Restriction fragment length polymorphism of the pMJ101-like plasmid and ribotyping in the fish pathogen *Vibrio ordalii*

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SUMMARY

A total of 32 *Vibrio ordalii* strains were studied for their plasmid content and shown to carry a plasmid of approximately 32 kb. This plasmid was subsequently subjected to restriction fragment length polymorphism (RFLP) studies. Using *Hind* III, three different restriction patterns were identified while *Bam*H I cleaved the plasmid into a single linear fragment. The results suggest that the 32 kb plasmid is highly conserved but that some variation in restriction pattern occurs. The same set of strains was subjected to ribotyping. Using *Mlu* I, six different restriction patterns were demonstrated. Strains from the USA and Canada shared profiles with strains from Australia and Japan. Strains from Australia generated a single pattern whereas strains from North America were subdivided into three patterns, and the Japanese strains fell into five patterns. The results suggest that ribotyping in combination with RFLP studies of the pMJ101-like plasmid may be useful in epidemiological studies of *V. ordalii*.

INTRODUCTION

Vibrio ordalii has been recognized as a cause of vibriosis among salmonids and some non-salmonid fish species, ayu (*Plecoglossus altivelis*) and rockfish (*Sebastes schlegeli*) in Japan, the Pacific Northwest of America, New Zealand, and Australia [1–3]. The bacterium has never been isolated in Europe. *V. ordalii* was previously described as *Vibrio anguillarum* biotype 2 [1, 4] but was subsequently renamed [5] due to significant differences in biochemical properties and DNA homology [6] compared with *V. anguillarum*. In addition, the clinical symptoms and pathological changes caused by this organism in fish are different from those recorded for *V. anguillarum* [7]. It was recently demonstrated that *V. ordalii* and *V. anguillarum* were easily distinguished by ribotyping [8]. *V. ordalii* cross-reacts serologically with *V. anguillarum* serogroup O2a [9] although minor antigenic differences have been demonstrated. Thus,

Mutharia and Amor [10] were able to generate monoclonal antibodies that recognized distinct species-specific epitopes in O-antigens of *V. anguillarum* and *V. ordalii* and the results of Mutharia and colleagues [11] demonstrated both common antigens and species-specific antigens using absorbed and unabsorbed polyclonal antisera, generated in fish and rabbits.

A plasmid of approximately 32 kb has been detected in all *V. ordalii* strains studied so far [3, 5, 8]. The plasmid of the *V. ordalii* type strain, DF$_3$K, has been designated pMJ101 [1]. In a recent study, it was shown by replicon typing that this plasmid was not related to any of a large number of plasmid incompatibility groups [12]. In most *V. anguillarum* serogroup O1 strains, isolated from dead fish, a 67 kb virulence plasmid is present [13] and it has been shown that restriction endonuclease analysis of this plasmid may be useful in epidemiological studies [14]. Ribotyping is a genotyping method, first proposed by Grimont and

Grimont [15] as a taxonomic or typing tool. This method has been applied to other *Vibrio* species, such as *Vibrio cholerae* [16], *Vibrio vulnificus* [17], and *Vibrio anguillarum* [18].

The present study was carried out to determine whether there were differences in ribotype among *V. ordalii* isolates from different parts of the world together with restriction fragment length polymorphisms of the 30–32 kb pMJ101-like plasmid and to evaluate these two methods for the typing of *V. ordalii*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A total of 32 *V. ordalii* isolates were examined (Table 1). Four isolates were from the USA or Canada, 5 from Australia, and 23 from Japan. The strains were considered epidemiologically unrelated, i.e. not originating from the same fish farm or the same outbreak of disease. All strains were stored at $-80\,°C$ until used and propagated on blood agar plates (Marine agar (Difco) supplemented with 5% sterile, citrate-stabilized calf blood), incubated at $20\,°C$ for 2 days.

O-serotyping

O-serotyping was carried out using the slide agglutination method as previously described [9, 19]. Absorbed O2a and O2b sera were obtained as described by Larsen and colleagues [9].

