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Non-O1 *Vibrio cholerae* NRT36S Produces a Polysaccharide Capsule That Determines Colony Morphology, Serum Resistance, and Virulence in Mice

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Non-O1 *Vibrio cholerae* produced two distinct colony types, designated as opaque and translucent. NRT36S, a clinical isolate shown to be virulent in volunteers, produced predominantly opaque colonies, but translucent colonies appeared on subculture. Opaque variants were recovered exclusively following exposure to normal human serum or animal passage. A nonreverting translucent mutant of NRT36S, JVB52, was isolated following mutagenesis with the transposon Tn*5* IS*50*$_L$::*phoA* (Tn*phoA*). Only translucent colonies were produced by a nonpathogenic environmental isolate, A5. Electron microscopic examination of the opaque form of NRT36S revealed thick, electron-dense, fibrous capsules surrounding polycationic ferritin-stained cells. The ferritin-stained material around translucent NRT36S or A5 was patchy or absent. JVB52 had a thin but contiguous capsular layer. The amount of ferritin-stained capsular material correlated with the amount of surface polysaccharide determined by phenol-sulfuric acid assay: opaque NRT36S had approximately three times as much polysaccharide as translucent NRT36S or A5 and four times as much as JVB52. The encapsulated, opaque variant of NRT36S was protected from serum bactericidal activity, while translucent non-O1 *V. cholerae* was readily killed. The encapsulated form also had increased virulence in mice. Our data provide the first indication that non-O1 *V. cholerae* strains can have a polysaccharide capsule. This capsule may be important in protecting the organism from host defenses and may contribute to the ability of some non-O1 *V. cholerae* strains to cause septicemia in susceptible hosts.

Vibrio cholerae of serogroup O1 is well known as the causative agent of cholera. It has become increasingly apparent in recent years that *V. cholerae* of other O groups (non-O1 *V. cholerae*) can also cause human disease. Non-O1 *V. cholerae* have been isolated from patients with diarrhea throughout the world (1, 3, 12, 16, 20, 26). Unlike O1 *V. cholerae*, non-O1 strains are also associated with ear infections, wound infections, and septicemia. Septicemia is most common in patients with underlying liver or immune system disorders and has a fatality rate exceeding 50% (4, 5, 14, 23). We currently do not have a good understanding of what factors are involved in virulence of non-O1 *V. cholerae* strains, particularly those factors that allow non-O1 but not O1 strains to cause septicemia.

Changes in colony morphology, such as changes in colony opacity, are often associated with changes in virulence (7, 35, 37, 38, 41, 43). The change may occur in only one direction, with permanent loss of virulence (9, 10, 27), or phase variation between two colony morphologies may occur as virulence factors are turned on and off (28, 33, 37, 38, 41). One such virulence factor is a polysaccharide capsule (28, 33, 35, 37, 43).

Complement-mediated bactericidal activity is an important defense against gram-negative sepsis (31). Many organisms capable of systemic infection possess a capsule which increases their resistance to complement-mediated killing (9, 10, 27). The role of capsule in the development of disseminated disease has clearly been demonstrated in *V. vulnificus*, an organism closely related to *V. cholerae*, which also causes wound infections and septicemia (8, 18, 24). In this report, we present evidence that non-O1 *V. cholerae* NRT36S produces a polysaccharide capsule that may play a role in virulence.

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MATERIALS AND METHODS

Non-O1 *V. cholerae* strains and culture conditions. NRT36S, a serotype O31 strain, was isolated from an adult with traveler's diarrhea at Narita Airport, Tokyo (25). A5, serotype O31, was isolated from frozen shrimp in Japan (2). Both strains have been administered to volunteers (25). NRT36S caused diarrhea ranging in severity from mild to severe: one volunteer ingesting 10^6 CFU produced more than 5 liters of diarrheal stool, comparable to the diarrheal purge seen in patients infected with O1 *V. cholerae*. NRT36S had two colony types, a creamy opaque type and a glassy translucent type. The opaque variant was designated NRT36S/O, and the translucent variant was designated NRT36S/T. A5 neither colonized nor caused disease in volunteers.

Cultures were maintained in L broth with 15% glycerol at −70°C. Frozen stocks were streaked for isolation and incubated overnight at 37°C on L agar (21) plates.

