number. The nucleotide sequence of R391 has been deposited in the GenBank database under accession number AY090559.

RESULTS AND DISCUSSION

General properties of the nucleotide sequence of R391. Assembly of the subclone sequences resulted in a circular DNA sequence of 88,532 nt. Ninety-six ORFs, corresponding to a total coding region of 90%, were identified (Table 1; Fig. 1). Six translated ORFs (6%) were found to show no significant similarity to protein sequences in the databases, while another

24 putative proteins (25%) are similar only to hypothetical proteins of unknown function. Putative genes which code for the backbone functions of R391 (integration/excision and conjugative transfer) and the phenotypic traits (kanamycin and mercury resistance) have been identified, as have a number of transposable elements.

The total G+C content of R391 is 46%. The G+C content varies significantly within R391, displaying a number of peaks and troughs (Fig. 2). These peaks and troughs correspond to different functional units, such as transposable elements and conjugative transfer elements, suggesting that R391 has a mosaic structure composed of elements of different origin. In particular, four putative transposase genes and the mercury resistance (*mer*) operon, which are visible as peaks in Fig. 2, have a notably higher G+C content than their surroundings. Four transfer regions, TRA I to IV, are homogeneous in G+C content but clearly differ from the adjacent regions. A region corresponding to a putative restriction enzyme (ORF 29) and a transposon-like region between TRA II and III show a lower G+C contents, with the exception of a putative transposase gene located within this region.

One-third of the ORFs found on R391 have homologs in *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Typhimurium, and 21 of those are associated with the multiple antibiotic resistance plasmid R27 (63) (see "Conjugative transfer" below), the remaining 12 being chromosomally associated ORFs. These homologies suggest evolutionary relatedness and a possible origin of large sections of R391 from these or closely related ancestral strains.

Remarkably, 67 of the ORFs (70%) are transcribed in the three forward frames (Table 1; Fig. 1). The exceptions are, first, two clusters of eight and six ORFs lying, respectively, upstream and downstream of the attachment site *attP*, which is located at the end of the linear map (Fig. 1). Second, a few ORFs belonging to the IS elements, as well as a number of dispersed ORFs, also lie on the reverse strand. This unidirectionality is even more surprising considering the different origins of this element.

Given that it has the ability to transfer by conjugation and that numerous antibiotic resistance markers are carried by plasmids, R391 had initially been thought to be a plasmid. However BLASTP analysis of the 96 ORFs failed to show similarity to any known plasmid replication gene; likewise, no similarity was found between R391 and any known origin of replication, suggesting the absence of a plasmid replicon on R391. It is likely, however, that major parts of R391 have been derived from a plasmid, as suggested by the high number of homologs to ORFs from *Salmonella* plasmids. If the ancestral R391 did possess a plasmid replicon, it might have been lost upon acquiring the integration mechanism.

Integration and excision. Integration of R391 into the host chromosome and its excision from it share similarities to the integration and excision of bacteriophages and genomic islands. Integration into the *prfC* gene is mediated by a phage-like integrase and results in short direct repeats at both ends of the element and in the reconstitution of *prfC* by the replacement of its 5' end (20, 22). However, this mechanism differs notably from those of the majority of genomic islands described to date in its use of an integration site that is not located in, or near, a tRNA gene and in the replacement of the

TABLE 1. Homology between ORFs in R391 and corresponding representative prokaryotic protein sequences

