Transport and anchoring of β -lactamase to the external surface of *Escherichia coli*

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ABSTRACT The outer membrane of Gram-negative bacteria presents an effective barrier that restricts the release of proteins from the cell. Virtually all extracellular proteins of Gram-negative bacteria are exported by specialized systems requiring the action of several gene products. We have constructed a tripartite fusion consisting of (i) the signal sequence and first nine N-terminal amino acids of the mature major *Escherichia coli* lipoprotein, (ii) amino acids 46–159 of the outer membrane protein OmpA, and (iii) the complete mature β -lactamase (EC 3.5.2.6) sequence. This protein had an enzymatically active β -lactamase and was found predominantly in the outer membrane. Immunofluorescence microscopy, the accessibility of the fusion protein to externally added proteases, and the rates of hydrolysis of nitrocefin and penicillin G by whole cells demonstrated that a substantial fraction (20–30%) of the β -lactamase domain of the fusion protein was exposed on the external surface of *E. coli*. In cells grown at 24°C the localization of β -lactamase on the cell surface was almost quantitative (>80% of the enzymatically active protein was exposed to the extracellular fluid) as determined by nitrocefin and penicillin G hydrolysis and trypsin accessibility. These results demonstrated that a soluble protein, β -lactamase, can be transported through—and become anchored on—the outer membrane by fusion to the proper targeting and localization signals.

The mechanisms of protein insertion within and translocation across the outer membrane of Gram-negative bacteria are not well understood. For some outer membrane proteins, such as the PhoE porin, the information necessary for proper localization and assembly is interspersed within the primary sequence (1, 2). Alternatively, the targeting signal may be contained within a single short continuous segment. For example, the signal sequence and first nine N-terminal amino acids of the major *Escherichia coli* lipoprotein (Lpp) are necessary for proper localization in the outer membrane. Fusion to this short sequence is sufficient to direct the normally soluble periplasmic protein β -lactamase (EC 3.5.2.6) to the outer membrane (3). Similarly, extensive studies with OmpA have suggested that the region between residues 154 and 180 is crucial for localization (4, 5). With OmpA, targeting and correct assembly into the outer membrane appear to be distinct events. Only large fragments containing the entire membrane-spanning sequence of OmpA are able to assemble into a conformation exhibiting native-like resistance to proteolytic digestion (4).

In general, amino acid substitutions or insertions within outer membrane loops exposed on the cell surface are well tolerated and do not interfere with the folding of the protein in the membrane. Peptides as large as 60 amino acids have been inserted within external loops of various outer membrane proteins and have been shown to be exposed on the surface of intact *E. coli* cells by immunochemical techniques (6–11). However, efforts to direct soluble reporter proteins, such as

alkaline phosphatase, to the cell surface by using fusions to outer membrane protein fragments have not been successful. These fusions either end up at incorrect cellular locations or become anchored in the membrane with the secreted protein domain facing the periplasm (12). In Gram-negative bacteria the outer membrane acts as a barrier to restrict the export of proteins from the cell. Normally only pilins, flagellins, specific enzymes, and a few toxins are completely transported across the outer membrane (13). Most of these proteins are first secreted into the periplasmic space via the general secretion pathway and then cross the outer membrane by a process that involves the action of several additional gene products (14). For example, 14 genes are specifically involved in the export of the enzyme pullulanase from *Klebsiella*. This protein is found in the growth medium and in association with the external side of the outer membrane. Kornacker and Pugsley (15) have shown that β -lactamase fused to a large N-terminal fragment of pullulanase can be translocated through the outer membrane of *E. coli* and becomes transiently associated with the outer membrane. This phenomenon, however, is dependent on the expression of the complicated pullulanase-specific export system.

