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NOTES

Protein Microarray for Profiling Antibody Responses to *Yersinia pestis* Live Vaccine

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A protein microarray representing 149 *Yersinia pestis* proteins was developed to profile antibody responses in EV76-immunized rabbits. Antibodies to 50 proteins were detected. There are 11 proteins besides F1 and V antigens to which the predominant antibody response occurred, and these proteins show promise for further evaluation as candidates for subunit vaccines and/or diagnostic antigens.

Plague, one of the most dangerous diseases, is caused by *Yersinia pestis*. The increasing possibility of antibiotic-resistant *Y. pestis* strains means that a vaccine effective against bubonic and pneumonic plague is urgently needed (12, 14, 18). The current interest is in developing plague vaccines that consist of purified protein subunits, with improved protection and reduced side effects (25, 31, 34). The F1 or V single-subunit vaccine and the F1 plus V combination vaccine have been shown to provide effective protection against bubonic and pneumonic plague in animal models (1, 31, 34). There may still exist other *Y. pestis* antigens that provide protection. These novel vaccine candidates in conjunction with F1 and V can be developed as multicomponent subunit vaccines (21). It may improve protection against F1- and/or V-antigen mutant but virulent strains. Therefore, evaluation of *Y. pestis* proteins beside F1 and V for their efficacy in inducing specific antibody in the infected animals or human patients is urgently needed for plague vaccine development.

Genomic sequences of *Y. pestis* CO92 (27), KIM (9), and 91001 (30) have been released in the past 3 years. Decoding of whole-genome sequence provides unprecedented opportunities for vaccine design. The microarray immobilized with multiple antigens, using a simple fluorescence analysis, allows high-throughput parallel detection and quantification of multiple specific antibodies in a miniaturized, low-sample-consumption format. In this study, a microarray representing 149 *Y. pestis* proteins was developed to profile the serum antibodies of rabbits that were immunized with live plague vaccine, providing an overall picture of the immunogenicity of the proteins tested.

Construction of an antigen microarray representing 149 *Y. pestis* proteins. In our present work, 202 genes were selected for cloning and expression (additional information is available at http://bioinflab.org/journals/ruifu/supplementary_TableS1.pdf/). The digested PCR products of specific genes were cloned into expression plasmid vector pET-32a (Novagen), and recombinant plasmids were transformed into BL21(DE3) cells. According to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, 172 genes were successfully expressed in *Escherichia coli*. The expressed proteins were subjected to purification in a 96-well format using Ni-NAT agarose (QIAGEN) according to Brauns' method with a few modifications (5). For quality control, the purified proteins were printed onto silylated glass slides (CEL) and incubated with Cy5-labeled antibody specific for the six-His tag. Only the proteins giving a signal-to-background ratio of ≥3.0 were thought to be acceptable for further analysis (26). After systematic optimization of purification conditions, 149 purified proteins were obtained. Western blotting was further conducted to examine 13 arbitrarily selected proteins, and the results confirmed that each purified protein gave a band with the expected size. The concentration of these 13 proteins was determined using a BCA protein assay reagent kit (Pierce) and fell in the range of 100 to 200 μg/ml. Then, the 149 proteins were printed in triplicate on the slides to fabricate the final version of the microarrays. Rabbit immunoglobulin G (IgG) was printed as a positive control, while cell lysate of *E. coli* BL21 transformed with PET-32a was used as a negative control. The printed slides were deposited at room temperature for at least 1 h and then stored at 4°C.

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Microarray profiling of serum antibody response in the EV76-immunized rabbits. The microarray was used as a tool to screen for the relative amounts of the corresponding antibodies present in the sera of EV76-immunized rabbits. Overnight cultures of the live vaccine EV76 were used for preparation of bacterial suspension with physiological saline. Four rabbits of 2 to 2.5 kg received a primary subcutaneous immunization of 2.5×10^8 EV76 adsorbed to the complete Freund's adjuvant and 2 weeks later a second immunization of the same quantity with the incomplete Freund's adjuvant. On days 29, 36, 43, and 50, each animal received a 1×10^9 booster intravenous dose. Sera were collected before immunization and 1 day before each booster since the second booster. Before being used for microarray profiling, all the sera were incubated with lysate of *E. coli* BL21 carrying pET-32a for 2 h so as to eliminate antibodies against *E. coli* in the tested sera.