ORF ^a		ORF product size (amino acids)	ORF location (start, stop) ^b	ORF product exhibits homology to:	Source	Identity ^c		Accession no.
No.	Name					%	Amino acids	
1		107	442, 119	ABC transporter permease protein	<i>Lactococcus lactis</i>	27	25/92	NP 267026
2		106	790, 1110	Y4dL; hypothetical 21.8-kDa protein	<i>Rhizobium</i> sp. strain NGR234	50	44/87	NP 443821
3		404	1103, 2317	Y4dM; putative protein	<i>Rhizobium</i> sp. strain NGR234	55	224/405	NP 443822
4		66	2528, 2728	Possible DNA-binding protein	<i>Mycobacterium leprae</i>	34	17/50	np 302574
5	<i>int</i>	413	3986, 2745	Integrase	SXT element; <i>Vibrio cholerae</i>	99	410/413	AAC72397
6		89	4257, 3988	Soluble lytic murein transglycosylase	<i>Brucella melitensis</i>	28	20/71	np 540005
7		324	5234, 4260	StbA; putative plasmid partitioning protein	Plasmid R27; <i>Salmonella enterica</i> serovar Typhi	24	62/252	Q9RGU9
10	<i>rumB</i>	435	7460, 6153	SamB; UV protection protein	<i>S. enterica</i> serovar Typhi CT18	61	256/419	np 458681
11	<i>rumA</i>	149	7878, 7429	RumA	SXT element; <i>Vibrio cholerae</i>	98	147/149	AAK64594
13		302	8518, 9426	DNA polymerase III (epsilon subunit)	<i>Agrobacterium tumefaciens</i> strain C58	39	117/300	AAL43906
14		104	9500, 9814	Polynucleotide phosphorylase	<i>Deinococcus radiodurans</i>	33	18/53	np 295786
15		308	9883, 10809	NMA1157; hypothetical protein	<i>Neisseria meningitidis</i>	35	93/261	np 283925
16	<i>tnpIS15</i>	238	11635, 10919	P22 protein	IS15; <i>Escherichia coli</i>	100	234/234	Q56369
17		279	11096, 11935	P11 protein	IS15; <i>Escherichia coli</i>	100	197/197	Q56370
18		202	12560, 11952	P11 protein	IS15; <i>Escherichia coli</i>	100	197/197	Q56370
19	<i>tnpIS15</i>	238	12021, 12737	P22 protein	IS15; <i>Escherichia coli</i>	100	234/234	Q56369
20	<i>aph</i>	271	12877, 13692	Aminoglycoside phosphotransferase	<i>Klebsiella pneumoniae</i>	100	271/271	S16630
21	<i>tnpIS15</i>	238	14588, 13872	P22 protein	IS15; <i>Escherichia coli</i>	100	234/234	Q56369
22		199	14049, 14648	P11 protein	IS15; <i>Escherichia coli</i>	100	197/197	Q56370
23		210	14567, 15199	Putative inner membrane protein	<i>S. enterica</i> serovar Typhimurium LT2	41	76/182	NP 463357
24		194	15196, 15780	Putative cytoplasmic protein	<i>S. enterica</i> serovar Typhimurium LT2	61	116/188	NP 463356
25		947	15799, 18642	Putative ATPase involved in DNA repair	<i>S. enterica</i> serovar Typhimurium LT2	62	585/943	NP 463355
26		317	18524, 19477	Putative ATPase involved in DNA repair	<i>S. enterica</i> serovar Typhimurium LT2	43	123/280	NP 463355
27		171	19634, 20149	Putative type II restriction enzyme, methylase subunit	<i>S. enterica</i> serovar Typhimurium LT2	56	96/171	NP 463354
28	<i>tnp391A</i>	225	20708, 21385	<i>OrfB</i> of IS911	IS911; <i>Escherichia coli</i>	88	198/224	AAC70114
29		1,083	21303, 24554	Putative type II restriction enzyme, methylase subunit	<i>S. enterica</i> serovar Typhimurium LT2	59	632/1060	NP 463354
30		894	24554, 27238	Putative cytoplasmic protein	<i>S. enterica</i> serovar Typhimurium LT2	50	455/896	NP 463351
31		746	27251, 29491	Putative ATP-dependent Lon protease	<i>S. enterica</i> serovar Typhimurium LT2	85	576/672	NP 463350
32		883	29535, 32186	Orf74	Plasmid pB171; <i>Escherichia coli</i>	25	235/920	NP 053136
33	<i>traI</i>	716	32341, 34491	Hypothetical protein	<i>Xylella fastidiosa</i> 9a5c	26	94/353	NP 299042
34		606	34540, 36360	TraG	Plasmid R27; <i>S. enterica</i> serovar Typhi	30	186/615	NP 458648
35		186	36370, 36930	Large repetitive protein	<i>S. enterica</i> serovar Typhi CT18	25	20/78	NP 458558
36		214	36908, 37552	Hypothetical protein	Plasmid R27; <i>S. enterica</i> serovar Typhi	29	29/98	NP 058330
37		195	38166, 37579	Hypothetical protein	<i>Listeria monocytogenes</i>	24	30/123	NP 464171
39	<i>traL</i>	93	38455, 38736	Sex pilus assembly and synthesis protein	Plasmid pNL1; <i>Novosphingobium aromaticivorans</i>	25	18/72	NP 049168
40	<i>traE</i>	130	38967, 39359	HtdE	Plasmid R478; <i>Serratia marcescens</i>	25	29/115	AAL27019
41	<i>traK</i>	300	39340, 40242	HtdP	Plasmid R478; <i>Serratia marcescens</i>	26	66/251	AAL27020
42	<i>traB</i>	429	40242, 41531	TrhB; putative transfer protein	Plasmid R27; <i>S. enterica</i> serovar Typhi	40	84/209	NP 058242
43	<i>htdD</i>	216	41528, 42178	HtdD	Plasmid R478; <i>Serratia marcescens</i>	28	45/158	AAD01915
44	<i>traA</i>	128	42175, 42561	TraA	Plasmid R27; <i>S. enterica</i> serovar Typhi	24	21/85	NP 058247
45		106	42922, 42602	Putative acetyltransferase	<i>S. enterica</i> serovar Typhimurium LT2	45	41/90	NP 463183
46		88	43365, 43099	Hypothetical protein	<i>Escherichia coli</i> O157:H7	44	40/89	NP 289961
47		125	43920, 43543	SMA0431, hypothetical protein	<i>Rhizobium meliloti</i>	27	31/114	Q930G9
48	<i>tnp391B</i>	305	45323, 44406	Transposase	<i>Listonella anguillarum</i>	66	201/301	AAA81774
49		107	46010, 45687	Glycosyl transferase	<i>Streptomyces fradiae</i>	22	21/95	AAF00214
50		82	46700, 46452	Hypothetical protein G	Tn1404 <i>Pseudomonas</i> sp. strain R9	60	15/25	AAD47995
51		508	45742, 47268	Sulfate permease	Tn1404 <i>Pseudomonas</i> sp. strain R9	80	401/497	AAD47994
52		283	47280, 48131	Hypothetical protein B	Tn1404 <i>Pseudomonas</i> sp. strain R9	38	109/283	AAD47991
53		149	48604, 48155	Conserved hypothetical protein	<i>Pseudomonas aeruginosa</i>	36	53/146	NP 250418
54		169	49144, 48635	Conserved hypothetical protein	<i>Pseudomonas aeruginosa</i>	35	39/109	NP 249266
56	<i>htdT</i>	230	49588, 50280	HtdT; putative transfer protein	Plasmid R27; <i>S. enterica</i> serovar Typhi	26	53/197	NP 058240
57	<i>traC</i>	799	50280, 52679	TraC	Plasmid R27; <i>S. enterica</i> serovar Typhi	27	239/869	NP 058238
59	<i>trhF</i>	163	53024, 53515	TrhF	Plasmid R27; <i>S. enterica</i> serovar Typhi	28	36/127	NP 058222
60	<i>traW</i>	375	53523, 54650	Putative transfer protein, pilus formation	Plasmid R27; <i>S. enterica</i> serovar Typhi	27	84/303	NP 058221
61	<i>traU</i>	342	54634, 55662	TraU	Plasmid pNL1; <i>Novosphingobium aromaticivorans</i>	36	125/339	NP 049157