In this work we have fused β -lactamase to the N-terminal targeting sequence of Lpp and an OmpA fragment containing five of the eight membrane-spanning loops of the native protein. We have demonstrated that this fusion protein is assembled on the cell surface with the β -lactamase domain stably anchored on the external side of the cell. This result showed that a periplasmic protein can be transported across the outer membrane in the absence of any specific export components. The expression of proteins on the bacterial surface has important implications for the development of whole cell adsorbents, live bacterial vaccines, and other biotechnological applications.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. *E. coli* strain JM109 (*endA1 recA1 gyrA thi-1 hsdR17* (r_k^- , m_k^+) *relA1 supE44* $\Delta(lac-proAB)/F'$ *traD36 proAB lacI^q lacZ* Δ M15) was used for all experiments except for the K3 phage resistance studies, which were performed in the *ompA* mutant strain UH203 (*lac supF ompA recA proA* or *proB rpsL/F' lacI^q lacZ* Δ M15) (16). Plasmids pJG311 (17) and pRD87 (18) were provided by M. Inouye and U. Henning, respectively. pJG311 contains a fusion of the signal sequence and first nine amino acids of Lpp and the complete mature β -lactamase gene. To construct plasmid pTX101, which codes for the Lpp-OmpA- β -lactamase fusion, a 342-base-pair (bp) *Sph* I-*Hpa* I fragment from pRD87 containing the sequence for five outer membrane-spanning domains of OmpA was blunt-ended with T4 DNA polymerase and ligated to *Eco*RI-digested pJG311 DNA that had been treated with the Klenow fragment of DNA polymerase.

Cultures were grown in either LB medium (Difco) supplemented with 0.2% glucose or M9 medium (26) supplemented

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FIG. 1. Amino acid sequence of the tripartite Lpp-OmpA- β -lactamase fusion protein derived from DNA sequences. Unlabeled residues are linkers. a.a., Amino acids.

with 0.2% casein amino acid hydrolysate and 0.2% glucose. Ampicillin (50 μ g/ml) and chloramphenicol (10 μ g/ml) were added as required.

Cell Fractionation and Protease Accessibility Experiments. The separation of inner and outer membrane fractions was carried out as follows (19). Cells grown at 37°C were harvested from 200 ml of LB medium containing 0.2% glucose at an OD₆₀₀ of 1.0, washed in 25 mM Tris-HCl at pH 7.4, and resuspended in 10 ml of the same buffer containing 1 mM EDTA and lysozyme at 100 μ g/ml at 4°C. After a 2-min incubation the cells were lysed by two passages through a French pressure cell at 10,000 psi (69 MPa). The cellular debris was removed by centrifugation at 2500 $\times$ *g* for 8 min and the total membranes were precipitated by centrifugation at 115,000 $\times$ *g* for 1 hr. Membranes were resuspended in 0.8 ml of 25 mM Tris-HCl at pH 7.4 containing 25% (wt/wt) sucrose and loaded onto a step gradient of 30, 35, 40, 45, 50, and 55% (wt/wt) sucrose in the same Tris-HCl buffer. After centrifugation at 165,000 $\times$ *g* for 16 hr in a Beckman SW41Ti rotor, 0.5-ml fractions were collected from the bottom of the tube. The density of the fractions was determined from refractive index measurements. The concentration of sucrose was lowered to <10% by diluting the samples with Tris-HCl buffer followed by centrifugation to pellet the membranes.

The effect of externally added proteases on the enzymatic activity of Lpp-OmpA- β -lactamase was measured essentially as described by Kornacker and Pugsley (15). Cultures grown in M9 medium were harvested at OD₆₀₀ = 1.0, washed with fresh medium, and resuspended in M9 salts without glucose or antibiotics. The β -lactamase activity in the whole cells was determined by using nitrocefin (Becton Dickinson) and penicillin G as substrates. The cells were incubated for 1 hr at 37°C in the absence of protease or in the presence of either proteinase K or trypsin at 0.1 mg/ml. The reactions were stopped by adding 10 mM phenylmethylsulfonyl fluoride or soybean trypsin inhibitor at 0.2 mg/ml, respectively. Subsequently, the cells were lysed in a French pressure cell and centrifuged at 2500 $\times$ *g* for 8 min to remove unbroken cells. The membranes were pelleted as described above and resuspended in 50 mM potassium phosphate buffer at pH 6.5, and the enzymatic activity of the membranes was measured.

