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Genetic Analysis of Virulence Plasmid from a Serogroup 9 *Yersinia enterocolitica* Strain: Role of Outer Membrane Protein P1 in Resistance to Human Serum and Autoagglutination

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Enteropathogenic strains of *Yersinia enterocolitica* harbor a virulence plasmid (70 kilobases) which specifies, at 37°C, a calcium requirement for growth, autoagglutinability, resistance to the bactericidal activity of human serum, and the expression of some outer membrane proteins (OMPs). To map the genes encoding these properties, the virulence plasmid of a serogroup 9 strain (W22708) was subjected to transposon mutagenesis. A set of 68 independent mutations was obtained in *Escherichia coli* by transposon Tn*813* (a *tnpR* mutant of Tn*21*)-mediated cointegration with the self-transmissible R388 plasmid. The resulting cointegrates were introduced and studied in *Y. enterocolitica* W22708. One mutant lost the calcium dependence property. Two other mutants presented a peculiar phenotype: they grew poorly at 37°C, especially in the presence of calcium. Lastly, two mutants were affected in the properties of autoagglutination and resistance to human serum. Analysis of the OMP pattern of these two mutants revealed the absence of the largest OMP, called P1 (I. Bölin, and H. Wolf-Watz, Infect. Immun. 43:72–78, 1984). Complementation of one of these mutations with the cloned structural gene of OMP P1 restored the wild-type phenotype. However, OMP P1 was not sufficient by itself to specify the serum resistance property and a rapid autoagglutination of the host.

The invasive enteropathogenic *Yersinia enterocolitica* strains predominantly belong to serogroups 3, 9, 5b (antigens O:5,27), and 8 (37), the latter being so far isolated only in the United States.

An IncFI (2) plasmid of 40 to 48 megadaltons has been shown to be involved in the virulence of three *Yersinia* species (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) (3, 14, 39).

Plasmids from serogroup 3, 9, and 5b strains share 90% homology among themselves (16, 24) and 75% with plasmids from serogroup 8 strains (16), the latter being 50% homologous with *Y. pestis* and *Y. pseudotuberculosis* plasmids (4, 7).

The *Y. enterocolitica* plasmid specifies several temperature-dependent properties. These include a calcium requirement for growth (14), autoagglutinability (AA) (23), modification of the outer membrane protein (OMP) pattern (34), resistance to the bactericidal activity of human serum (SR) (16, 30), hydrophobicity, and a change of surface charge (21).

The virulence, conditioned by the plasmid, is monitored for serogroup 8 strains by invasion of the conjunctival epithelium of guinea pigs (Sereny test) or by lethality for mice (14, 39). For serogroup 3, 9, and 5b strains, orally infected mice only show a colonization of the intestine (28, 31), mild diarrhea (23), and an invasion of the spleen (R. Bakour, G. Balligand, Y. Laroche, G. Cornelis, and G. Wauters, J. Med. Microbiol., in press).

The calcium dependency (CD) region has been mapped in plasmid pYV019 of *Y. pestis* (33). A comparison of the restriction patterns of pYV019 and virulence plasmids from serogroup 3, 5b, 8, and 9 strains indicates that this region is particularly conserved (16, 24, 34).

One of the temperature-dependent OMPs, called protein 1 (P1) (molecular weight of 240,000) (6) was found to be correlated with AA in *Y. pseudotuberculosis* (35). An immunologically related protein has been detected in *Y. enterocolitica* (35).

To understand the various plasmid-encoded properties in *Y. enterocolitica* and to localize the genes involved, plasmid pVYE22708 (from a serogroup 9 strain) was subjected to transposon mutagenesis.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *Y. enterocolitica* W22708 (serogroup 9, biogroup 2, streptomycin resistant) is a restriction mutant (Res$^-$ Mod$^+$) isolated earlier in this laboratory (9). The *Escherichia coli* strains used were HB101 (26), JC6310 (38), and C600 (26). R388 is a self-transmissible plasmid of 32. 4 kilobases encoding trimethoprim and sulfonamide resistance (11). Plasmids pBR322::Tn*21* and pACYC184::Tn*813* (13) are gifts from J. Grinsted. pVYE22708 and pVYE439-80 are the virulence plasmids (70 kilobases) of *Y. enterocolitica* W22708 and 439–80 (serogroup 9), respectively (24). Their restriction patterns appear to be identical (24). pYL4 is a derivative of pVYE22708 labeled with Tn*3* (17), obtained after mobilization with pMR5 (data not shown). Tn*3* is inserted in *Bam*HI restriction fragment B2 of pVYE22708.