Continued on following page

TABLE 1—Continued

No.	ORF ^a Name	ORF product size (amino acids)	ORF location (start, stop) ^b	ORF product exhibits homology to:	Source	Identity ^c		Accession no.
						%	Amino acids	
62	<i>traN</i>	1,230	55665, 59357	TraN	Genomic island STI1; <i>S. enterica</i> serovar Typhimurium	33	418/1253	AAK02035
64		220	59951, 60613	DNA ligase	<i>Escherichia coli</i> O157:H7	25	31/124	NP 288973
65		200	61306, 60704	Lipoprotein	<i>Haemophilus ducreyi</i>	21	30/139	AAAB48950
66		112	61662, 62000	AnsG oxidoreductase	<i>Streptomyces collinus</i>	27	19/68	AAD31836
67		139	62016, 62435	Single-strand binding protein	Plasmid pLP231a; <i>Escherichia coli</i>	38	52/135	C38487
68		272	62515, 63333	Recombination protein Bet	Bacteriophage 933W; <i>Escherichia coli</i> O157:H7	55	108/195	NP 049474
69		338	63621, 64637	Hypothetical protein	<i>Xylella fastidiosa</i> 9a5c	32	51/155	NP 298936
70		358	64730, 65806	Probable cobalamin biosynthesis protein	<i>Sinorhizobium meliloti</i>	30	87/281	NP 386778
71		255	65806, 66573	Molybdate metabolism regulator- related protein	<i>Deinococcus radiodurans</i>	31	33/105	NP 386778
72		317	66672, 67625	Hypothetical protein	Plasmid R27; <i>S. enterica</i> serovar Typhi	20	41/205	NP 058418
74		551	68197, 69852	Hypothetical protein	Plasmid R27; <i>S. enterica</i> serovar Typhi	21	138/636	NP 058417
75		165	69938, 70435	RadC; putative DNA repair protein	<i>Vibrio cholerae</i>	56	86/151	NP 231421
78		357	70867, 71940	Hypothetical protein	Plasmid R27; <i>S. enterica</i> serovar Typhi	30	113/375	NP 058415
79		235	72030, 72737	Hypothetical protein	SXT element; <i>Vibrio cholerae</i>	80	61/76	AAK95994
80		180	73088, 73630	Probable oxidoreductase	<i>Agrobacterium tumefaciens</i>	27	35/126	NP 396434
81	<i>traF</i>	314	74368, 75312	TraF	SXT element; <i>Vibrio cholerae</i>	97	306/314	AAK 95986
82	<i>traH</i>	462	75315, 76703	TraH	Genomic island STI1; <i>Salmonella</i> <i>enterica</i> serovar Typhimurium	42	189/449	AAK02038
83	<i>traG</i>	1,189	76707, 80276	Putative pilus assembly protein	Genomic island STI1; <i>Salmonella</i> <i>enterica</i> serovar Typhimurium	30	375/1214	AK02037
84	<i>merR</i>	129	80374, 80763	MerR	IncJ element pMERPH; <i>Shewanella</i> <i>putrefaciens</i>	100	557/557	
85	<i>merT</i>	107	80848, 81171	MerT	IncJ element pMERPH; <i>Shewanella</i> <i>putrefaciens</i>	97	99/102	Q54462
86	<i>merP</i>	91	81256, 81531	MerP	IncJ element pMERPH; <i>Shewanella</i> <i>putrefaciens</i>	98	90/91	Q54463
87	<i>merC</i>	137	81541, 81954	MerC	IncJ element pMERPH; <i>Shewanella</i> <i>putrefaciens</i>	98	135/137	CAA89056
88	<i>merA</i>	557	81992, 83665	MerA; mercuric reductase	IncJ element pMERPH; <i>Shewanella</i> <i>putrefaciens</i>	100	557/557	Q54465
89		143	84202, 83771	Hypothetical protein	<i>Listeria innocua</i>	26	23/86	CAC42036
90		177	84790, 84257	FlhC; flagellar transcriptional activator	<i>S. enterica</i> serovar Typhimurium LT2	27	41/150	AAB96640
91		99	85086, 84787	FlhD protein	<i>Serratia liquefaciens</i>	28	28/97	S61276
92		182	85631, 85083	Hypothetical protein	<i>S. enterica</i> serovar Typhi CT18	32	39/121	NP 458645
93		202	86226, 85618	Hypothetical transmembrane protein	<i>Ralstonia solanacearum</i>	39	26/66	CAD17270
94		289	87136, 86267	Unknown protein	Cryptic prophage CP-933M; <i>Escherichia coli</i> O157:H7	35	27/76	NP 286859
96		215	87561, 88208	Putative prophage repressor protein; similar to cI repressor	<i>Yersinia pestis</i>	38	87/227	NP 404832

^a ORFs not showing significant homology to corresponding proteins in the public databases are not shown.

^b Nucleotide position from start to stop codon in the R391 sequence.