Immunofluorescence. Cultures grown in M9 medium were harvested at midexponential phase and resuspended in phosphate-buffered saline (PBS) with or without trypsin at 0.1 mg/ml and incubated at 37°C. Soybean trypsin inhibitor was added at different times to stop the reaction and incubation at 37°C was continued for a total of 1 hr. All subsequent procedures were conducted at room temperature. The cells were washed with PBS, incubated for 45 min with PBS and 1% bovine serum albumin (BSA), washed, and then incubated in the same PBS/BSA solution with rabbit anti- β -lactamase antibodies at 1:1,000 dilution for 45 min. After another three washes with PBS/BSA the cells were mixed with rhodamine-conjugated goat antibody to rabbit immunoglobulin, incubated for 45 min, and then washed three more times. Finally, the cells were resuspended in PBS and examined by phase-contrast and video-enhanced fluorescence microscopy.

General Procedures. SDS/PAGE on 11% and 15% acrylamide gels and Western blotting were performed as described (20, 21). The enzymatic activity of β -lactamase was measured spectrophotometrically at 240 nm with penicillin G (22) or at 482 nm with nitrocefin (23) as substrates. With penicillin G, one unit of β -lactamase activity is defined as that which

hydrolyzes 1 mg of penicillin G per min. For phage spot tests, UH203(pTX101) cells were plated on LB agar plates containing ampicillin at 100 μ g/ml. Drops of various concentrations of phage were placed over the cells and the plates were incubated overnight at 37°C. The plates were then examined for areas of lysis where the phage were added. Protein concentrations were measured by the Bio-Rad assay.

RESULTS AND DISCUSSION

We constructed a tripartite fusion consisting of the signal sequence and first nine N-terminal amino acids of Lpp, residues 46–159 of OmpA, and the entire mature β -lactamase (Fig. 1). Since Klose *et al.* (4) have shown that the OmpA fragments lacking the sequence between amino acids 154 and 180 are not transported to the outer membrane, the targeting signal from Lpp was used to mediate proper localization of the fusion protein. A schematic of the expected conformation of the Lpp-OmpA- β -lactamase in the outer membrane is shown in Fig. 2. The positions of the membrane-spanning domains and the externally exposed loops of OmpA are based on the model of Vogel and Jähnig (24).

E. coli JM109 containing the plasmid pTX101, which carries the fusion protein gene, was grown in LB medium supplemented with glucose. The plasmid pTX101 carries the *lpp* promoter and the *lac* promoter-operator region so that the expression of the Lpp-OmpA- β -lactamase is inducible by isopropyl β -D-thiogalactoside (IPTG) (3). The plasmid also carries the gene for the *lac* repressor. Although induction with IPTG was found to be lethal, a high level of expression was nevertheless obtained in the absence of inducer. Cultures were harvested in late exponential phase and the cells were lysed in a French pressure cell and separated into soluble and cell envelope fractions by high-speed centrifugation. Approximately 84% of the total β -lactamase activity from JM109(pTX101) lysates was found in the cell envelope fraction. Essentially all the remaining activity was present in the soluble fraction of the cell lysates, with less than 0.5% in the extracellular fluid. Even after prolonged incubation of stationary-phase cells (24 hr) there was no increase in the percentage of β -lactamase in the extracellular fluid, indicating the fusion protein was not released from the cells. Qualitatively similar results were observed when the distribution of the fusion protein in the different fractions was

FIG. 2. Schematic of the expected structure of the Lpp-OmpA- β -lactamase fusion in the outer membrane. Grey rectangles represent membrane-spanning β -strands of OmpA based on the model of Vogel and Jähnig (24). aa, Amino acid(s).