Transposon mutagenesis. JVB52, a translucent mutant of NRT36S/O that did not revert to the opaque morphology at detectable levels, was obtained by mutagenesis with Tn*5* IS*50*$_L$::*phoA* (Tn*phoA*). Tn*phoA* contains a gene for alkaline phosphatase which lacks the promoter and signal sequences.

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In-frame fusion of this gene to a target gene encoding a secreted protein results in an active alkaline phosphatase enzyme which is detected by the production of blue color on L agar plates containing 5-bromo-4-chloro-3-indolyl phosphate (XP) (22). Tn*phoA* was introduced into NRT36S/O by conjugation with *Escherichia coli* Sm10λ*pir* containing the suicide vector pRT733 (40), with selection on L agar containing polymyxin B (50 U/ml), kanamycin (50 μg/ml), glucose (0.2%), and XP (40 μg/ml). Translucent transconjugants were examined for reversion following exposure to 65% normal human serum (NHS) or by intraperitoneal injection of mice. Tn*phoA* insertion was confirmed by Southern blot analysis (21).

Rates of conversion. To determine the rate of conversion from opaque to translucent, we inoculated an isolated opaque colony of NRT36S into 5 ml of L broth. After overnight incubation with shaking at 37°C, the culture was diluted with sterile Dulbecco's phosphate-buffered saline (PBS) and plated on L agar incubated at 37°C. Because serum is bactericidal for translucent strains, the rate of conversion of translucent to opaque was determined by mixing approximately 10^7 bacteria from an overnight culture with 65% NHS, incubating at 37°C for 30 min, and plating on L agar.

Animal passage. Six- to eight-week-old outbred CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were injected intraperitoneally with 10^7 CFU of each strain in 0.5 ml of sterile PBS. At death, heart blood and peritoneal cavity swabs were collected and cultured on L agar. Typical isolates from positive cultures were subcultured on thiosulfate citrate bile sucrose agar (Difco Laboratories, Detroit, Mich.). Identification of putative *V. cholerae* isolates was confirmed by API 20E. Colony morphology was evaluated on L agar.

Electron microscopy. Bacteria were grown on L agar overnight at 37°C, harvested, and washed once with cacodylate buffer (0.1 M, pH 0.7). Bacterial cells were fixed with 5% glutaraldehyde in cacodylate buffer for 2 h at room temperature. Fixed bacteria were washed and resuspended in cacodylate buffer containing 1 mg of polycationic ferritin (Sigma Chemical Co., St. Louis, Mo.) per ml. After 30 min at room temperature, samples were diluted 1:10 with buffer. The ferritin-labeled organisms were washed three times, pelleted, and immobilized with 4% agar (17). Postfixation was done over 2 h with 2% osmium tetroxide and then three washes. Samples were dehydrated in a graded series (30 to 100%) of ethanol solutions, washed twice with propylene oxide, and embedded in Epon by a rapid embedding technique (29). Thin sections were cut and placed on 300-mesh Formvar-carbon grids, stained with uranyl acetate and lead citrate, and examined under a JEOL EX transmission electron microscope operating at an accelerating voltage of 60 kV.

To determine the presence or absence of pili, bacteria were grown in L broth, washed with PBS, stained with phosphotungstic acid, and examined as above.

Capsule purification. For the determination of the amount of capsular polysaccharide (CPS) for each strain, bacteria were grown on L agar in 150-mm petri dishes. Cells were harvested, suspended in 10 ml of 0.5 × PBS in 40-ml polypropylene centrifuge tubes, and shaken at 200 rpm on a rotary shaker for 2 h at room temperature. Serial dilutions of the bacterial suspensions were spread for plate counts. The bacterial suspensions were centrifuged to remove cell debris (16,000 × *g*, 20 min, 4°C), and the supernatant was dialyzed with multiple changes of distilled water. The samples were ultracentrifuged (154,000 × *g*, 2 h, 20°C), and the supernatant was retained and digested with RNase A (100 μg/ml) and DNase I (50 μg/ml plus 1 mM $MgCl_2$) (17). The concentration of CPS was determined by phenol-sulfuric acid assay (11). A_{490} was compared to a standard curve prepared with galactose. Concentrations were determined per 10^8 cells, and at least three samples were averaged. A larger sample of CPS from NRT36S/O was prepared and further purified by digestion with pronase (250 μg/ml) and then sequential phenol-chloroform extraction. Purity was assessed by wavelength scanning spectrophotometric analysis (Gilford Instrument Laboratories, Inc., Oberlin, Ohio), bicinchoninic acid protein assay (MicroBCA; Pierce Chemical Co., Rockford, Ill.) (36), and *Limulus* amoebocyte lysate assay (Sigma Chemical Co., St. Louis, Mo.).