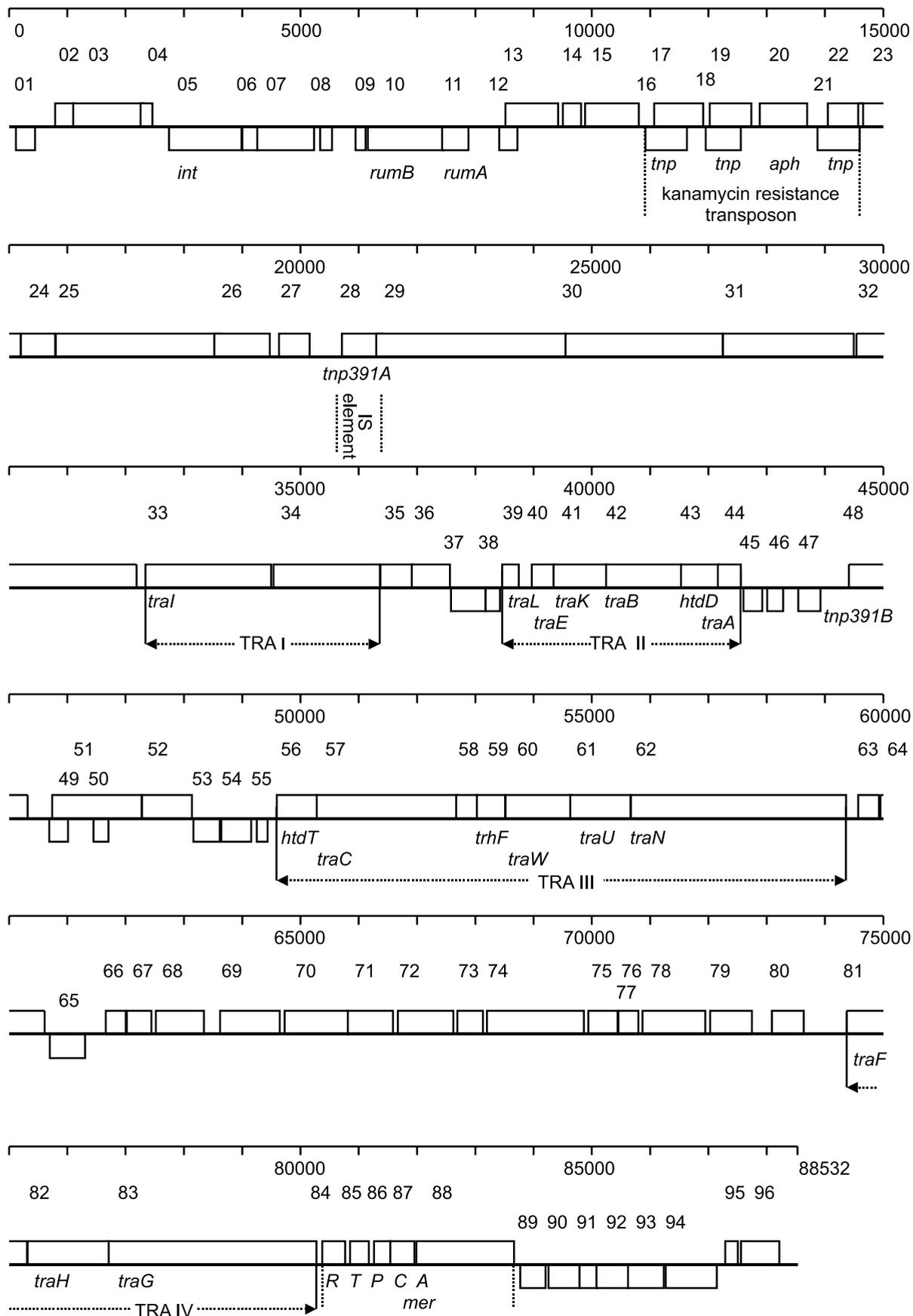
^c Identity in percentage and amino acids (number identical relative to total number examined) as determined with BLAST and FASTA.

5' end of the interrupted gene. Other mobile elements using phage-like integrases normally carry a 3' terminus of the interrupted gene, presumably because upstream regulatory sequences such as the promoter region are more complex and difficult to replace (74).

The integrase gene *int* (ORF 5) is located from 2.7 to 4 kb from the left end of R391 (Fig. 1) and is translated towards the left junction *attL* of the element, unlike genomic islands, where the integrase gene is typically located directly adjacent to the junction with the chromosome and is translated divergently from the junction.

The IncJ elements R997 and pMERPH probably possess an

integration mechanism closely related to those of R391 and the SXT element. In both R997 and pMERPH PCR, amplification generates a PCR product of the expected size for the integrase gene and the integration sites are nearly identical (B. McGrath and J.T. Pembroke, unpublished data). In addition, restriction digestion analysis revealed many restriction fragments common to both R391 and R997 (45). R391 and the SXT element can integrate into the same chromosome in tandem fashion, with an incoming element probably using one of the junction sites of the resident element with the chromosome *attL* or *attP* as the integration site (21). Conversely, in *recA* mutant strains containing both R391 and R997, the incoming element could



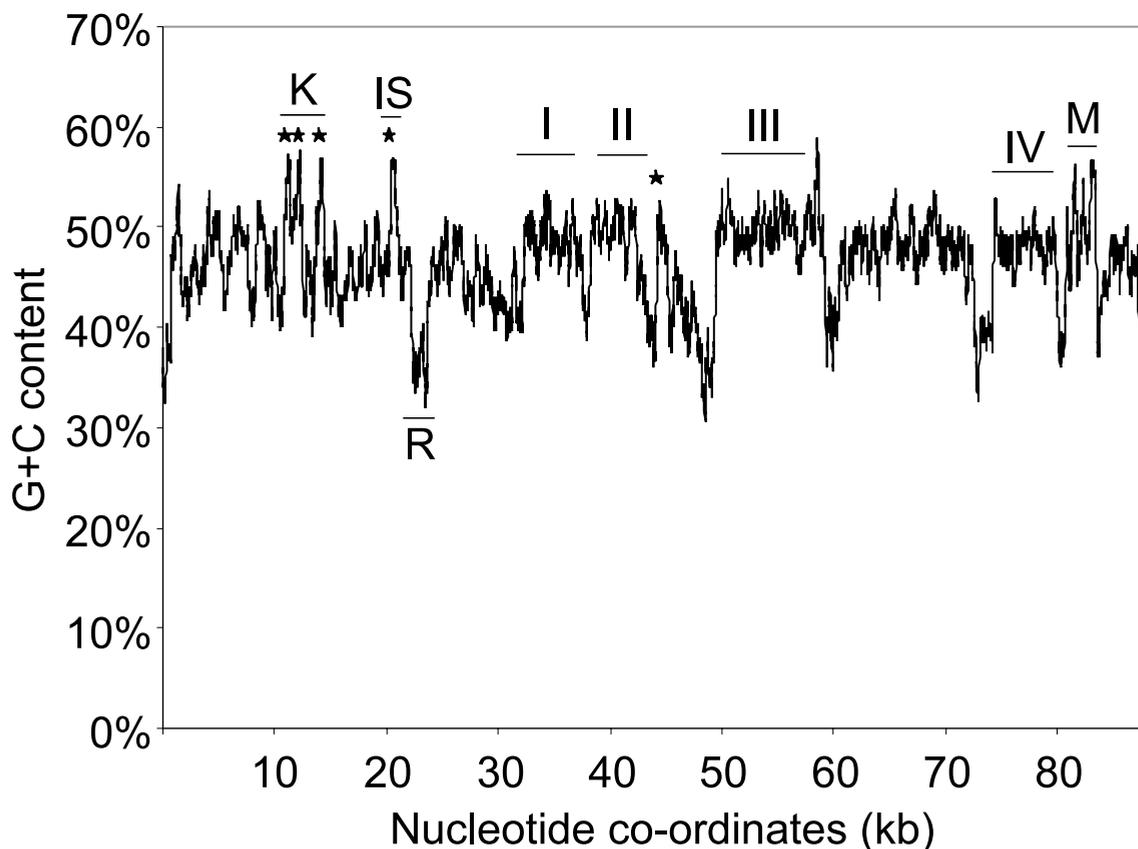


FIG. 2. G+C plot of the DNA sequence of R391. Regions indicated are the transfer regions TRA I to IV (I to IV), the kanamycin resistance transposon (K), the IS element (IS), the putative restriction enzyme methylase subunit ORF 27 (R), and the mercury resistance operon (M). Asterisks indicate putative transposase genes.