Microarrays used for serum profiling were blocked using bovine serum albumin–0.01 mM phosphate-buffered saline (PBS), pH 7.5, for 1 h at room temperature. Then, 200 μl of diluted serum (1:200) was incubated with them for another 1 h. After washing one time with PBS-Tween 20 and two times with PBS, the protein chips were incubated with Cy5 dye-labeled goat antirabbit IgG (1:1,000 dilution) generated by using a Cy5 antibody labeling kit (Amersham Biosciences) for 1 h. Slides were washed as described above and imaged with an Axon 4100A scanner (Axon Instruments). Image and data analysis was performed using the GenePix pro 4.0 software (Axon Instruments). The fluorescence signal of each spot was calculated as the median fluorescence intensity subtracted from the local background median intensity. The spot signals for each protein in three replicated hybridizations were averaged. In order to reduce differences produced in the operation process, we used the fluorescence signal of rabbit IgG as the criterion to normalize the fluorescence signal of each spot. The negative values of spot intensity were set to zero to reflect that local background intensity is equal to spot signal. The normalized data sets were logarithm transformed (base 2) and displayed with TreeView software (10).

Since there was a good correlation between the fluorescence intensity and the IgG concentration captured by the printed antigen according to our previous study (7), which was confirmed in this study (data not shown), the averaged fluorescence intensity is considered as an indicator of the concentration of antibodies. Given that all the proteins were printed in roughly equal amounts, the technology afforded a screening strategy that was relatively unbiased in terms of the effect of protein concentration on sensitivity of detection. Figure 1 gives a schematic representation of the serum antibody profiles induced in the immunized rabbits for the 149 *Y. pestis* proteins on the protein chip. The immunized rabbits made antibody responses to 50 of the 149 proteins, and each antibody titer gave an increment tendency with the development of immunizing times (Fig. 1 and Table 1). However, 37 of these 50 proteins showed cross-reaction with the preimmunized sera. According to the fluorescence intensity-displaying relative concentration of specific antibody (IgG) presented in the immune sera, a group of 12 proteins (labeled with an asterisk in Table 1) to which antibody response appeared to be similar to or stronger than that to the known strongly immunogenic V antigen can be identified. Notwithstanding, our studies show that 12 proteins described in Table 1 are immunodominant when rabbits are immunized with live EV76 and suggest these proteins are expressed during the course of a plague infection.

The remaining proteins tested in our study were unable to evoke antibody signals for several reasons. First, some of these proteins are putative or hypothetical, and actually they may not be expressed by the bacterium. Second, some proteins are not expressed under our culturing and immunizing conditions. Third, the amount of proteins expressed may be too low to induce an immune response. Fourth, the immunogenicity of these proteins might be too weak to contact antigen-presenting cells to raise antibodies, or this breed of rabbit may lack the genetic ability to respond to certain epitopes. Finally, the proteins printed on slides are recombinant and therefore differ in conformation from their naive forms, so the corresponding antibodies cannot efficiently recognize them.

Evaluation of the immunogenicity of plasmid-encoding proteins. *Y. pestis* strains typically carry three virulence plasmids, i.e., pCD1, pPCP1, and pMT1. Seventy plasmid-encoding proteins with presumed virulence-related functions were included in the microarray analysis, and antibody responses to 26 proteins were recorded (Table 1). As show in Fig. 1, antibody response to F1 antigen was the most dramatic in terms of both speed and magnitude of the increment in antibody titers, while V antigen was assigned to a group of 13 proteins with very strong antigenicity (see above). F1 and V, when tested as vaccine antigens, provide a high level of protection against experimental plague (31, 34), especially when used in combination (1, 16). Our results confirm that even in the earlier stages of plague infection, both F1 and V constitute the immunodominant antigens in *Y. pestis*. As a *Y. pestis*-specific, strongly antigenic protein, F1 is the ideal candidate for plague serodiagnosis (29).

