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Large Conjugative Plasmids from Clinical Strains of *Salmonella enterica* Serovar Virchow Contain a Class 2 Integron in Addition to Class 1 Integrons and Several Non-Integron-Associated Drug Resistance Determinants

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Two large conjugative resistance (R) plasmids from clinical strains of *Salmonella enterica* serovar Virchow carried a class 2 integron with the 5′ conserved sequence (5′CS)-*dfrA1-sat1-aadA1*-3′CS gene array, which is associated with defective Tn*7* transposons. In addition, each contained a different class 1 integron (with 5′CS-*aadA1*-3′CS or 5′CS-*sat-smr-aadA1*-3′CS gene arrays) linked to Tn*21*-Tn*9* sequences, and several non-integron-associated R determinants. An intact copy of Tn*7* (including the class 2 integron) was present in the chromosome of each strain.

Integrons are gene expression elements that play an important role in the recruitment of antimicrobial drug resistance (R) determinants via site-specific recombination events catalyzed by the integron-encoded integrase (15). Based on the sequence of the integrase genes, several classes of integrons have been described (10, 12).

Integrons of classes 1 and 2 were simultaneously found in six isolates of *Salmonella enterica* serovar Virchow that were recovered as causal agents of acute gastroenteritis in a northern region of Spain. PCR amplifications (4, 20) and/or sequence analysis revealed 1,000-bp/*aadA1* and 2,300-bp/*sat-smr-aadA1* variable regions in the class 1 integrons of four and two isolates, respectively. The class 2 integrons from the six isolates were apparently identical (EMBL accession number AM055749) and carried the *dfrA1-sat1-aadA1* gene array and the inactive integrase gene characteristic of Tn*7* (5, 17, 19). Two serovar Virchow isolates (LSP 231/90 and 205/98), each containing one of the detected integron combinations, were selected for further characterization (Table 1). Both were resistant to six nonrelated antimicrobial drugs but displayed different R patterns. By conjugation experiments using *Escherichia coli* K-12 J53 as the recipient, transconjugants (Tc) with R phenotypes identical to those of the parental strains were obtained. The extraction of plasmid DNA using the S1-pulsed-field gel electrophoresis (PFGE) method (1) (Fig. 1A) revealed the presence of a large conjugative R plasmid (pUO-*Sv*R1, ca. 275 kb) in LSP231/90 and its transconjugant (Tc-231), while a different conjugative plasmid (pUO-*Sv*R2, of about the same size) was found in LSP205/98 and its transconjugant (Tc-205). PCR amplifications using Tc-231, Tc-205, and the recipient *E. coli* as the sources of template DNA confirmed the location of the expected R genes and integrons in the two identified plasmids. Of note, as far as we are aware, this is the first report on the presence of class 2 integrons in self-transferable plasmids of *Salmonella*.

In order to investigate a possible association between integrons and transposons (7, 17, 19, 22), the presence of Tn*21*-, Tn*9*-, and Tn*7*-related sequences in the two serovar Virchow strains and their transconjugants was investigated by PCR amplifications of individual genes (using already-described primers and primers designed for the present work) (4) (Table 2). The four strains, but not *E. coli* K-12 J53, yielded right-sized amplicons with primers that were specific for the *tnpA*, *tnpR*, and *merEDACPTR* genes of Tn*21*. The same result was obtained for the *catA1* gene, which was used as an indicator of Tn*9*. With regard to Tn*7*, all tested genes (*ybfA*, *ybfB*, *ybgA*, *tnsE*, *tnsD*, *tnsC*, *tnsB*, and *tnsA*) could be amplified from LSP231/90 and LSP205/98. However, genes downstream of *ybfA* and *tnsD* were not detected in Tc-231 and Tc-205, respectively. Additional amplifications of overlapping fragments (Fig. 2) demonstrated (i) the insertion of the two class 1 integrons within Tn*21*, which was in turn associated to Tn*9*, forming complex structures that differed from Tn*2670* (7) by the absence of IS*1353* (Fig. 2A), and (ii) the existence of an apparently intact copy of Tn*7*, including the class 2 integron, on the chromosome of each of the serovar Virchow strains and of a second copy of the integron, associated to a truncated Tn*7*, in each of the conjugative plasmids carried by these strains (Fig. 2B).