be isolated in the extrachromosomal circular form (45). The existence of a circular extrachromosomal form raises the possibility of mutual exclusion from integration between these two elements and the probable requirement for extrachromosomal replication. However, this study found no indication of a plasmid replicon, suggesting that the circular form may be a non-replicative transposition or transfer intermediate.

Evolutionary relationships between phage-related integrases. The R391 integrase belongs to a diverse family of integrase proteins within a wider group of tyrosine recombinases with a range of functions in DNA metabolism (71; <http://mywebpages.comcast.net/domespo/trhome.html>). It has previously been reported that the closely related integrase from the SXT element was itself closely related to that from bacteriophage $\phi 80$ (23). BLAST analysis of the R391 integrase showed it to be most closely related (excluding SXT) to that from the Gifsy-1 prophage from *Salmonella enterica* serovar Typhimurium LT2 (36% identity over 413 amino acids).

To further investigate the evolutionary relationships within the integrase family, representative integrases from R391, the

SXT element, phages, and pathogenicity and other genomic islands that were identified by a BLAST similarity search were aligned and a phylogenetic tree was constructed (Fig. 3). From the several distinct clusters identified, the integrases from R391 and the SXT element belong to a group containing the prophage $\phi 80$ (29) and other cryptic prophages. This $\phi 80$ group branches next to a cluster of integrases which is related to phage lambda (28) and which contains two *Staphylococcus* pathogenicity islands (13, 30) and the *Salmonella* genomic island SGI1 (6). The relatively large evolutionary distances within both groups suggest early and considerable divergence of most of their members. A third distinct subgroup contains the satellite phage P4 (49) and a number of pathogenicity islands from *Yersinia* spp. (18, 61) and *E. coli*. A number of pathogenicity islands from *E. coli*, *Shigella flexneri* (35, 69), and *Haemophilus influenzae* (7), as well as the *clc* element from *Pseudomonas putida* (54) and a symbiosis island from *Mesorhizobium loti* (64), cannot be assigned unambiguously to any of these three groups, but the relatively smaller evolutionary distances suggest a relationship to the P4 subgroup.

FIG. 1. Linear map of R391 showing all ORFs and their orientations in the chromosomally integrated state, from the left junction *attL* to the right junction *attR*. The boxes above and below the axis represent ORFs in the forward and reverse frames, respectively. ORF numbers, names, and sequence features are indicated; the scale bar is given in kilobases. See also Table 1.