Plasmid pCD1 harbors a gene cluster named LCRS (low calcium response stimulon) that encodes a type III secretion system (4). Through the type III secretion system, *Y. pestis* injects the YOP effector proteins into the cytosol of eukaryotic cells when docking at the surface of the host cell, mediating resistance to phagocytosis (8). The antigenicity and protective efficacy of a portion of LCRS components have been tested in previous studies; beside V antigen, only YopD was found to provide partial protection against nonencapsulated *Y. pestis* subcutaneous challenge (2, 3, 21). Twenty-three of the 43 LCRS proteins were included in the microarray analysis. Table 2 shows the comparison of our results and those of the previous studies. Five new proteins (YscB, SycE, YscE, LcrG, and YscL) were found to be immunogenic in the current work. No antibody response to YopN, YscO, YscP, or TyeA was observed in our study, which is totally opposite to the previous results (17, 21). The discrepancy may be due to the fact that we used the live attenuated vaccine but not the purified recombinant proteins or the fully virulent strain for immunization.

Interestingly, a group of proteins that have no homology with any known or hypothetical proteins currently in the databases was found to induce antibody response. Detection of antibody confirms that they are indeed accessible to the humoral response system.

Evaluation of the immunogenicity of chromosomal proteins. A total of 79 chromosomal proteins including the proven virulence factors, putative adhesins/invasins, outer membrane