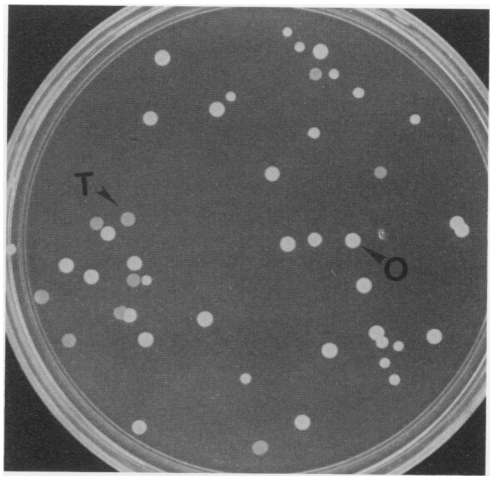

FIG. 1. Opaque (O) and translucent (T) colonies of NRT36S.

Complement resistance. Human serum was pooled from four or more donors, mixed with 30 μl of guinea pig complement (Whittaker M. A. Bioproducts, Walkersville, Md.) per ml, and stored in aliquots at −70°C. A 0.35-ml aliquot of bacteria, containing approximately 10^7 CFU in PBS, was mixed with 0.65 ml of freshly thawed serum and incubated at 37°C; control aliquots were mixed with serum that had been heated to 56°C for 30 min. The number of viable cells was determined by plate counts at 30 min. A drop in CFU of 1 $\log_{10}$ or greater in the unheated samples but not in the heated samples was considered meaningful (8, 39). An encapsulated *V. vulnificus* strain, MO6-24/O, and *V. cholerae* 395, a serogroup O1 Ogawa strain, served as controls.

LD_{50} in mice. Six- to eight-week-old outbred CD-1 mice (Charles River) were shaved over the right thigh and injected intradermally with 0.1 ml of serial dilutions in PBS of each strain (34). Five mice were used per group. The 50% lethal dose (LD_{50}) for each strain was calculated by the method of Reed and Muench (30).

RESULTS

Colony morphology. Two distinct colony morphologies were seen with non-O1 *V. cholerae* strains. NRT36S formed both creamy, smooth, opaque colonies and glassy, translucent colonies (Fig. 1). The environmental isolate, A5, formed only translucent colonies.

Transposon mutagenesis. A translucent mutant of NRT 36S/O that did not revert to opaque under any conditions