Plasmid profiling

Cultures for isolation of plasmids were propagated in Marine broth (Difco), agitated vigorously for 2 days at $20\,°C$. Plasmid DNA for profiling was extracted by the method of Kado and Liu [20] and subjected to electrophoresis in 0·8% agarose gels (SeaKem GTG, FMC BioProducts, Rockland, ME, USA). Gels were stained with ethidium bromide and photographed in UV light. Plasmids from *Escherichia coli* V517 and 39R861 were used as molecular weight size markers [21, 22].

Restriction enzyme digestion of plasmids

Plasmid DNA for RFLP studies was extracted by the method of Olsen [23]. Bacterial cultures were propagated in Marine broth as described above whereafter

1·0–1·5 ml broth culture was centrifuged and the pellet resuspended in 40 μl TE buffer 50:10 (tris 50 mM, EDTA 10 mM) pH 8·0. After the addition of 400 μl lysis buffer (tris 50 mM, SDS 3%, pH adjusted to 12·52–12·54 with fresh 2 M NaOH), lysates were incubated at 56 °C for 30 min. Then 300 μl potassium acetate, 1·5 M, pH 5·2, were added and the mixture incubated at 5 °C for 30 min. After centrifugation at 13000 rpm for 10 min, the supernatant was filtered through sterile gauze into a new microfuge tube, 600 μl isopropanol was added and the tube incubated at $-20\,°C$ for 30 min. The mixture was centrifuged at 13000 rpm for 10 min, whereafter, the pellet was washed in 70% (vol/vol) ethanol and resuspended in TE 10:1, pH 8·0. The quality of the plasmid preparation was checked by electrophoresis and staining as described above. Plasmid DNA was digested with *Hin*d III and *Bam*H I according to the instructions of the manufacturer (Promega Corp. Madison, WI, USA). Fragments were separated by electrophoresis in 1·0% agarose gels in TAE buffer, stained with ethidium bromide or SYBR Green I DNA Gel Stain (FMC BioProducts), and photographed. *Hin*d III digested λ phage DNA (Boehringer, Mannheim, Germany) was used as a molecular weight marker.

Ribotyping

Ribotyping was carried out using the method described by Austin and colleagues [24] and Grimont and Grimont [25]. Briefly, chromosomal DNA was extracted using an AutoGen 540 (AutoGen Instruments, Bioclear, Potterne, UK) automate, and 2–5 μg DNA were digested with *Mlu*I (Pharmacia, Saint-Quentin-Yvelines, France). Fragments were separated by agarose gel electrophoresis in TBE buffer (tris 89 mM, boric acid 89 mM, EDTA 2 mM, pH 8·3) and blotted onto nylon membranes (Hybond-N, Amersham International, Amersham, England) using a VacuGene System (Pharmacia). After transfer, DNA was fixed to the membranes by baking at 80 °C for 15 min and hybridized with an acetylaminofluorene (AAF)-labelled 16S and 23S rRNA probe from *E. coli* (Eurogentec, Seraing, Belgium) as described by Grimont and colleagues [26]. Membranes were then incubated with mouse anti-AAF monoclonal antibody followed by alkaline phosphatase labelled anti-mouse IgG whereafter hybridized fragments were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma) as substrates. Digitalization and interpretation of ribotypes was