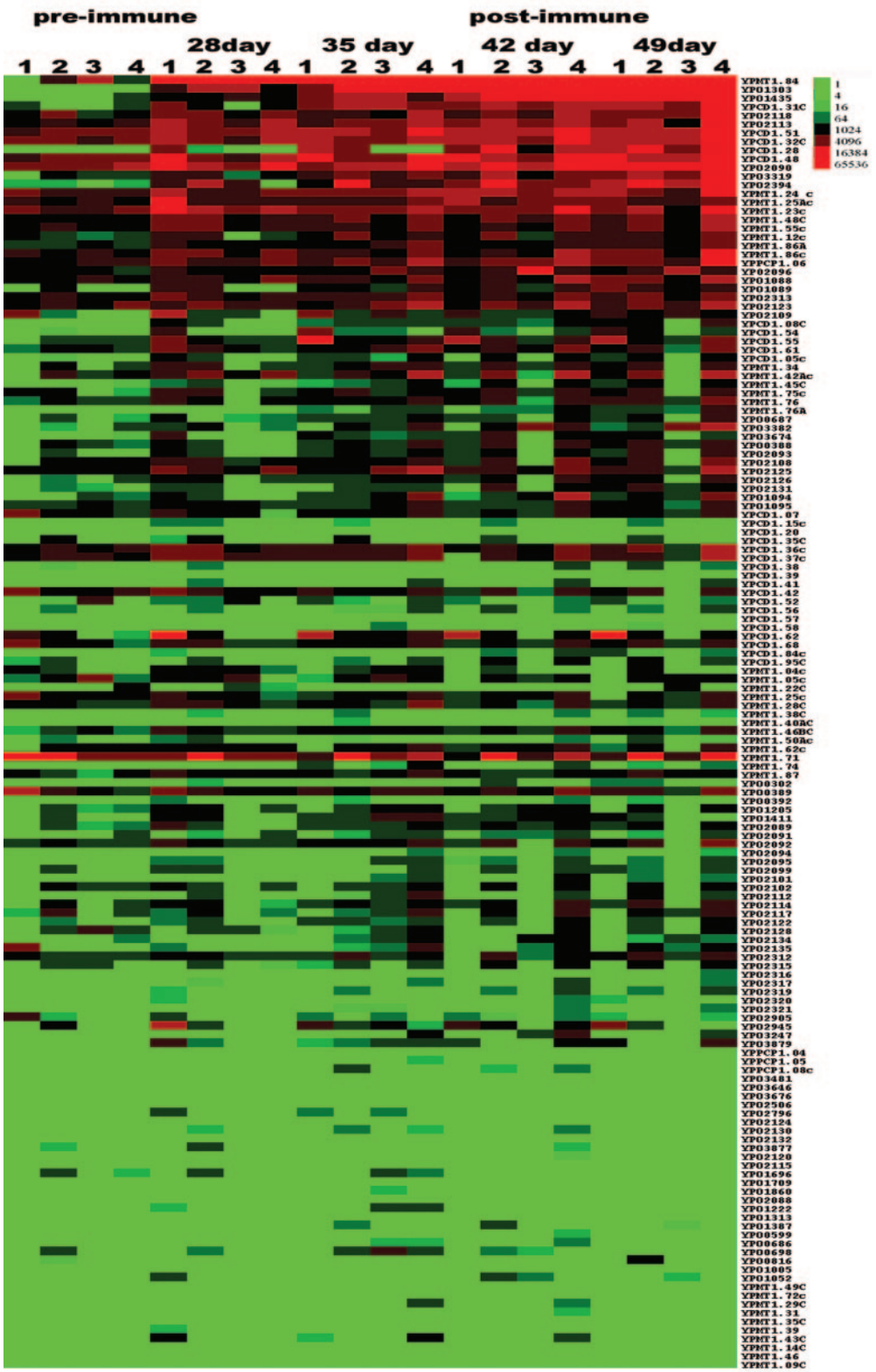

FIG. 1. Profiles of serum antibodies against different proteins. The fluorescence values were normalized, logarithm transformed (base 2), and viewed with TreeView. Genes are listed at right, while the time points of immunization and identification numbers of rabbits tested are listed at the top. The color key shows the normalized absolute fluorescence values. The genes were designated with the CO92 gene definition (e.g., YPO0302).

TABLE 1. Proteins that induced antibody response in EV76-immunized rabbits[a]

Protein characteristic	Gene ID in CO92	Protein	Function	Cross-reaction
Plasmid encoding	YPMT1.84*	Caf1	F1 capsule antigen	Yes
	YPCD1.31c*	LcrV	V antigen	No
	YPCD1.51*	YscB	Type III secretion apparatus component	Yes
	YPCD1.54	YscE	Type III secretion apparatus component	No
	YPCD1.55	YscF	Type III secretion apparatus component	Yes
	YPCD1.61	YscL	Type III secretion apparatus component	Yes
	YPCD1.32c*	LcrG	Yop regulator	Yes
	YPCD1.28*	YopD	Yop negative regulation/targeting component	No
	YPCD1.48*	VirG	Targeting protein of the YscC complex	Yes
	YPCD1.05c	SycE	YopE chaperone	No
	YPMT1.12c		Hypothetical protein	Yes
	YPMT1.23c		Hypothetical protein	Yes
	YPMT1.24c		Hypothetical protein	Yes
	YPMT1.25Ac		Hypothetical protein	Yes
	YPMT1.34		Hypothetical protein	Yes
	YPMT1.42Ac		Hypothetical protein	Yes
	YPMT1.45c		Hypothetical protein	No
	YPMT1.48c		Hypothetical protein	Yes
	YPMT1.55c		Hypothetical protein	Yes
	YPMT1.75c		Hypothetical protein	Yes
	YPMT1.76		Hypothetical protein	Yes
	YPMT1.76A		Hypothetical protein	No
	YPMT1.86A		Hypothetical protein	Yes
	YPMT1.86c		Hypothetical protein	Yes
	YPCD1.08c		Hypothetical protein	No
	YPPCP1.06		Hypothetical protein	Yes
Chromosomal	YPO1303*	PsaA	pH 6 antigen	No
	YPO0687		Putative adherence protein	No
	YPO1435*	OmpA	Outer membrane porin A protein	Yes
	YPO2394*	MlpA	Major outer membrane lipoprotein	No
	YPO3319*	KatY	Catalase-peroxidase	Yes
	YPO3382	GsrA	Global stress requirement protein	Yes
	YPO3674	TccC	Putative insecticidal toxin	No
	YPO0388		Conserved hypothetical protein	Yes
	YPO2090*		Putative phage protein	Yes
	YPO2093		Putative phage protein	Yes
	YPO2096		Hypothetical phage protein	Yes
	YPO2108		Hypothetical phage protein	Yes
	YPO2109		Hypothetical phage protein	Yes
	YPO2113*		Hypothetical phage protein	Yes
	YPO2118*		Hypothetical phage protein	Yes
	YPO2123		Putative phage minor tail protein	Yes
	YPO2125		Putative phage-regulatory protein	Yes
	YPO2126		Putative phage protein	Yes
	YPO2131		Putative phage host specificity protein	Yes
	YPO1088		Putative DNA-binding prophage protein	Yes
	YPO1089		Putative regulatory prophage protein	No
	YPO1094		Hypothetical protein	No
	YPO1095		Hypothetical protein	Yes
	YPO2313		Hypothetical protein	Yes