LSP231/90, LSP205/98, and their transconjugants were also analyzed by macrorestriction using XbaI-PFGE (11). The two serovar Virchow strains generated distinctive profiles (Jaccard's coefficient of similarity, 0.79), which included 16 matching and 7 nonmatching fragments (Fig. 1A). Selected probes for classes 1 (*qacEΔ1*, *sul1*, and *aadA1*) and 2 (*aadA1*, *dfrA1*) integrons, for Tn*21* (*merA*), Tn*9* (*catA1*), and Tn*7* (*tnsE* and *tnsA*) transposons, and for R genes that were not associated to integrons or transposons [*aacC2*, *strA*, *strB*, bla_{TEM}, and *tetA*(A)] were mapped on the XbaI and S1-PFGE profiles of the four strains (8, 13).

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TABLE 1. Characteristics of serovar Virchow strains and their transconjugants

Bacterial strain	R phenotype[a]	R genotype[b]	VR size/R gene integron(s)		Transposons[d]		
			Class 1	Class 2	Tn*21*	Tn*9*	Tn*7*
LSP231/90	Amp Chl Gen Sul Str Spt Tmp	*bla*$_{TEM}$ *catA1 aacC2 suI1 strA strB aadA1 dfrA1 sat sat1*	2,300/*sat-smr-aadA1; qacEΔ1-sul1*	2,300/*dfrA1-sat1-aadA1*	+	+	+
Tc-231	Amp Chl Gen Sul Str Spt Tmp (Rif)[c]	*bla*$_{TEM}$ *catA1 aacC2 suI1 strA strB aadA1 dfrA1 sat sat*	2,300/*sat-smr-aadA1; qacEΔ1-sul1*	2,300/*dfrA1-sat1-aadA1*	+	+	Δ
LSP205/98	Chl Kan Sul Str Spt Tet Tmp	*catA1 aphA1 suI1 strA strB aadA1 tetA dfrA1 sat sat1*	1,000/*aadA1; qacEΔ1-suI1*[e]	2,300/*dfrA1-sat1-aadA1*	+	+	+
Tc-205	Chl Kan Sul Str Spt Tet Tmp (Rif)[c]	*catA1 aphA1 suI1 strA strB aadA1 tetA dfrA1 sat sat*	1,000/*aadA1; qacEΔ1-sul1*[e]	2,300/*dfrA1-sat1-aadA1*	+	+	Δ

[a] The strains were tested for susceptibility to antimicrobial drugs by disk diffusion according to the method of the CLSI (formerly NCCLS) (2).
[b] Resistance determinants were identified by PCR amplification using previously described primers and conditions (4, 6). Resistance to streptothricin has not been tested, but the presence of *sat* genes was established by nucleotide sequencing.
[c] Resistance from the *E. coli* recipient strain.
[d] +, positive for the indicated transposon; Δ, truncated Tn*7*.
[e] Genes located in the 3′ conserved sequence region of the integron(s).