FIG. 3. Fractionation of membranes from JM109(pTX101) and JM109(pJG311) cells. (A) Percent of total membrane protein and β -lactamase activity in different fractions from the sucrose gradient of JM109(pTX101). The β -lactamase activity was determined from the rate of hydrolysis of penicillin G. Fractions 2–7 had an average density (ρ) of 1.22 g/ml; fractions 11–13, ρ = 1.19 g/ml; and fractions 16–20, ρ = 1.15 g/ml, corresponding exactly to the values for outer membrane, intermediate, and inner membrane vesicles determined by Osborn *et al.* (25). (B) SDS/PAGE of membrane fractions. Samples from every three fractions were pooled and loaded in consecutive lanes. Lanes 2–8, pTX101; lanes 9–15, pJG311. Lanes: 1, molecular mass markers; 2, fractions 1–3; 3, fractions 4–6; 4, fractions 7–9; 5, fractions 10–12; 6, fractions 13–15; 7, fractions 16–18; 8, fractions 19–21; 9, fractions 1–3; 10, fractions 4–6; 11, fractions 7–9; 12, fractions 10–12; 13, fractions 13–15; 14, fractions 16–18; 15, fractions 19–21; 16, molecular mass markers. The molecular mass standards (BRL) are as follows: myosin H chain, 200 kDa; phosphorylase *b*, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 29 kDa. Arrows indicate the fusion proteins Lpp-OmpA- β -lactamase (lane 2) and Lpp- β -lactamase (lane 9). (C) Western blot of the JM109(pTX101) fractions from the sucrose gradient (B, lanes 2–8). The primary antibody was used at a concentration of 1:20,000. The gel was overloaded to

examined by immunoblotting with β -lactamase or OmpA-specific antisera (data not shown). The Lpp- β -lactamase protein from plasmid pJG311 had 2-fold higher total activities compared with the three-part fusion, which contained the OmpA insert. The former protein was also found predominantly in the membrane pellet (98% of the total activity), in agreement with previous observations.

Cell envelope pellets from JM109(pTX101) were separated into inner and outer membrane fractions by isopycnic sucrose gradient centrifugation as described in *Materials and Methods*. Two distinct protein peaks were obtained in fractions having the expected densities for inner and outer membrane fractions (25). Virtually all of the β -lactamase enzymatic activity was found in the higher-density fractions, which correspond to the outer membrane fractions (Fig. 3A). A protein band migrating at a molecular mass of approximately 43 kDa, the expected size of Lpp-OmpA- β -lactamase, co-fractionated with the major outer membrane proteins OmpC/F and OmpA on SDS/PAGE gels. This protein was not present in JM109 cells containing pJG311. The intensity of the Lpp-OmpA- β -lactamase band was comparable to that of the abundant major outer membrane proteins (Fig. 3B). The fusion protein was subjected to some degradation, which resulted in the appearance of lower molecular mass bands that crossreacted with β -lactamase-specific antibodies in immunoblots (Fig. 3C). However, the relatively small proportion of degradation fragments indicated that most of the fusion protein had not been subjected to proteolysis.

The localization of the β -lactamase domain with respect to the external surface of *E. coli* was determined by various immunocytochemistry methods, activity assays, and protease accessibility experiments. For immunofluorescence studies, cells grown in M9 medium were labeled with rabbit β -lactamase-specific antibodies followed by secondary rhodamine-conjugated goat rabbit-antibody-specific antibodies. In control experiments no fluorescence above background was detectable with JM109(pJG311) cells expressing the Lpp- β -lactamase fusion protein. As expected, the Lpp moiety directed β -lactamase to the outer membrane but was not sufficient to allow export to the cell surface. Fig. 4 shows a comparison of the same field of JM109(pTX101) cells viewed with phase-contrast and fluorescence microscopies. Nearly all of the cells became fluorescent, indicating that they contain sequences recognized by the anti- β -lactamase antibodies. Incubation with trypsin for various times prior to antibody labeling resulted in a gradual decrease in the fluorescent signal. No signal could be detected after 1 hr of incubation.

The above results demonstrated that polypeptides cross-reacting with the β -lactamase antibodies were present on the surface of the cells expressing the tripartite fusion protein. It is possible, however, that the molecules that were able to cross the membrane contained only short β -lactamase sequences derived by proteolytic degradation during export and not the complete, enzymatically active protein domain. The presence of enzymatically active β -lactamase on the cell surface was determined from protease accessibility experiments and the rates of hydrolysis of substrates that do not readily diffuse through the outer membrane. Cultures grown in M9 medium at 37°C were harvested in midexponential phase, washed, and incubated with proteinase K or trypsin for 1 hr. In cells containing plasmid pTX101, about 20% of the

show the presence of degradation products. There were no degradation products below the 32-kDa molecular mass standard. As with the native β -lactamase, the Lpp-OmpA- β -lactamase migrates as two bands, depending on the oxidation of the single disulfide bond (36). The prestained molecular mass markers (Bio-Rad) have apparent molecular masses as follows: phosphorylase *b*, 106 kDa; bovine serum albumin, 80 kDa; ovalbumin, 49 kDa; and bovine carbonic anhydrase, 32 kDa.