Table 1. *Vibrio ordalii strains used in the investigation*

Reference number	Collection/strain number	Country	Fish species
VIB 172	241-S	USA	Salmon
VIB 173	DF$_3$K	USA	Salmon
VIB 174	7448	Canada	Salmon
VIB 307	LMG 13544	USA	Salmon
VIB 449	Eaves F378	Australia	Salmon
VIB 450	Eaves F379	Australia	Salmon
VIB 451	Eaves F380	Australia	Salmon
VIB 452	Eaves F381	Australia	Salmon
VIB 453	LMG 10951	Australia	Salmon
VIB 678	Muroga RF-2	Japan	Rainbow trout
VIB 679	Muroga PT-81025	Japan	Ayu
VIB 680	SsH-8301	Japan	Rockfish
VIB 714	Tajima V-11	Japan	Rainbow trout
VIB 715a	Tajima V-91a	Japan	Amago trout
VIB 715b	Tajima V-91b	Japan	Amago trout
VIB 716a	Tajima V-249a	Japan	Rainbow trout
VIB 716b	Tajima V-249b	Japan	Rainbow trout
VIB 717a	Tajima V-250a	Japan	Rainbow trout
VIB 717b	Tajima V-250b	Japan	Rainbow trout
VIB 733	Tajima V-297	Japan	Rainbow trout
VIB 734	Tajima V-298	Japan	Rainbow trout
VIB 735a	Tajima V-299a	Japan	Rainbow trout
VIB 735b	Tajima V-299b	Japan	Rainbow trout
VIB 736	Tajima V-300	Japan	Rainbow trout
VIB 737	Tajima V-302	Japan	Rainbow trout
VIB 738a	Tajima V-303a	Japan	Rainbow trout
VIB 738b	Tajima V-303b	Japan	Rainbow trout
VIB 739	Tajima V-306	Japan	Amago trout
VIB 740	Tajima V-307	Japan	Rainbow trout
VIB 741	Tajima V-308	Japan	Rainbow trout
V-331	Tajima V-331	Japan	Unknown
V-333	Tajima V-333	Japan	Unknown

done using programs of the Taxotron® package (Taxolab, Institute Pasteur, Paris, France). The membranes were scanned using One-Scanner (Apple Computers, Cupertino, CA, USA) and the resulting TIFF images were searched for lanes and bands by RestrictoScan®. Fragment sizes were interpreted from migration data by RestrictoTyper®, with error setting chosen as 3·5–5·0% (allowed variation in fragment length in the 1–20 kb range). The complement of the Dice coefficient (distance) and single linkage [27] were used by Adanson® and Dendrograf® to produce a dendrogram. Discriminatory index was calculated according to Hunter and Gaston [28].

RESULTS

All *V. ordalii* strains reacted serologically with absorbed O2a but not with absorbed O2b antiserum.

Additionally, all isolates contained a plasmid of 30–32 kb (Fig. 1). In most strains, a second, very faint band was frequently, but not always, detected very close to the chromosomal band. This band corresponded to a size of approximately 27–29 kb.

One restriction site for *Bam*H I was detected in the 30–32 kb plasmid (Fig. 1). *Hind* III cleaved the plasmid into 11 visible fragments (Fig. 2) and some variation in the restriction patterns was noticed. Three different patterns were detected and designated profile A, B and C. All North American strains and all Japanese strains, except one, belonged to profile A whereas all isolates from Australia showed profile B. A single isolate from Japan had profile C.

Ribotyping using *Mlu* I yielded six different patterns (Fig. 3). Strains from North America shared ribotypes with strains from Australia and Japan. Patterns 4 and 6 were each represented by 1 Japanese strain. All
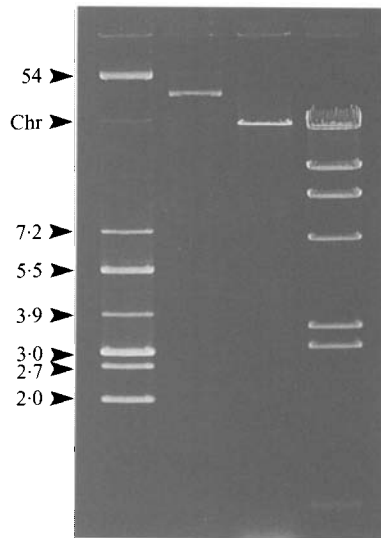
Fig. 1. Plasmid profile of *Vibrio ordalii*. Lane 1, Plasmid molecular weight reference molecules from *E. coli* V517; lane 2, 32 kb plasmid from *V. ordalii* DF₃K; lane 3, 32 kb plasmid from *V. ordalii* DF$_3$K, digested with *Bam*H I; lane 4, molecular weight reference molecules, *Hin*d III digested λ phage DNA. Chr: chromosomal band. Molecular weights of the *E. coli* V517 plasmids and the λ DNA fragments are shown to the left and to the right, respectively.
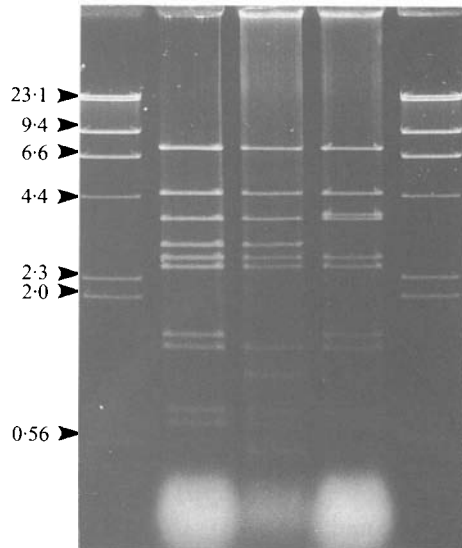