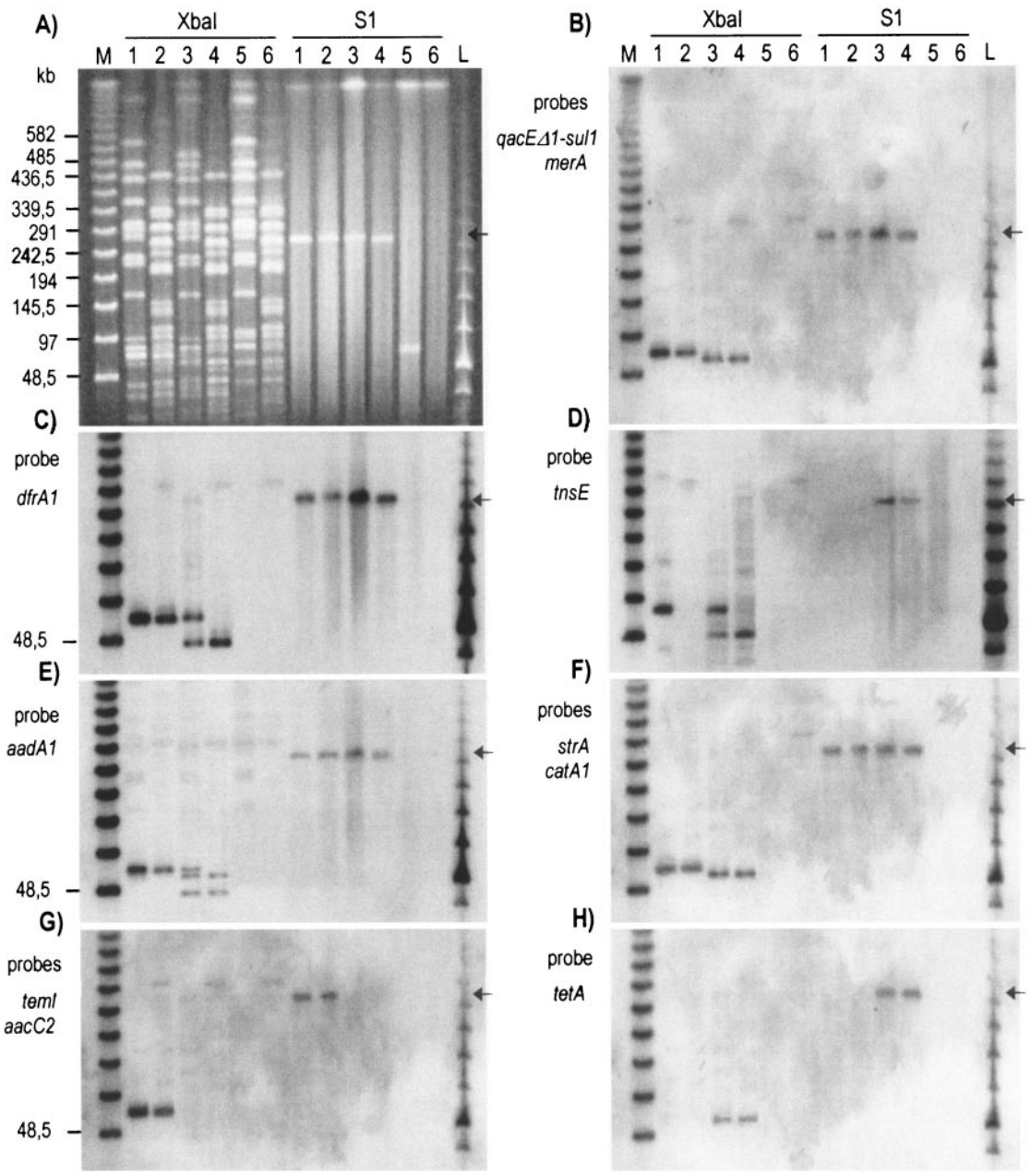

TABLE 2. Primers designed for this work

Region or gene	Name	Sequence (5′ to 3′)	Temp (°C)	Accession no.
IS1 from Tn*9*	IS1a-F	CTGACGGGGTGGTGCGTAACGGC/	65	NC_002134
	IS1a-R	GTAAAACAGCCAGCGCTGGCGCG		
tnpA	tnpA-R100-F	GATCGGCGCGGGGAAGTTCAGGC	65	NC_002134
	tnpA-R100-R	AGCCGGTTGCAGAGGCCGTAGC		
tnpR	tnpR-R100-F	GAGCGTCAGCGCGAGGGTATTG	—[a]	AF_071413
istA	istA-F	GACAGCGTTGGCAAGTTAAGTCC	60	AF_071413
	istA-R	GGCATGCCAGTGGGCATCAAACA		
tniA	tniA-F	GATCCTCCGGCGCACGCTGACCC	65	AF_071413
	tniA-R	AATCTGGTCGAACGGTTTGGCGG		
urf2	urf2-F	GGAGATCGAACTGACCGAATCGG	65	AF_071413
	urf2-R	CAGGAGCTGGCTGCACAACAGC		
merE	merE-F	CGCCCCTGACAAACTGCCGCCC	60	AF_071413
	merE-R	CGAGCGCGGCAACACCCCAATG		
merD	merD-F	TACGGCCGGTGGCCTGCACCACG	60	AF_071413
	merD-R	CGCTCGGCTGGCATGGAGGCCAG		
merA	merA-F2	AAGGCGCACGTGTCACGCTGATC	70	AF_071413
	merA-R2	CCGCTGCCTTCTTCAACCACCAG		
merC	merC-F	AGCGTCGTTTCCGCGATGGGCTG	60	AF_071413
	merC-R	CCGGTCCGCAACGGCGATGCGCC		
merP	merP-F	CTCCCTTGCCCTCGCCGCCGCTG	65	AF_071413
	merP-R	GGACGGATAGCCGGCGTCTGCGG		
merT	merT-F	GCGCCACGCGAGAAGCGGCCGC	60	AF_071413
	merT-R	GAAATCCAAGCGCGACCAGGAC		
merR	merR-F	GGGGTCAACGTGGAGACAATCCG	65	AF_071413
	merR-R	CCTTGAGCTTGTGTTCGGCCAGG		
ybfA	ybfA-F	CGCCCAAGTAAGTATCCAGCTGTG/	60	NC_002525
	ybfA-R	CTTGACATCTCATCAATACCACC		
ybfB	ybfB-F	CGATGTGGCTACGAGTCTGATC/	55	NC_002525
	ybfB-R	CTTCTCTCTAGATAGCAGGG		
ybgA	ybgA-F	CTATGCACTTTGTGACAAGTTGG/	60	NC_002525
	ybgA-R	CTTGAAGTGCAGGCGAGCACG		