FIG. 4. Micrographs of the same field of JM109(pTX101) cells viewed by fluorescence (A) and phase-contrast (B) microscopy.

total membrane-bound activity was reproducibly lost after a 1-hr incubation with either trypsin or proteinase K, compared with only a 3% decrease in JM109(pJG311) (Table 1). A comparable, although somewhat higher, percentage of surface-exposed activity was obtained from the rates of hydrolysis of nitrocefin and penicillin G in intact versus lysed cells grown in M9 medium. While nitrocefin and penicillin G can penetrate the outer membrane (27, 28), the rate of hydrolysis by periplasmic β -lactamase is limited by the rate of diffusion through the membrane. As demonstrated by the low rate of hydrolysis of nitrocefin and penicillin G by intact JM109(pJG311) cells, the rate of diffusion of these β -lactamase substrates into the periplasm and the subsequent hydrolysis by the periplasmic enzyme was low (Table 1). With intact JM109(pTX101) cells, the rate of hydrolysis of the

β -lactam antibiotics was considerably higher, due to the localization of β -lactamase on the external face of the outer membrane. These results suggested that between 20% and 30% of the enzymatic activity of Lpp-OmpA- β -lactamase was exposed on the surface of cells grown at 37°C, a significant increase over the background in control cultures (1–3%; Table 1). Thus, a substantial fraction of the intact β -lactamase must be fully exported across the outer membrane and become anchored on the *E. coli* surface.

Studies in our laboratories have demonstrated that the rate of folding of β -lactamase decreases drastically with temperature (20). We reasoned that growing JM109(pTX101) at a lower temperature might be able to retard the premature folding of the fusion protein during export and thus favor the correct insertion of the protein in the membrane. Indeed, in cultures grown at 24°C the β -lactamase activity was almost quantitatively present on the cell surface. The rates of nitrocefin and penicillin G hydrolysis and trypsin accessibility indicated 80–90% surface exposure (Table 1). It is noteworthy that the efficiency with which the β -lactamase domain was anchored on the cell surface under these conditions was even higher than has been reported for fusion proteins exported by the pullulanase-specific system (15).

OmpA serves as a receptor for several bacteriophages. Morona *et al.* (29) have shown that phage K3 binds to the second exposed loop of the native protein. The *ompA* mutant strain UH203 is completely resistant to infection by K3. Since the tripartite fusion protein contains the OmpA sequences that serve as K3 receptors, the possibility that it restored phage sensitivity to UH203 was investigated. UH203 cells transformed with pTX101 were completely resistant to infection with various concentrations of the phage as determined by spot tests. The most plausible explanations for the resistance to phage infection are either (i) the OmpA domain is not properly folded in the outer membrane or (ii) the bulky β -lactamase on the cell surface sterically shields the receptor domains in OmpA and prevents access of the phage. We favor the latter hypothesis for the following reason. The fact that β -lactamase is localized on the surface indicates that the C terminus of the OmpA fragment must be exposed on the cell, in agreement with the proposed conformation of the native protein (24). It is hard to see how an improperly folded OmpA domain could be able to traverse the membrane and provide the driving force for the export of a 28-kDa protein.

The above results show that the outer membrane of *E. coli* does not present an inherent barrier to protein transport. In the case of β -lactamase, exposure on the cell surface requires the presence of two distinct sequences that serve as signals for outer membrane (i) targeting and anchoring and (ii) translocation. The signal sequence and first nine amino acids of Lpp fulfill the targeting and anchoring function and allow

Table 1. Percent β -lactamase exposed on the surface as determined by protease accessibility and enzymatic activity

Plasmid	Growth temperature, °C	% decrease in penicillin G hydrolysis after incubation*		Nitrocefin or penicillin G hydrolysis activity in intact cells, % of total lysate activity	
		With proteinase K	With trypsin	Nitrocefin	Penicillin†
pJG311	37	3	3	1	1 (0.03/3.45)
pJG311	24	—	—	6	3 (0.10/3.74)
pTX101	37	23	18	33	56 (0.67/1.19)
pTX101	24	—	89	93	100 (1.85/1.84)

The standard deviation for all experiments was less than $\pm 5\%$ of the reported mean values.