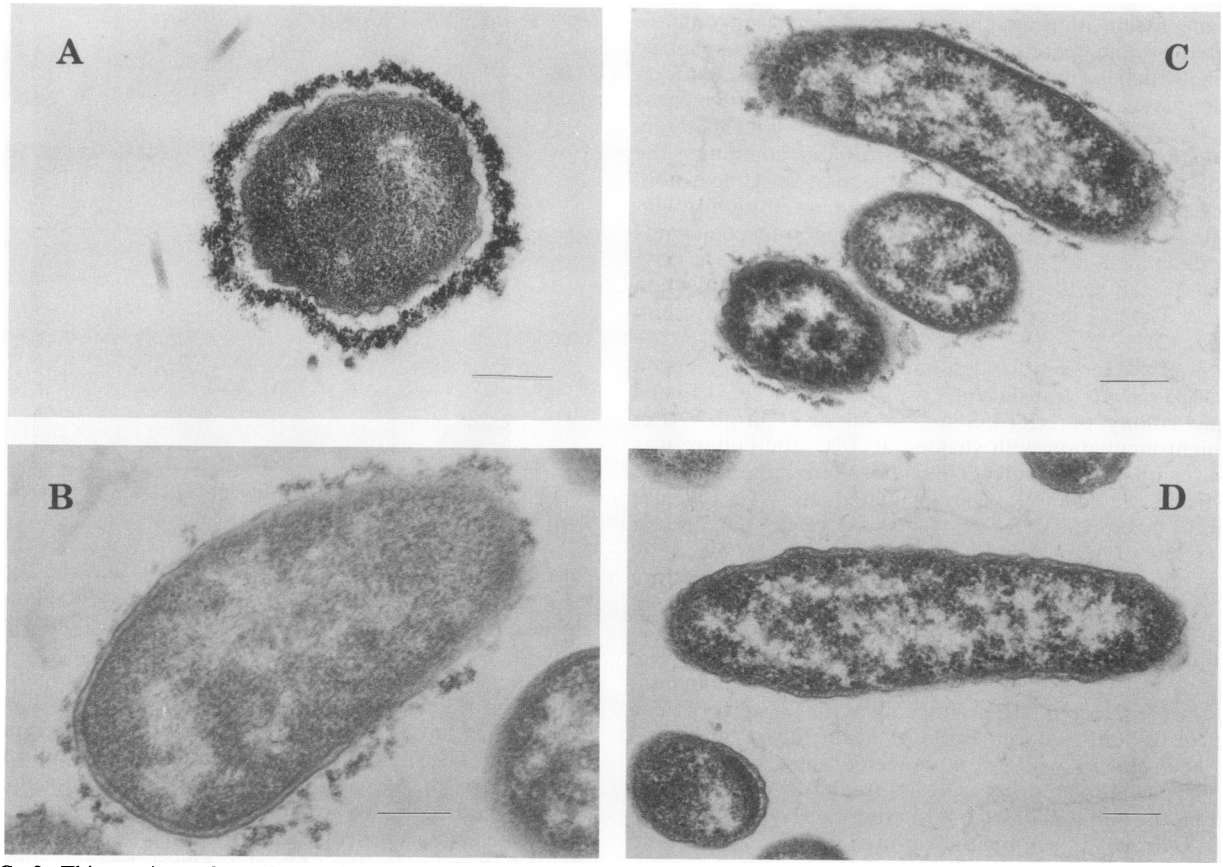

FIG. 2. Thin sections of non-O1 *V. cholerae* stained with polycationic ferritin. (A) NRT36S/O; (B) NRT36S/T; (C) JVB52; (D) A5. Bar, 200 nm.

tested was isolated following mutagenesis with pRT733, a suicide vector carrying Tn*phoA*. A second transposon mutant, JVB23, an opaque strain with a cryptic Tn*phoA* insertion, was selected as a control. Neither JVB52 nor JVB23 had alkaline phosphatase activity, but a chromosomal Tn*phoA* insertion in both strains was confirmed by Southern blot analysis (21) (data not shown).

Phase variation. NRT36S underwent phase variation, converting from opaque to translucent and back again at a low rate. Opaque colonies of NRT36S (NRT36S/O) inoculated into L broth and subcultured on L agar produced translucent colonies (NRT36S/T) at a frequency of 1.2×10^{-5}. Conversion of translucent to opaque variants occurred at a rate of 1.9×10^{-6} in serum. No opaque variants of A5 or JVB52 were seen in $>10^9$ colonies plated on L agar after exposure to serum.

Animal passage. Colonies isolated from mice inoculated with NRT36S/O or JVB23 were opaque. Colonies recovered from the blood or peritoneum of animals given NRT36S/T were also opaque, indicating that the opaque phenotype was induced or selected for in vivo. *V. cholerae* was recovered from the peritoneal cavities but not the blood of mice given A5 or JVB52; all colonies were translucent. Both the translucent and kanamycin-resistant phenotypes of JVB52 were retained following mouse passage.

Electron microscopy. To determine the nature of the colony opacity differences, we evaluated thin sections of bacteria stained with polycationic ferritin by electron microscopy. Representative profiles are shown in Fig. 2. NRT36S/O showed a heavy, fibrous, electron-dense layer completely surrounding the cells. NRT36S/T and JVB52 cells had much thinner, patchy, ferritin-stained layers. A5 cells all lacked a complete capsule, but occasional aggregated patches of ferritin were seen in the vicinity of the cells.