tnsA	tnsA-F	GCAGCAGCCTTACAAGACGAGCG	60	NC_002525
	tnsA-R	GGCTAATACAGAGATCTGCACAC		
tnsB	tnsB-F	TGTAGGTTTGCCAGATGTGTTGC	60	NC_002525
	tnsB-R	TGACCCCGAGTAATACAAACCCC		
tnsC	tnsC-F	GGCAATTACAGCTTTTACAACGC	60	NC_002525
	tnsC-R	CTGCGATATCTAGCTGAAGTTGG		
tnsD	tnsD-F	ACAGGGATTGGCTAGTTCACTGG/	60	NC_002525
	tnsD-R	CTTTGTGCTTCCTCAGTTATCCG		
tnsE	tnsE-F	GGCGGATCGTGTGATTGAGTTTG/	60	NC_002525
	tnsE-R	GCGTACTACCATCACCTGCCTCC		

[a] tnpR-R100-F was used together with tnpA-R100-R at an annealing temperature of 65°C.

With LSP231/90 and Tc-231, all probes except *tnsE* mapped on a ca. 75-kb fragment of the XbaI profiles and on the ca. 275-kb band corresponding to pUO-*Sv*R1 (Fig. 1B, C, and E through G). The *tnsE* probe mapped on a 75-kb fragment from LSP231/90 but failed to hybridize with the XbaI profile of Tc-231 and with pUO-*Sv*R1 (Fig. 1D). These results located the class 1 integron that was linked to Tn*21*-Tn*9* sequences and the class 2 integron inserted into a defective Tn*7*, as well as all independent R genes within a ca. 75-kb XbaI fragment that was generated from pUO-SvR1. The hybridization of *tnsE* on a fragment of about the same size suggests the existence of two comigrating bands on the macrorestriction profile of LSP231/90, one from the chromosome and one from pUO-*Sv*R1. In the former, the intact copy of Tn*7* (including the class 2 integron) would be located. The high intensity of the relevant fragment on the agarose gel (Fig. 1A) supports this possibility.