Fig. 2. Restriction fragment length polymorphism of the 30–32 kb plasmid of *V. ordalii*, digested with *Hin*d III. Lanes 1 and 5, molecular weight reference molecules, *Hin*d III digested λ phage DNA (molecular weights are indicated to the left); lane 2, profile A (strain VI B 678); lane 3, profile B (strain VI B 451); lane 4, profile C (strain VI B 716b).

patterns were characterized by 8 fragments (except for pattern 4 and 6 which had only 7) between 18 and 8 kb, 4–6 fragments between 6 and 3·5 kb, and one

fragment around 1·6 kb. Patterns 1–4 additionally had a fragment of 1·3 kb. Strains from North America were subdivided into 3 ribotypes (pattern 2, 3 and 5). Australian strains corresponded to ribotype 3 which was shared by two American strains but not with any Japanese strains. The Australian isolates had identical plasmid RFLP profiles. The 23 strains from Japan were subdivided into 5 ribotypes and 2 plasmid RFLP profiles (Fig. 3). They shared ribotype 2 and 5 with some American strains but no relationship was apparent with Australian strains. Ribotypes 1, 4 and 6 were found only among Japanese strains.

DISCUSSION

In the present study, all of 32 *V. ordalii* strains were found to carry one plasmid, approximately 30–32 kb, equivalent to the pMJ101 plasmid of the type strain, *V. ordalii* DF₃K [1]. This is in accordance with previous reports [1, 3, 5, 8, 12].

The pJM1-like plasmid of *V. anguillarum* serogroup O1 has been shown to encode an iron-sequestering system consisting of an outer membrane protein, OM2, and a siderophore, and has been shown to be important for the virulence of this organism [29–32]. In contrast, no phenotypic characters correlate with the presence of the pMJ101-like plasmid. So far, this plasmid is cryptic. Recently, it was discovered that within *V. anguillarum* serogroup O1, distinct populations could be recognized on the basis of ribotype and plasmid profile. Strains isolated from the kidney of dead fish possessed the pJM1-like plasmid and distinct ribotypes whereas environmental strains did not have this plasmid and belonged to quite different ribotypes [33]. Similar studies have not been carried out on *V. ordalii*; so far no strains lacking the pMJ101 plasmid have been isolated.

Although *V. ordalii* cross-reacts sereologically with *V. anguillarum* serogroup O2, pMJ101-like plasmids have never been detected among *V. anguillarum* serogroup O2 isolates.