Media. The bacteria were grown on tryptic soy broth and tryptic soy agar (TSB-TSA) (Difco Laboratories) enriched with 0.3% yeast extract (Difco), brain heart infusion (BHI) (GIBCO Laboratories), and MacConkey agar (Difco). The minimal medium was that described by Burrows and Gillet (8) supplemented with 5 μg of thiamine per ml for *Yersinia* strains. Magnesium oxalate agar consisted of TSA with 20 mM $MgCl_2$ and 20 mM sodium oxalate. Selective agents were ampicillin (25 μg/ml), chloramphenicol (10 μg/ml), merbromine (150 μg/ml), streptomycin (200 μg/ml), sulfathiazole (250 μg/ml), and trimethoprim (50 μg/ml). Except when otherwise stated, *Y. enterocolitica* strains were grown at 28°C, and *E. coli* strains were grown at 37°C.

DNA manipulations. The plasmids were visualized and sized by the method of Kado and Liu (20). The mapping of the mutations was done by *Bam*HI and *Pst*I restriction on plasmid DNA prepared by a modification of the method of Grosveld et al. (15) and of Ish-Horowicz and Burke (19) (E. Beck, personal communication). Restriction, gel electrophoresis, and nick translation were done according to the methods described by Cornelis and Saedler (10).

Colony hybridization was done as described by Maas (25). The cloning of *Bam*HI restriction fragments of pVYE439-80 has been described previously (24).

Transformation of *Y. enterocolitica* W22708. *Y. enterocolitica* W22708 was transformed by a modification of the method of Dityakin et al. (12) and Holsters et al. (18). Bacteria were grown with shaking at 28°C in 100 ml of TSB supplemented with 10 mM $CaCl_2$ to a cell density of $5 \cdot 10^8$ bacteria per ml. Cells were harvested, washed with 20 ml of Tris-hydrochloride (10 mM; pH 7.5), and resuspended in TSB supplemented with 10 mM $CaCl_2$ and 20 mM $MgCl_2$ at a cell density of 10^{10} bacteria per ml. An amount of 200 μl of this bacterial suspension was mixed with 100 μl of DNA solution (1.5 to 3 μg of DNA). The mixture was frozen in liquid nitrogen for 5 min, warmed for 25 min at 37°C, diluted fivefold with TSB, incubated at 28°C for 1.5 h, and plated on selective media.

Transposon mutagenesis. The mutagenesis was carried out with Tn*813*, a *tnpR* derivative of class II transposon Tn*21* (13). This element generates cointegrates between the transposon vector and target that do not resolve into normal transposition products. The transposition vector was a R388::Tn*813* derivative obtained after mobilization of pACYC184::Tn*813* by R388. The cointegrate selected was afterwards resolved by complementation with pBR322::Tn*21*. The *Yersinia* plasmid pYL4 (i.e., pVYE22708::Tn*3*) was introduced by transformation into *E. coli* HB101. Ten doubles containing R388::Tn*813* and pYL4 were then constructed by conjugation. Cointegrates between R388::Tn*813* and pYL4 were constructed by mating the 10 doubles with *E. coli* JC6310 and selecting for ampicillin-resistant transconjugants. This strategy also selects for transposition of Tn*3* onto R388::Tn*813*. The discrimination between the cointegrates R388::Tn*813*::pYL4 and R388::Tn*813*::Tn*3*, transposition products, was done by gel electrophoresis and colony hybridization. A fifth of the transconjugants were of the cointegrate type. They were subsequently introduced into *Y. enterocolitica* W22708 by conjugation.

CD. The calcium requirement for growth at 37°C was determined as described previously (Bakour, et al., in press).