In LSP205/98 and its transconjugant, the *qacE*Δ*1-sul1*, *merA*, and *catA1* probes (and hence the Tn*2670*-like transposon with the class 1 integron), as well as the probes for independent R genes, mapped on the ca. 275-kb pUO-*Sv*R2 and on a ca. 65-kb XbaI-fragment that was common to the macrorestriction profiles of the two strains and therefore of plasmid origin (Fig. 1B, F, and H). The *dfrA1* probe hybridized with the 275-kb plasmid, a ca. 40-kb XbaI fragment from the transconjugant, and

FIG. 1. Mapping of integron, transposon, and R genes on the *S. enterica* serovar Virchow genome. (A) XbaI and S1 profiles of serovar Virchow strains and their transconjugants. (B through H) Hybridizations of panel A with the probes indicated at the left of each panel. Lanes M and L, PFGE marker I (New England BioLabs). Lanes 1 and 2, serovar Virchow LSP231/90 and Tc-231, both carrying pUO-*Sv*R1. Lanes 3 and 4, serovar Virchow LSP205/98 and Tc-205, both carrying pUO-*Sv*R2. Lane 5, serovar Virchow CECT 4154, susceptible to antimicrobial drugs. Lane 6, *E. coli* K-12 J53, plasmid free. The arrow indicates the ca. 275-kb plasmids.