[a] *, antibody response similar to or stronger than that to the known strongly immunogenic V antigen. The proteins, whose signal/noise ratio was ≥3.0 (i.e., spot signal against background signal) in the preimmune serum of at least three rabbits, were considered to have cross-reaction with the preimmune sera.

proteins, insecticidal toxins, and genomic island-related proteins were included in the microarray analysis; 24 of them were found to induce an antibody response (Table 1). pH 6 antigen, whose expression is induced in infected macrophage in acidic environment (23), can bind to glycosphingolipids that can be found on a range of host cell types (28) and apolipoprotein B-containing lipoproteins in human plasma (24), and it was recently identified as an antiphagocytic factor (19). In all preimmunized animals, we could not detect the specific antibody to this protein, but in the EV76-immunized rabbits at 42 days after immunization, the fluorescence intensity is increased to the level even higher than that of V antigen at the same time point. The microarray still included seven other putative adhesins/invasins, while only antibody to the protein encoded by YPO0687 was detected in the immune sera.

Outer membrane protein A (OmpA) is highly represented in the bacterial cell wall, conserved among the *Enterobacteriaceae*, and involved in bacterial virulence and growth (20). OmpA appears as a new pathogen-associated molecular pattern that interacts with antigen-presenting cells, suggesting that

TABLE 2. Evaluation of LCRS components as immunogenic and protective proteins[a]

Protein	Present in microarray	Immunogenic		Protective efficacy in bubonic/pneumonic model (reference[s])
		Previous study	Present study	
LcrV	Yes	Yes	Yes	Bubonic and pneumonic—protective (22, 35)
YopD	Yes	Yes	Yes	Bubonic—partially protective (2)
YscF	Yes	Yes	Yes	Bubonic—not protective (17)
VirG	Yes	Yes	Yes	Bubonic—not protective (17)
YopN	Yes	Yes	No	Bubonic—not protective (2, 21)
YscO	Yes	Yes	No	Bubonic—not protective (17)
YscP	Yes	Yes	No	Bubonic—not protective (17)
TyeA	Yes	Yes	No	Bubonic—not protective (17)
YopH	No	Yes	ND	Bubonic—not protective (2)
YopE	No	Yes	ND	Bubonic—not protective (2, 21)
YopK	No	Yes	ND	Bubonic—not protective (2, 21)
YopM	No	Yes	ND	Bubonic—not protective (2, 21)
YpkA	No	Yes	ND	Bubonic—delayed time to death (2)
YscJ	No	Yes	ND	Bubonic—not protective (17)
YscB	Yes	ND	Yes	ND
YscE	Yes	ND	Yes	ND
YscL	Yes	ND	Yes	ND
LcrG	Yes	ND	Yes	ND
SycE	Yes	ND	Yes	ND

[a] ND, not determined. A total of 10 Yop proteins were actually subjected to cloning and expression. The genes *yopB* and *yopM* could not be successfully amplified with *Y. pestis* 82009 DNA as a template. YopE, YopK, YopJ, and YopH were successfully expressed, but unfortunately, after being purified through 96-well format, they could not pass the quality control process. Therefore, only YopD, YopN, and YopR were included in the final version of the protein microarrays.

the immune system has acquired the ability to recognize this type of protein (20). MlpA is a major outer membrane lipoprotein that contributes to the structural integrity of the outer membrane along with OmpA (32). Both OmpA and MlpA represent a new type of candidate for vaccine design. In our study, OmpA (YPO1435) and MlpA (YPO2394) induced a strong increment of antibody titers with a pattern similar to that of V antigen.