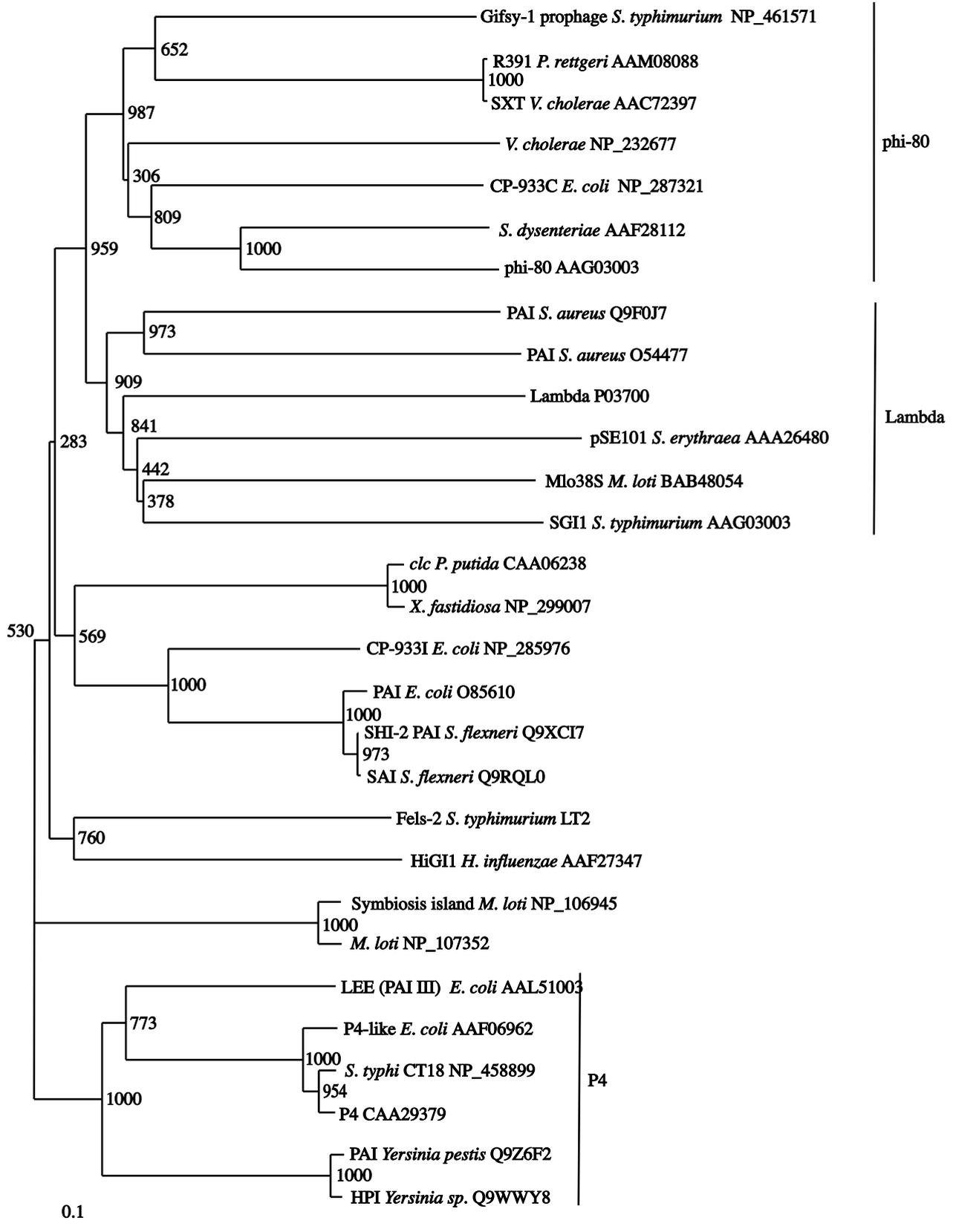


FIG. 3. Bootstrap neighbor-joining tree of integrases from R391 and SXT, including genomic islands (SGI1, *clc*, and HiGI), pathogenicity islands (PAI, SHI, SAI, LEE, and HPI), phages (Gifsy1, CP-933C, phi-80, lambda, Fels-2, and P4), and uncharacterized putative integrases. Host organisms, accession numbers of protein sequences, and bootstrap values (1,000 replicates) are indicated.

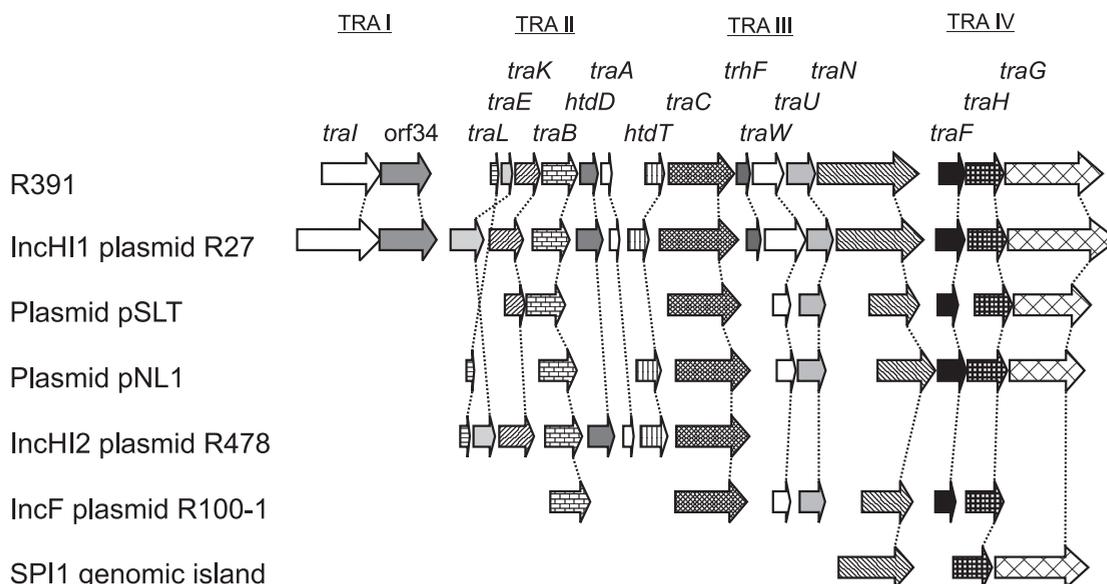


FIG. 4. Relationship between the putative transfer genes of R391 and homologs present in related plasmids and genomic islands. The names of the R391 transfer genes and transfer regions are indicated. Homologous proteins are depicted by arrows with the same shading and connected by dotted lines. Clustered genes are shown as linked. Note that the structural organization and gene order in the different plasmids and genomic islands differ from those of R391. Both *traI* and *orf34* have no homologs with significant similarity in plasmids except R27. *orf34* is a homolog of *traG* on R27 (based on IncP nomenclature); however, in R391, ORF 83 has been designated *traG*, in accordance with the IncF nomenclature.

These results indicate multiple origins of mobile integrating elements, such as pathogenicity islands and other genomic islands, by independent acquisition of the integration module of a prophage.

Conjugative transfer. A total of 17 putative transfer genes, located in four clusters (TRA I to IV) spanning 24 kb of R391, were identified (Fig. 1). Given the lack of consistency in the nomenclature of transfer genes among different incompatibility (Inc) groups, in this study the putative R391 transfer genes were named by their homologs in the IncF plasmids (14) and, for ORFs not having close relatives in F plasmids, according to their closest relative.

The first cluster, TRA I, is 4 kb in size and is located 32 kb from the left end of the R391 sequence, following a region containing mainly ORFs whose putative function is related to DNA repair or modification. TRA I codes for two putative transfer proteins. The first (encoded by ORF 33) is a homolog of the *traI* product from plasmid R27 (63), which is thought to be a relaxase, responsible for nicking the DNA during conjugative transfer. ORF 34 is related to a putative coupling protein from R27. Such proteins usually contain a transmembrane domain and an ATP-binding domain. The R391 homolog similarly carries motifs of this protein family.

Separated from TRA I by four ORFs of unknown function, the second cluster, TRA II, is also about 4 kb in size and contains homologs of *traL*, *traE*, *traK*, *traB*, *htdD*, and *traA* (ORFs 39 to 44, respectively), which are probably all involved in pilus synthesis and assembly.

TRA II and TRA III are separated by a 7-kb region containing a number of ORFs which are related to those found on transposons and which possibly represent the remnants of such an element. TRA III spans nearly 10 kb and contains homologs of *htdT* (ORF 56), *traC* (ORF 57), *trhF* (ORF 59), *traW* (ORF

60), *traU* (ORF 61), and *traN* (ORF 62). From the function of their closest relatives, it can be assumed that the first five of these ORFs are involved in pilus synthesis and assembly while *traN* is probably involved in aggregate stability. Additionally, this cluster contains an ORF (ORF 58) of unknown function.