AA. The ability of *Y. enterocolitica* strains to autoagglutinate at 37°C was tested by a modification of the method of Laird and Cavanaugh (23). Two 20-ml universal bottles containing 10 ml of TSB were inoculated with 100 μl of an overnight culture grown at 28°C and incubated at 37 and 28°C, respectively. The turbidity of the culture was examined after 12 h.

SR. The human sera used were first tested for the absence of specific antibodies to *Y. enterocolitica* (serogroup 9) (36). The serum resistance was assayed by a modification of the method of Heeseman et al. (16). Approximately 10^7 bacteria of an overnight culture grown at 28°C in BHI were inoculated in 10 ml of BHI, contained in a 100-ml conical flask, and grown at 37 or 28°C with shaking at 180 rpm to a density of 10^8 cells per ml. Cells were harvested by centrifugation for 10 min at 1,500 × *g*, suspended in 10 ml of BHI containing 0.02 M sodium oxalate and 0.02 M $MgCl_2$, and incubated for 2 h at 37 or 28°C with shaking. Bacteria were then harvested as previously described, suspended in saline (pH 7.4) at a cell density of 10^6 cells per ml, and incubated at 37°C in the presence of 5% (vol/vol) human serum. Viable counts were made by plating appropriate dilutions onto TSA after 0, 30, 60, and 90 min.

Isolation of bacterial OMPs and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The Triton X-100-insoluble OMPs were isolated as described by Achtman et al. (1), suspended in sample buffer (62.5 mM Tris-hydrochloride [pH 6.8], 3% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, 0.03% bromophenol blue) and stored frozen at −20°C.

The proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22). Running gels were 10 to 20% acrylamide-bisacrylamide (ratio, 29:1) gradients and staking gels were 3% acrylamide-bisacrylamide (29:1). Gels were stained for 30 min in 0.2% Coomassie blue in 50% methanol–7% acetic acid and destained in 20% methanol–7% acetic acid with several changes.

Molecular weight standards were α_2-macroglobulin (170,000 reduced), phosphorylase *b* (97,400), glutamate dehydrogenase (55,400), and lactate dehydrogenase (36,500) (Boehringer).

RESULTS

Mutagenesis. Transposition of class II elements occurs through transient cointegration. Since the virulence plasmid appears not to be self-transmissible, we decided to carry out mutagenesis with an altered class II transposon to generate a conjugative cointegrate. The conjugative plasmid, carrying the resolution-defective transposon Tn*813*, was R388. For selection purposes, the mutagenesis was carried on a pVYE22708::Tn*3* derivative called pYL4 (see above). The insertion of Tn*3* did not impair CD, AA, SR, and OMPs. A collection of 80 cointegrates between pYL4 and R388::Tn*813* was constructed as described above. According to their origin and restriction pattern, 68 mutants were clearly independent. The number of insertions in each *Bam*HI fragment is given in Fig. 1. After construction in *E. coli*, the cointegrates were introduced by conjugation into the restrictionless *Y. enterocolitica* W22708 strain. The analysis of the phenotype was done in the latter strain.

Mutations affecting CD and growth. All the *Y. enterocolitica* W22708 strains bearing the cointegrates were tested for the calcium requirement for growth at 37°C. Only one clone carrying the cointegrate pGB51 grew well at 37°C on a calcium-deficient medium (magnesium oxalate agar). In this mutant, R388::Tn*813* is inserted in the *Bam*HI fragment B7 of pYL4. This fragment belongs to a region highly conserved in the virulence plasmids of the three *Yersinia* species (*Bam*HI restriction fragments B5, B6, and B7) (16, 24, 34). The presence of two other cointegrates (pGB09 and pGB67) clearly hindered the growth of their *Y. enterocolitica* host at 37°C on TSA. Surprisingly, the clones containing these cointegrates seemed to grow better on magnesium oxalate agar than on TSA at 37°C. In the two cointegrates the integration site of R388::Tn*813* is located in *Bam*HI fragment B9, a fragment neighboring the conserved region.