Electron microscopic examination of phosphotungstic acid-stained cells showed that all strains had flagella but no pili (data not shown).

Capsule extraction. The degree of encapsulation seen in thin sections correlated with the amount of CPS isolated from cells, as determined by phenol-sulfuric acid assay (Table 1). CPS solutions had a peak absorbance at 490 nm, which is typical of hexoses, methyl hexoses, and polysaccharides. NRT36S/O had approximately three times as much PBS-extractable polysaccharide as NRT36S/T or A5 and four times as much CPS as JVB52.

Spectrophotometric scanning of purified CPS from NRT36S/O showed a broad peak centered at 190 nm (data not shown). The lack of absorbance at 260 or 280 nm indicated an absence of contaminating protein or nucleic acids. Protein was undetectable (<2 μg/ml in a 10-μg/ml CPS sample) by MicroBCA assay. Lipopolysaccharide detected by the *Limulus* amoebocyte lysate assay was present at a trace level of 0.0003%.

Complement resistance. Translucent strains of non-O1 *V. cholerae* were much more sensitive to the bactericidal activity of NHS than the opaque strains, showing a drop in titer more than 2 logs greater than that seen with NRT36S/O (Table 1). A5 and JVB52 were even more sensitive to serum

TABLE 1. Comparison of the amount of CPS, bactericidal effect of NHS, and mouse lethality for different strains of *V. cholerae* non-O1

Strain	ng of saccharide/10^8 cells ± SE[a]	Bactericidal activity of 65% NHS[b]	Mouse LD_{50}[c]
V. cholerae non-O1			
NRT36S/O	13.1 ± 2.5	0.74	7.0×10^7
NRT36S/T	4.2 ± 5.1	2.98	2.5×10^9
JVB23	12.9 ± 3.2	0.42	6.3×10^7
JVB52	2.6 ± 2.9	4.56	$>1.4 \times 10^{10}$
A5	4.9 ± 4.5	3.95	1.8×10^9
V. cholerae O1 395	ND[d]	4.88	ND
V. vulnificus MO6/O	ND	0.23	ND

[a] Determined by phenol-sulfuric acid assay of small-scale extractions. Results are averages of at least three samples.
[b] Data are reported as $\log_{10}$ decline in CFU during a 30-min incubation.
[c] Mice were injected intradermally.
[d] ND, not determined.

than NRT36S/T. Only opaque colonies were recovered from serum incubated with NRT36S/T. Occasional translucent colonies were recovered from assays with A5. No colonies were recovered from assays with JVB52.

Mouse lethality. The colony morphology also correlated with mouse lethality. The LD_{50} of NRT36S/O was 1.6 logs lower than that of NRT36S/T and at least 2.2 logs lower than that of JVB52 (Table 1).

DISCUSSION

Our data clearly indicate that at least one strain of *V. cholerae* non-O1 produces a polysaccharide capsule that confers an opaque colony morphology, resistance to serum killing, and increased mouse lethality. This is the first report of capsule production by *V. cholerae*.

Changes in colony opacity may be due to changes in the expression of pili (7, 33, 38), outer membrane proteins (33, 38), or capsule (28, 35, 37, 38, 43). Our data indicate that differences in the opacity of non-O1 *V. cholerae* NRT36S and A5 colonies are dependent on the amount of CPS surrounding the cells (shown by electron microscopy analysis and phenol-sulfuric acid assay). No differences in piliation were seen between translucent and opaque variants of the same strain.

As is common in bacteria (6, 17, 27, 41), the capsule of NRT36S/O is primarily polysaccharide in nature. The staining of the extracellular fibrous layer by polycationic ferritin strongly suggests the presence of acidic polysaccharide (18). The reaction of extracted capsular material with phenol and sulfuric acid confirms the presence of sugars and suggests that these sugars are hexoses and methyl hexoses (11).