In addition to the 30–32 kb CCC plasmid band, a smaller DNA band of approximately 27–29 kb was detected in some but not all strains. At present we do not know whether this band represents a second plasmid or an artefact. The band had a position very close to the chromosomal band upon gel electrophoresis but was clearly distinct from this. However, its position was very close to that of the *Bam*H I digested pMJ101-like plasmid, and thus may represent
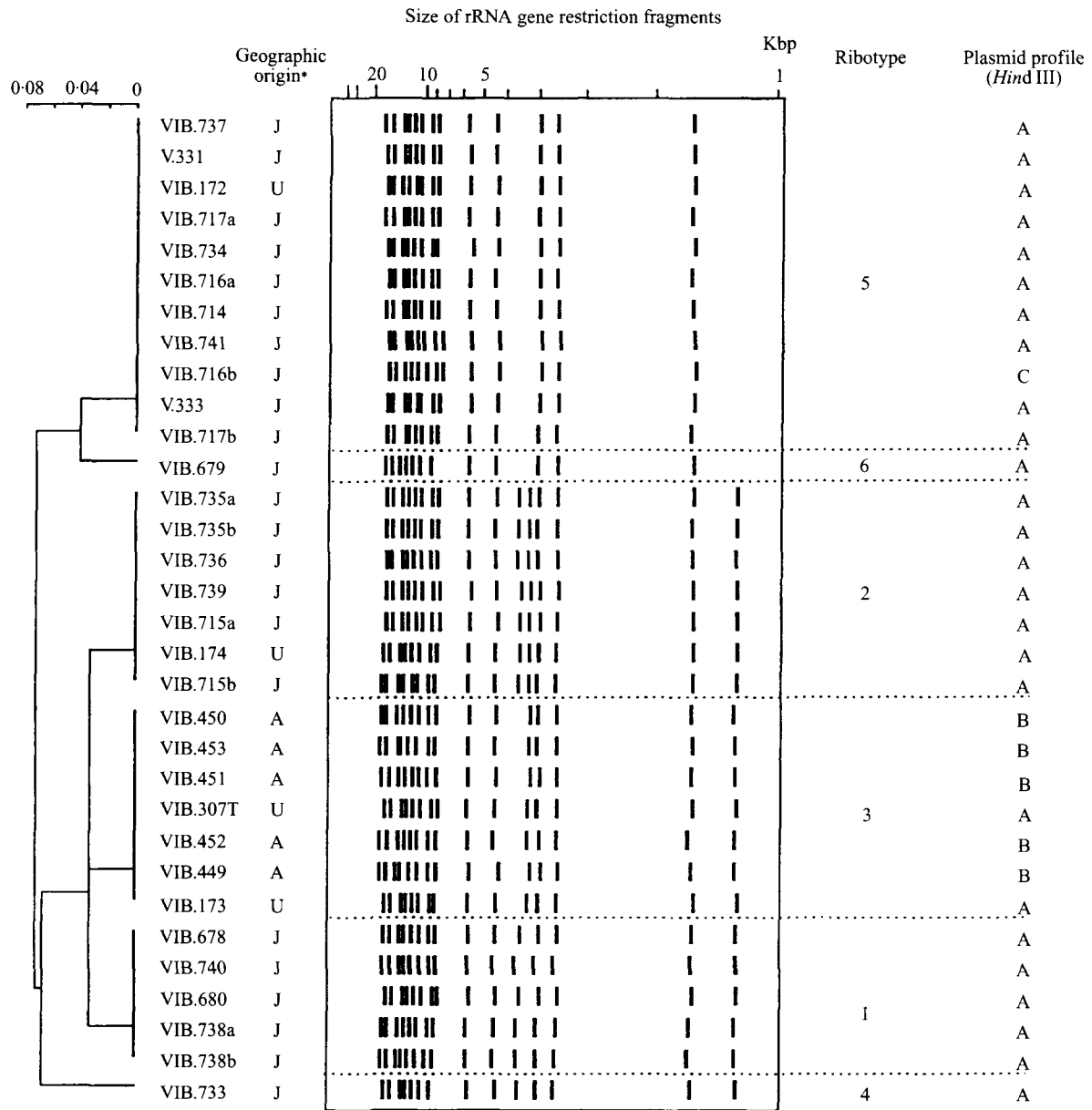
Size of rRNA gene restriction fragments

	Strain	Geographic origin*	rRNA gene restriction fragments	Ribotype	Plasmid profile (*Hind* III)
	VIB.737	J			A
	V.331	J			A
	VIB.172	U			A
	VIB.717a	J			A
	VIB.734	J			A
	VIB.716a	J		5	A
	VIB.714	J			A
	VIB.741	J			A
	VIB.716b	J			C
	V.333	J			A
	VIB.717b	J			A
	VIB.679	J		6	A
	VIB.735a	J			A
	VIB.735b	J			A
	VIB.736	J			A
	VIB.739	J		2	A
	VIB.715a	J			A
	VIB.174	U			A
	VIB.715b	J			A
	VIB.450	A			B
	VIB.453	A			B
	VIB.451	A			B
	VIB.307T	U		3	A
	VIB.452	A			B
	VIB.449	A			B
	VIB.173	U			A
	VIB.678	J			A
	VIB.740	J			A
	VIB.680	J		1	A
	VIB.738a	J			A
	VIB.738b	J			A
	VIB.733	J		4	A

Dendrogram scale: 0·08 0·04 0. Fragment size scale (Kbp): 20 10 5 ... 1.