Mutations affecting AA and OMP pattern. Of the 68 cointegrates, 2 (pGB08 and pGB910) did not confer the property of AA to *Y. enterocolitica* W22708. Comparison between the OMP profiles from the two AA$^-$ strains and the strains bearing pVYE22708 or its derivative, pYL4, revealed the absence of the largest thermosensitive OMP (P1; molecular weight of 240,000) in the two mutants (Fig. 2). This result is in agreement with that of Skurnik et al. (35) and confirms that protein P1 plays a role in the AA of *Y. enterocolitica*. We also observed the presence of a new protein with a molecular weight of ca. 55,000 in the *Y. enterocolitica* W22708 (pGB910) OMP pattern. To localize the structural gene of OMP P1, the Tn*813* insertions were mapped into pGB08 and pGB910. The two mutations occur in *Bam*HI fragment B4 at 2,500 and 1,600 base pairs, respectively, from the right extremity of this fragment.

Mutations affecting the SR. The ability of the 68 cointegrate-bearing strains to resist the bactericidal activity of human serum was tested as described above. As expected, strain W22708 (R388::Tn*813*) cultivated at 28 or 37°C and strain W22708 (pYL4) cultivated at 28°C were unable to survive in human serum at 37°C: 60 min after the exposure to 5% human serum, less than 1% of the bacteria survived (Table 1). On the other hand, strain W22708 (pYL4), grown at 37°C, presented a high degree of resistance: 70% of the bacteria survived after 90 min of exposure to the serum. Among the mutants, 66 of 68 behaved like the pYL4$^+$ strain, but 2 of 68 (containing pGB08 and pGB910) were as sensitive as the pYL4$^-$ strain. The two *omp* P1 mutants are thus also affected in their resistance to the bactericidal activity of human serum.

Cloning of *Bam*HI fragment B4 of the virulence plasmid of serogroup 9 strains and complementation experiments. The fusion site of the two cointegrates pGB08 and pGB910 is localized in *Bam*HI fragment B4. This fragment, from

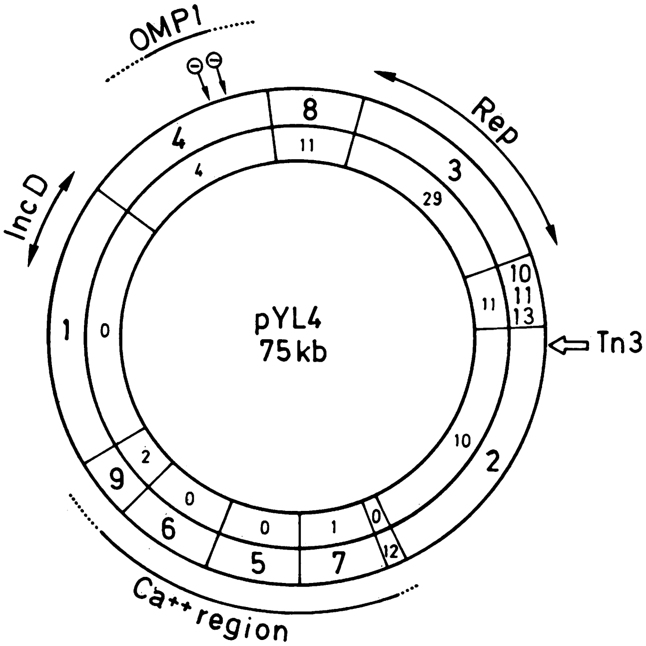

FIG. 1. *Bam*HI restriction map of pYL4 (pVYE22708::Tn*3*) (24). The insertion site of Tn*3* within pVYE22708 is indicated by an open arrow. The external circle numbers refer to *Bam*HI restriction fragments. The number of insertions of Tn*813* in each *Bam*HI fragment is given in the internal circle. Arrows marked with the symbol ⊖ indicate the Tn*813* insertion that abolishes the OMP P1 expression. OMP1 represents the approximate region that encodes for OMP P1. The Ca^{2+} region represents the highly conserved plasmid region that was shown in the plasmid pYV019 of *Y. pestis* to contain the Ca^{2+} dependence locus (33). The Rep and IncD regions, respectively, contain the replication genes and the *incD* determinant of incompatibility (2).