Many encapsulated pathogenic bacteria produce, at a low frequency, acapsular variants with reduced virulence. This is most often due to a mutational block in capsule synthesis, and reversion to the encapsulated form is rare (6, 9, 27). *V. vulnificus* switches from opaque to translucent at a frequency of about 10^{-4}. Several reports suggest that this organism does not revert from translucent to opaque (35, 43), but Wright et al. (42) found a reversion rate of 9.2×10^{-3} for strain MO6-24. This rate is similar to the rate seen with phase variation in *Bordetella pertussis* (10^{-3} to 10^{-6} depending on the strain) (33) or *Citrobacter freundii* (2×10^{-4} to 7×10^{-3}) (28, 37). Phase variation in *B. pertussis* involves expression of filamentous hemagglutination, pertussis toxin, adenylate cyclase toxin, dermonecrotic toxin, hemolysin, and several outer membrane proteins in addition to capsule and is controlled by the *trans*-acting *vir* gene (41). *C. freundii* phase variation involves Vi capsular antigen expression and is controlled by insertion and deletion of an IS element (28, 37).

The rate of switching of NRT36S in L broth does not rule out a mechanism for colony morphology variation involving either simple mutation or a control mechanism such as seen with the *B. pertussis* or *C. freudii* systems. The reversibility of the phase variation does suggest that the mechanism does not involve extensive loss of genetic material, as would be the case if switching was due to loss of a prophage or a plasmid carrying the structural genes for capsule synthesis. Opaque variants were recovered exclusively following mouse passage of NRT36S/T. The recovery of the opaque form may be due to improved survival of the encapsulated form; it may also involve induction of capsule formation in vivo.

The absence of opaque variants of A5, even after animal passage or exposure to serum, suggests that this strain has permanently lost the ability to produce a complete capsule. We do not know the frequency of occurrence of encapsulated strains and whether these strains differ only in the amount and distribution of the CPS or whether the chemical structure of the CPS also differs between strains. Differences in CPS structure may alter virulence and may be useful in identifying strains having increased potential for causing severe disease.

Although the organisms are closely related, there are clear differences in the disease spectra of O1 and non-O1 strains. Serogroup O1 *V. cholerae* almost never cause extraintestinal disease. In contrast, close to half of the non-O1 strains submitted to the U.S. Centers for Disease Control are from extraintestinal sites, including blood (21% of isolates), ears (12%), and wounds (7%) (13). Capsule production, and the resultant protection from host defenses, may help to explain the ability of some non-O1 *V. cholerae* to cause systemic infection. As shown in this study and as reported by other investigators (39), *V. cholerae* of serogroup O1 are unencapsulated and exquisitely sensitive to the bactericidal activity of normal human serum. In contrast, we found that an encapsulated non-O1 strain was resistant to serum bactericidal activity. As is the case with *V. vulnificus*, many patients with disseminated *V. cholerae* non-O1 disease have underlying liver disease, frequently cirrhosis (8, 32). Cirrhotic sera often have suboptimal complement activity (15), suggesting that resistance to complement-mediated killing is important for the survival of this organism in the blood of an infected host.

Further support for the role of capsule in pathogenicity was provided by the mouse LD_{50} data. The encapsulated strain, NRT36S/O, had a lower LD_{50} than either NRT36S/T or A5. The animal data are complicated by the isolation of opaque variants of NRT36S from mice injected with the translucent form. To clarify this situation, a translucent mutant of NRT36S, JVB52, was isolated following Tn*phoA* mutagenesis. JVB52 does not appear to be a simple translucent variant of NRT36S, as it did not revert to the opaque phenotype in vitro or in vivo and was more serum sensitive than NRT36S/T. The increase in the LD_{50} compared with that seen with the spontaneous translucent variant and the opaque parent may have been due to the inability of this mutant strain to revert to the opaque, encapsulated form. However, we cannot exclude the possibility that the Tn*phoA*

insertion in JVB52 is in a regulatory gene and that virulence was also affected by changes in other, as yet unidentified, phenotypic characteristics.

Encapsulation is a crucial virulence determinant for a number of bacterial species, providing protection from serum killing and phagocytosis (9, 10, 19, 27). Increased resistance of some non-O1 *V. cholerae* strains to serum killing, provided by a capsule, may allow the spread of this organism from the site of entry. While other factors undoubtedly contribute to virulence, our observation that non-O1 *V. cholerae* NRT36S can assume an encapsulated form may help to explain the virulence of some strains of this organism in susceptible hosts.

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