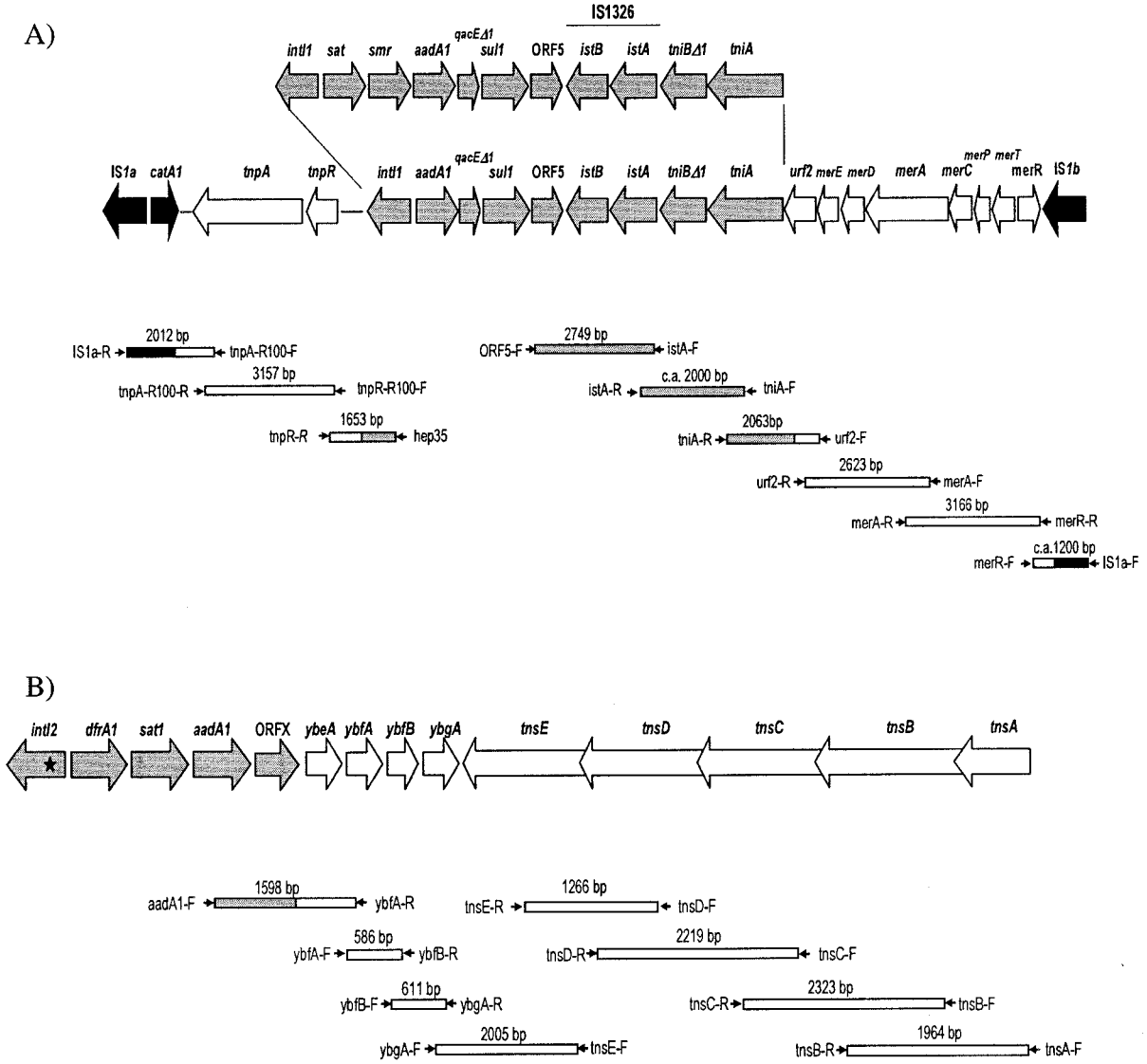

FIG. 2. Integron-transposon associations. (A) Physical linkage between the class 1 integrons, Tn*21*, and Tn*9*. The scheme is based on sequencing data from R100 *Shigella flexneri* plasmid (NC_002134). The class 1 integron, Tn*21*, and Tn*9* sequences are indicated by gray, white, and black arrows, respectively. All of the overlapping fragments that are depicted below the scheme could be amplified from the two serovar Virchow strains and their transconjugants. ORF5, open reading frame 5. (B) Physical linkage between class 2 integrons and Tn*7*. The schematic representation is based on sequencing data from the R721 plasmid of *E. coli* (NC_002525). Integron and transposon genes are indicated by gray and white arrows, respectively. All of the overlapping amplicons were generated from the two serovar Virchow strains, except the last three from Tc-205 and the first two from Tc-231. Primers used in PCR amplifications are compiled in Table 2.

two XbaI fragments (of ca. 75 and 40 kb) from the donor strain (Fig. 1C). These results are consistent with the presence of the class 2 integron in both the plasmid and the chromosome of LSP205/98. This was confirmed by hybridizations with probes for *aadA1* (Fig. 1E), a gene cassette shared by the classes 1 and 2 integrons of LSP205/98, and *tnsE* (Fig. 1D). The former gene mapped on the 275-kb plasmid and on two XbaI fragments (65 and 40 kb) from Tc-205. By comparison with results that were obtained with the *qacEΔ1-sul1* and *dfrA1* probes, the 65-kb fragment would contain the *aadA1* cassette of the class 1 integron and the 40-kb fragment would contain the *aadA1* cassette of the class 2 integron. In the XbaI profile of LSP205/98, an additional fragment of ca. 75 kb (where *dfrA1* was previously located) hybridized with *aadA1* and *tnsE*, hence verifying the existence of the chromosomal copy of the Tn*7* integron (Fig. 1D and E). The association of the extrachromosomal copies of the class 2 integron with truncated Tn*7* transposons was finally corroborated by the absence of hybridization of a *tnsA* probe with the two plasmids and with the XbaI profiles of the transconjugants (not shown).

With regard to public health, serovar Virchow has emerged as the third or fourth most common *Salmonella* serovar that causes human gastroenteritis in Europe (3, 18) and its incidence is even higher in countries like Australia and Israel,

where it has been reported as an important cause of bacteremia in children (9, 14, 16, 21). Taking this into account, the emergence of multiple drug-resistant strains, such as those reported here, is an obvious cause of concern, as it is this wide range of mobile genetic elements that can efficiently contribute to the acquisition, maintenance, and spread of resistance determinants.

Nucleotide sequence accession number. The sequence for *sat-smr-aadA* was deposited in the EMBL database under accession number AM055748.

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