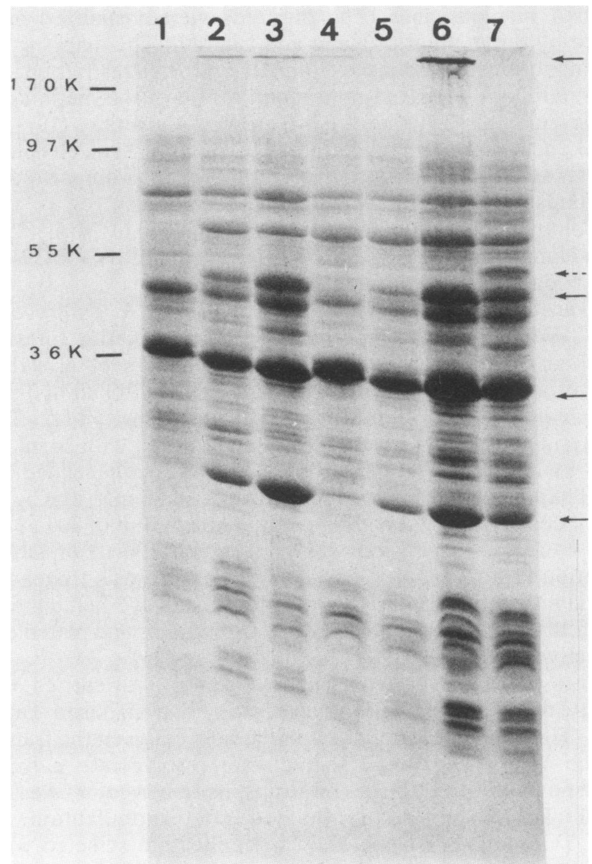

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 to 20% polyacrylamide gradient gel) of OMPs of *Y. enterocolitica* W22708 bearing different plasmids after growth in BHI at 37°C. Lane 1, W22708 (R388::Tn*813*); lane 2, W22708 (pVYE22708); lane 3, W22708 (pYL4); lane 4, W22708 (pACYC184-B4); lane 5, W22708 (pGB08); lane 6, W22708 (pGB08) (pACYC184-B4); lane 7, W22708 (pGB910). The arrows point to the plasmid-mediated OMPs. The dashed arrow points to a protein only observed in W22708 (pGB910).

pVYE439-80, the virulence plasmid for another serogroup 9 strain, was previously cloned into the plasmid vector pACYC184 (24). The recombinant plasmid (pACYC184-B4) was introduced by transformation into *E. coli* C600 and *Y. enterocolitica* W22708. *E. coli* C600 bearing pACYC184-B4 did not harbor protein P1 in its outer membrane and did not manifest AA or SR. *Y. enterocolitica* W22708 (pACYC184-B4) synthesized protein P1 when grown at 37°C, even with the presence of 1 to 5 mM of calcium (data not shown). However, this strain was as sensitive as the plasmidless strain to the bactericidal activity of the serum. As far as AA is concerned, the results are not clearcut: the strain autoagglutinated at 37°C, but the phenomenon was much "slower" in the sense that it was apparent after 24 h of growth instead of 12 h of growth. The results clearly show that OMP P1 is involved in AA and SR. However, the cloning of the relevant gene indicates that the presence of P1 is not sufficient to confer either AA or SR within 12 h.

To confirm these findings, plasmid pGB08 was introduced into strain W22708 (pACYC184-B4). The resulting strain clearly was resistant to the bactericidal activity of human serum and autoagglutinated in 12 h at 37°C. The mutation altering the SR is thus fully complemented by the presence of an intact B4 fragment.

TABLE 1. Sensitivity to human serum of *Y. enterocolitica* W22708 harboring different plasmids after growth at 28 and 37°C

Plasmid	Growth temp (°C)	% Survival in 5% human serum at 37°C after incubation time (min)[a]:		
		30	60	90
pVYE22708	28	0.1	0.025	<0.01
	37	100	75	70
pYL4	28	0.15	0.025	<0.01
	37	93	110	75
R388::Tn*813*	28	0.15	0.025	<0.01
	37	45	0.6	0.03
pGB08	28	0.1	0.025	<0.01
	37	60	9	1.6
pGB910	28	0.05	0.025	<0.01
	37	55	12	0.06
pACYC184-B4	28	0.5	0.025	0.03
	37	66	2	0.7
pGB08, pACYC184-B4	28	3.3	0.5	0.3
	37	94	80	55

[a] All organisms survived growth at 28 and 37°C before incubation in 5% human serum at 37°C.