The fourth cluster, TRA IV, is about 6 kb in size and is separated from TRA III by a 15-kb region. It contains homologs of *traF* (ORF 81) and *traH* (ORF 82), again putatively involved in pilus synthesis and assembly, and of *traG* (ORF 83), putatively involved in pilus assembly and mating pair stabilization.

Generally, in terms of individual homology and overall structure, the R391 transfer genes are most closely related to homologs from large plasmids from *Salmonella* strains (Fig. 4). Of the 17 transfer genes identified, 16 have homologs (average identity, 27%) in the *Salmonella enterica* serovar Typhi plasmid R27 (63) and its derivative pHCM1 (43). Both of these plasmids are very large (180 and 218 kb, respectively) and carry multiple antibiotic resistance genes. The order and structural organization of transfer genes, however, are significantly different in R391 and R27. The transfer genes might have been derived from a common ancestral plasmid, having subsequently diverged and undergone rearrangements. Other conjugative plasmids sharing transfer gene homologs with R391 are the 90-kb *Salmonella enterica* serovar Typhimurium plasmid pSLT (33), with nine homologs, and the 184-kb plasmid pNL1 from *Novosphingobium aromaticivorans* (55), having 10 homologs, all of them located within clusters TRA II, III, and IV of R391. The F plasmid derivative R100 (2) shares seven homologs within these clusters. The IncHI2 plasmid R478 from *Serratia marcescens* (42) has a total of eight homologs, including all of the putative transfer genes from TRA II and the first two of TRA III. Furthermore, two genomic islands

from *Salmonella enterica* serovar Typhimurium (6) and *Neisseria gonorrhoeae* (11) have three and two homologs, respectively.

The location of the origin of transfer (*oriT*), at which a single-strand nick is introduced and transfer to the recipient is initiated, could not be located precisely. Five families of homologous *oriT* core nick sites have been identified in different plasmid groups (73). None of the signature sequences of these *oriT* families could be found in R391, apart from the 8-bp consensus sequence of the IncP plasmid family (YATCCTGY), which is present in eight copies. Six of these copies are placed within coding regions and are thus unlikely to be the *oriT*. Of the remaining two copies, one is located in an untranslated region upstream of TRA IV, although this is not sufficient evidence for it being the *oriT*. No regulatory element of the putative transfer genes has been identified, and for that reason it remains unclear if the expression of the transfer genes is constitutive or linked to excision of R391 from the chromosome.

Phenotypic traits. (i) Kanamycin resistance. R391 confers a kanamycin resistance phenotype. This trait is conferred by an aminoglycoside phosphotransferase gene (ORF 20) which is 100% identical to a number of such kanamycin resistance genes from different strains (Table 1). The putative gene is carried by a transposon flanked by one copy of IS15-Δ1 downstream and two copies upstream, as similarly reported by Hochhut et al. (22). The composite transposon, which is about 3.5 kb in size, is inserted in a region containing several ORFs that are related to genes involved in DNA modification and DNA repair.

(ii) The mercury resistance (*mer*) operon. Narrow-spectrum mercury resistance is mediated by a 3.5-kb *mer* operon consisting of four structural genes, *merT*, *merP*, *merC*, and *merA* (ORFs 85 to 88) and the regulatory gene *merR* (ORF 84). The structural genes of the *mer* operon of R391 were found to be highly similar (97 to 100% nucleotide identity) to those of the IncJ element pMERPH from *Shewanella putrefaciens* (40). The *merR* gene of pMERPH had not been sequenced previously, but PCR amplification and sequence analysis of the pMERPH *merR* gene in this study proved that the predicted amino acid sequence is 100% identical to the R391 MerR sequence (D. Böltner and A. M. Osborn, unpublished data). Unusually for a *mer* operon from a gram-negative organism, there is no indication of the operon being carried by a transposon, and furthermore, the *merR* gene is transcribed unidirectionally with the structural genes, a characteristic of gram-positive *mer* operons (41).

(iii) Mutagenic DNA repair and UV sensitization. Previously, R391 had been shown to carry genes involved in mutagenic DNA repair. These genes, *rumA* and *rumB* (ORFs 11 and 10, respectively), had been cloned, sequenced, and shown to be homologs of the *E. coli umuDC* genes and to function in damage-inducible mutagenic DNA repair (27). In contrast to these results, R391 more recently has been reported to confer a UV sensitizing phenotype, apparently compromising the host's DNA repair capability and leading to decreased postirradiation survival (46, 47, 70). The UV-sensitizing phenotype is probably conferred by ORF 13, located adjacent to *rumA*, which encodes a putative exonuclease domain similar to the DNA polymerase III epsilon subunit. This protein is likely to

interact with RumA and/or RumB, altering their activity and dramatically reducing the DNA repair capacity (C. MacMahon and J. T. Pembroke, unpublished data).

Sequence analysis of R391 revealed a number of additional ORFs whose homologs are involved in DNA modification or repair or which contain motifs corresponding to such proteins: ORF 23 (DNA mismatch repair), ORFs 25 and 26 (putative ATPases involved in DNA repair), ORFs 27 and 29 (putative type II restriction enzyme), ORF 64 (DNA ligase), ORF 67 (single-strand binding protein), and ORF 75 (DNA repair protein RadC). While the restriction enzyme is probably not functional due to insertion of an IS element (see "Transposable elements" below), the role of the other ORFs remains unclear. Homology of ORFs 23 to 27 and 29 to 31 to a 10-kb region from *Salmonella enterica* serovar Typhimurium LT2 suggests that they may be derived from the same origin.

Transposable elements. A number of transposable elements have been identified on R391. The kanamycin resistance gene (ORF 20) is flanked by two copies of IS15-Δ1 (68) in inverse orientation upstream and one copy downstream in inverse orientation to the second element. Each of the three identical copies of IS15-Δ1 is 820 nt in size. The 5' end of the adjacent ORF 23 spans the right end of the transposon, and thus it is likely to be a pseudogene. IS15-Δ1 and the nearly identical IS26 have been found in a number of enteric bacteria, including *Salmonella* and *Proteus* spp. (<http://www-is.biotoul.fr/is.html>).