Both KatY (13) and HtrA (33) are produced in great abundance after growth in vitro at 37°C but not at 26°C. KatY (antigen 5) with catalase-peroxidase activity is thought to mediate resistance to killing by professional phagocytes (13). Our results showed that the EV76-immunized rabbits made a strong antibody response to KatY with a pattern similar to that with V antigen. In contrast to the wild-type strain, the *htrA* mutant fails to grow at an elevated temperature of 39°C but shows only a small increase in sensitivity to oxidative stress and is only partially attenuated in the animal model (33). Antibody to HtrA was detected in the immunized rabbits.

Horizontal gene transfer entails the direct integration of genetic elements into the bacterial genome to form "genomic islands" with functions of increasing bacterial fitness (15). Three genomic islands (YPO0387 to YPO0397, YPO2087 to YPO2135, and YPO1087 to YPO1098) were tested in our present work because they appear to be newly acquired. YPO0387 to YPO0397 and YPO2087 to YPO2094 are uniquely conserved in *Y. pestis* (6, 37). If strongly antigenic, proteins encoded by this locus will have huge promise as serodiagnostic markers. Unfortunately, there was cross-reaction with the negative sera, although the three proteins encoded by YPO0388, YPO2090, and YPO2093 were found to induce significant antibody responses in the immunized rabbits. YPO1087 to YPO1098 were present in only some *Y. pseudotuberculosis* strains but in all *Y. pestis* strains tested (6). Antibodies to four proteins encoded by this locus (YPO1088, YPO1089, YPO1094, and YPO1095) were detected in the immunized sera. Genes YPO2095 to YPO2135 constitute a 33-kb chromosomal fragment that was absent from the biovar *Microtus* strains (30, 38). *Y. pestis* 91001, a member of the biovar *Microtus* strains, has a 50% lethal dose of 23.2 for mice by subcutaneous challenge; meanwhile, 10^9 live cells of strain 91001 failed to cause any infectious symptoms in rabbits. The most striking characteristic of strain 91001 is that 1.5×10^7 cells challenging through the subcutaneous route caused neither bubonic plague nor pneumonic plague in a volunteer trial (11). *Microtus* strains are supposed to be avirulent to humans, although they are highly lethal to mice, so this fragment likely contributes the ability to infect humans in fully virulent strains. Twenty-nine putative proteins encoded by this fragment were tested, and nine of them were found to induce antibody response.

Sequence analysis predicted several genes, probably acquired by horizontal gene transfer, to be homologues of insecticidal toxins (TcaA, TcaB, TcaC, TccC, viral enhancin, etc.) of insect pathogens, and they were implicated in the adaptation of *Y. pestis* to the flea life cycle (27, 36). This study showed that at least the TccC (YPO3674) homologue could induce the host antibody response, which clearly demonstrated that this antigenic protein was expressed in the infected mammalian host.

Conclusions. Microarray profiling of the host immune response to *Y. pestis* EV76 has identified immunodominant proteins of this live plague vaccine strain. Our results revealed a set of *Y. pestis* proteins to which the predominant antibody response occurred in immunized rabbits, raising a wealth of new candidates for us to further test as vaccines and diagnostic antigens. For example, proteins that can induce strong anti-

body response during immunization—such as those encoded by YPO2394, YPO1435, YPO2090, YPO2118, YPO3382, YPO3319, and YPCD1.48—are currently being investigated in our laboratory for their protective potency against bubonic and pneumonic plague in animal models.

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