Fig. 3. Schematic graph showing migration patterns of rRNA gene restriction fragments of DNA from *Vibrio ordalii* strains from North America, Australia, and Japan after *Mlu*I cleavage. Restriction profiles of the pMJ101-like plasmid after cleavage with *Hind* III are indicated to the right. Similarity between strains is represented by a dendrogram to the left indicating at the top the distance between strains. *, A, Australia; J, Japan; U, USA and Canada.

a linearized plasmid, produced during the process of isolation.

Likewise, a more diffuse band with a molecular weight higher than 32 kb was often present (Fig. 1), consistent with open circular plasmid DNA. This is in agreement with previous results that described the presence of a single plasmid in *V. ordalii* strain DF$_3$K [1, 5, 8].

Schiewe and Crosa [1] and Wards and colleagues [3] digested purified plasmid DNA from one *V. ordalii* strain. Both groups reported that *Bam*H I had a single restriction site whereas *Hind* III yielded several fragments. These results are in accordance with those from the present study. However, none of these groups studied any differences in cleavage patterns among isolates of the pMJ101-like plasmid from various geographical regions.

V. anguillarum serogroup O1 is a very homogeneous group of bacteria, and shares most biochemical and immunological properties. To distinguish between strains, molecular biological methods have been introduced, such as ribotyping [18], pulsed-field gel

electrophoresis [34], and restriction fragment length polymorphism studies of the pJM1-like plasmid [14]. *V. ordalii* is also a very homogeneous and highly conserved organism. However, in the present study we showed that, like the pJM1-like plasmid of *V. anguillarum* serogroup O1, the pMJ101-like plasmid of *V. ordalii* showed some restriction fragment length polymorphism. A total of three RFLP patterns were detected. It is interesting that all North American and most Japanese isolates shared the same profile whereas all Australian strains shared a separate profile. This indicates that the plasmids of the North American and the Japanese isolates are more closely related to each other than to the Australian strains. However, this was not entirely supported by the results of the ribotyping: The five strains from Australia shared ribotype with two American strains although they had different plasmid RFLP profiles. Remaining American strains shared ribotypes with some of the Japanese isolates. Plasmid RFLP profile C was represented by only a single isolate and may represent incidental mutations of profile A. Nevertheless, the identification of only three cleavage patterns indicates a remarkable stability of this plasmid and confirms the homogeneity of *V. ordalii*.

Using the single linkage clustering, only one tree is possible to generate from the data. The present dendrogram (Fig. 3) has little phylogenetic pretention but mainly serves to emphasize the high similarity of *V. ordalii*. In addition, together with the patterns of each strain, the dendrogram serves to give an objective indication of pattern identity, i.e. it tells which patterns are regarded as identical. On the basis of ribotyping, the overall banding pattern similarity among the 32 strains was 92% (distance = 0·08) (Fig. 3), pointing out the great homology in this species. The discriminatory index was 0·82, confirming this characteristic. Ribotyping had a higher discriminatory power than plasmid RFLP profiling (discriminatory index 0·33). Plasmid RFLP profiles did not – in contrast to ribotyping – indicate any relationship between Australian and American strains. North American strains (4 strains, 3 ribotypes, 1 plasmid RFLP profile) and Japanese strains (23 strains, 5 ribotypes, 2 plasmid RFLP profiles) seemed to represent more diversity than did Australian strains (4 strains, 1 ribotype, 1 plasmid RFLP profile). These results suggest that a combination of ribotyping and plasmid RFLP analysis may be preferable for clonal studies of *V. ordalii*.

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