A novel IS element related to IS911 (82% identity) from *Shigella dysenteriae* (50) has been identified. Located 20 kb from the start of the sequence, the IS element is 1,223 nt in length and is flanked by 26-nt imperfect inverted repeats. The two ORFs upstream and downstream of the IS element (ORFs 27 and 29) are both related to the same putative restriction modification enzyme from *Salmonella enterica* serovar Typhimurium (GenBank accession no. AAL23313). Insertion of the IS element has probably interrupted the original ORF in R391. Although the right end of the IS element contains a new start codon for ORF 29, both ORF 27 and 29 are probably pseudogenes. Between the transfer regions TRA II and TRA III is a region containing the gene for a putative transposase, *tnp391B* (ORF 48, with 66% identity to the IS5 transposase gene) and, additionally, three ORFs (ORFs 50 to 52), one of which encodes a sulfate permease (ORF 51) and which is related to the *Pseudomonas* sp. strain R9 transposon Tn1404 (60). This region might be a transposon or the remnant of such an element, although no inverted repeats, marking the ends of transposons, were found.

Putative regulatory elements. ORF 96, located upstream of the right end of R391, is related to both the *cI* repressor from phage lambda and the *lexA* repressor. Both these repressors carry a DNA-binding helix-turn-helix motif and an autoprotease domain, which cleaves the protein in the presence of RecA and ATP. In the case of the *cI* repressor, cleavage inactivates the protein, resulting in prophage induction (25). LexA controls the expression of a number of DNA repair proteins and is likewise inactivated upon cleavage. The *rumA* gene carried by R391 (ORF 11) is one such DNA repair gene, and it is preceded by a LexA binding site (27). Interestingly, RumA itself also contains an autoprotease domain, but in this case the protein is activated when cleaved (25). The putative

repressor encoded by ORF 96 could possibly control the expression of the integrase and inhibit excision, given that both the repressor and the integrase are related to phage lambda proteins. However, the presence of a LexA binding site may also suggest a role in regulation of the *rumAB* operon. Alternatively, the repressor could belong to the remains of a cosedent phage, for which a number of phage-like ORFs on R391 might be an indication (see below). At present, the exact role of ORF 96 remains unknown but clearly deserves further attention.

While being most closely related to ORFs of unknown function from *Rhizobium* sp. strain NGR234, ORFs 2 and 3 also show similarity to *hipB* and *hipA*, respectively, which in *E. coli* act as a pair of transcriptional regulators that affect lethality due to inhibition of peptidoglycan or DNA synthesis (4, 5). ORFs 90 and 91 are homologous, respectively, to *flhC* and *flhD*, the transcriptional regulators of flagellar synthesis. These form a master operon at the top of the regulatory hierarchy of flagellar synthesis while themselves being regulated by a number of molecules, including the cyclic AMP-catabolite activator protein complex and OmpR. *flhD* is also a repressor of cell division (31, 51–53). ORF 15 may be another transcriptional regulator, since it contains a motif encoding such proteins.

Conclusions. Sequence analysis of R391 revealed a composite structure of elements of different origins (phage, plasmid, and transposable elements). While the integrase is clearly phage related, there are a number of further phage-related ORFs, including the *cI* repressor homolog (ORF 96), an upstream ORF (ORF 94) whose function is unclear, ORF 67 (related to single-strand binding proteins, which are common to phages and plasmids), ORF 68 (related to phage recombination proteins), and ORF 75 (related to DNA primases). Since these ORFs are dispersed within a 30-kb region, it is unclear if all or some of them are the remains of one prophage or if they are of multiple origins. The conjugative transfer functions are related to those from a number of plasmids and probably share a common origin with the plasmids R27, pHCM1, and pSLT from *Salmonella* and pNL1 from *Novosphingobium*. One-third of the 96 ORFs of R391 are related to homologs from *Salmonella*, in particular the transfer regions and a further 15-kb region containing 8 ORFs which is homologous to a chromosomal region of *Salmonella enterica* serovar Typhimurium LT2. Further elements contributing to the mosaic nature of R391 are the transposable elements and the mercury resistance operon, whose origin remains unknown because of the versatility and widespread distribution of these elements.

R391 and the related IncJ elements were originally described as transmissible resistance factors (8) and were initially assumed to be plasmids. They were reclassified as conjugative transposons (CTNs) on the basis of their RecA-independent transfer and chromosomal integration (36, 37). More recently, the demonstration of the chromosomal integration of R391 by its use of a phage-like integration system closely related to that of the SXT element from *Vibrio cholerae* led to a proposal that they should be included in the new group of constins (conjugative, self-transmitting, integrating elements) (21).

R391 and the SXT element share functional and structural similarities to genomic islands, conjugative transposons, and bacteriophages. R391 and its relatives carry specific pheno-

types that are also found in genomic islands (including symbiosis and pathogenicity islands). Moreover, many genomic islands similarly carry phage-related integrase determinants and terminal direct repeats (15, 16). R391 likewise shares a number of common features with CTNs (for example, Tn916 and the *Bacteroides* CTnDot). CTNs represent a diverse group of self-transmissible elements that are normally integrated into the chromosome but are capable of excision, integration, and transfer by conjugation. While exhibiting a transposon-like phenotype in terms of their ability to excise from, and integrate into, the chromosome, these elements have molecular mechanisms that clearly differ from those of classical transposons such as Tn5 and Tn10 and are instead related to those of lambdoid phages (57, 62). With conjugative transfer functions related to those of plasmids, CTNs are thus hybrid elements comprising phage integration and plasmid conjugation functions. The primary difference between CTNs and R391 is that the latter shows a strong specificity with regard to integration site, integrating into the *prfC* gene of *E. coli* while CTNs integrate relatively randomly into the chromosome (57, 62). While genomic islands exhibit nonrandom target integration, as does R391, their preferred integration targets are tRNA genes (15, 16). Finally, in contrast to prophages, R391 lacks the genes that are essential for the formation of a bacteriophage. Our future analysis will further investigate the relationships between, and evolutionary origins of, these different classes of mobile genetic elements.

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