DISCUSSION

A set of 68 different mutations in the virulence plasmid pVYE22708::Tn*3*, pYL4, was constructed by cointegration with R388::Tn*813*. The insertion points of R388::Tn*813* within pYL4 were not randomly distributed. Most of the insertions occurred in *Bam*HI fragment B3, a fragment previously shown to contain the replication function of the plasmid (2).

Insertion of R388::Tn*813* within *Bam*HI fragment B7 of pYL4 abolishes the CD property of the strain. This fragment is part of a particularly conserved region of the virulence plasmid in the genus *Yersinia* (16, 24, 34). This region was previously shown to encode CD and to be essential for virulence in *Y. pestis* and *Y. pseudotuberculosis* (6, 33). As expected, this highly conserved region thus also encodes CD in *Y. enterocolitica*.

Two clones harboring plasmids mutated in *Bam*HI fragment B9, a fragment neighboring the conserved region, present a phenotype never described so far in the genus *Yersinia*: they grow poorly at 37°C on TSA. Surprisingly, instead of promoting growth of these mutants at 37°C, calcium seems to reduce it even more. The characterization of these mutants and others presenting the same phenotype will be presented in a separate paper.

Two *Y. enterocolitica* W22708 strains carrying plasmids mutated in *Bam*HI fragment B4 (pGB08 and pGB910) lost the property of AA. The analysis of their OMP pattern reveals that they have lost the OMP P1 (240,000 molecular weight). The OMP pattern of the clone harboring one of these cointegrates (pGB910) presents a new protein of ca. 55,000 molecular weight. Since it was suggested that P1 could be a polymerized structure (35), the observed new protein could be a slightly truncated form of the monomer. As already shown for *Y. pseudotuberculosis* (35), this result indicates that OMP P1 is involved in the AA phenomenon. To confirm this, we cloned the relevant *Bam*HI fragment onto pACYC184. *E. coli* carrying this recombinant plasmid does not express P1 in its outer membrane, even at 37°C. This contrasts with the expression of the *Y. pseudotuberculosis* OMP P1 in *E. coli* (6). *Y. enterocolitica* W22708 carrying the same plasmid expresses P1 in its outer membrane at 37°C and becomes, albeit slowly, autoagglutinable.

Recently the OMPs of *Y. enterocolitica* strains carrying virulence plasmid have been shown to be involved in the resistance to the bactericidal activity of human serum (27). The implication of OMPs in SR is not unprecedented: some plasmid-encoded OMPs have been shown to be responsible for plasmid-specified SR in *E. coli* (5, 29). In agreement with this, the two mutants defective in P1 production are sensitive to the bactericidal action of serum. Surprisingly, *Y. enterocolitica* W22708 carrying the recombinant clone and expressing P1 in its outer membrane is not resistant to human serum. However this clone fully complements the insertion mutation in the OMP P1 gene. These results suggest that OMP P1 is a necessary but not sufficient condition for SR. All the other mutants were thus tested to define a second plasmid gene involved in SR, but no such mutant was found.

In *Y. pseudotuberculosis*, a mutant defective in OMP P1 was shown to be fully virulent (6). Moreover, in *Y. pestis* and *Y. pseudotuberculosis*, SR is not plasmid encoded (32). These two elements clearly suggest that in these two species, SR and the presence of OMP P1 is not required for virulence. It would have been most interesting to test the virulence properties of the two SR^- AA^- CD^+ clones. Unfortunately, virulence in *Y. enterocolitica* strain O:9 is difficult to assess (Bakour et al., in press). Moreover, *Y. enterocolitica* harboring pYL4 seems to be even less virulent than the other O:9 plasmid-bearing strains, though pYL4 specifies CD, SR, AA, and OMPs. This reduction in virulence, presumably due to the Tn*3* insertion, made it irrelevant to assay the virulence of the mutants. A new set of mutants is presently being constructed in our laboratory.

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