*Cells were incubated with proteinase K or trypsin for 1 hr and the total membrane fractions were isolated. The percent of exposed β -lactamase corresponds to the activity remaining after incubation with proteases relative to untreated membranes.

†The numbers in parentheses are the ratios of whole cell specific activity to lysate specific activity (units/ml of cells normalized to an optical density of 1.0 at 600 nm).

even a complex protein such as the tripartite fusion to reach the correct subcellular location. Our hypothesis is that the Lpp moiety is the key component of our construct. Lpp differs fundamentally from other outer membrane proteins in that all of the requisite information for its localization and insertion is present in its amino-terminal region. Previous studies have shown that fusions to this short Lpp sequence become covalently modified by the attachment of diglyceride and a fatty acid to the N-terminal cysteine (3). The lipid moiety is presumed to be inserted into the membrane (17) as shown in Fig. 2. Because the hybrid Lpp- β -lactamase protein encoded by pJG311 is modified, processed, and localized in the same way as the normal Lpp, we anticipate that the same events occur with the Lpp-OmpA- β -lactamase, given its stable outer membrane association and close relationship to Lpp- β -lactamase.

It is impossible to use fusions to Lpp alone for cell surface localization, as Lpp has no surface-exposed domain and, indeed, the majority of the polypeptide is in the periplasm. Therefore, to provide an outer membrane translocation sequence, an odd number of OmpA transmembrane segments were inserted between the Lpp and β -lactamase sequences. If the OmpA domain folds in a native-like conformation similar to the one proposed by Vogel and Jähnig (24), the fusion junction between the nine Lpp amino acids and OmpA must be facing the periplasmic side of the outer membrane, whereas the OmpA- β -lactamase junction must be exposed on the cell surface. [Similar sequences from other outer membrane proteins might translocate β -lactamase equally well. The effect of varying the number of transmembrane segments and the significance, if any, of the fact that the nonomer sequence at the junction between OmpA and β -lactamase contains three proline and two glycine residues (Fig. 1) have not been tested.] The free energy change decrease that accompanies the folding of OmpA-46-159 is apparently sufficient to drive the β -lactamase domain at its C terminus across the outer membrane. The efficiency of the export process may be related to the conformation of the protein prior to the interaction with the outer membrane.

In contrast to Lpp, the most important assembly region for other outer membrane proteins is probably in their last membrane-spanning segment; this segment is usually at the C terminus, is directed toward the periplasm, and is conserved in amino acid sequence (30). [In OmpA, a 325-residue protein with a large periplasmic tail, this sequence occurs at amino acids 162-170, a β -strand previously identified as being essential for association with the outer membrane (26) and absent from our construct.] The importance of the last membrane-spanning segment may at least partially explain the failure of soluble proteins fused to typical outer membrane proteins to be exposed on the cell surface. In libraries of bipartite fusions such as FepA-PhoA (31) and FhuA-PhoA (32), fusions downstream of the crucial transmembrane segment would orient PhoA toward the periplasm and fusions upstream of the segment would result, as a consequence of the loss of the segment, in distorted and inefficient outer membrane insertion at best.

Whether or not polypeptides other than β -lactamase can be readily exposed in active form on the cell surface remains to be determined. Surface expression of other polypeptides may require changes in the growth environment, fine tuning of fusion protein expression, host cell mutations that prevent premature folding or proteolysis in the periplasmic space, or, finally, mutations resulting in alterations in the composition of the outer membrane so that regions of phospholipid bilayers become prevalent. The ability to export a soluble protein across the outer membrane has considerable implications for the biogenesis of the cell envelope of Gram-negative bacteria. Further experiments are necessary to determine more precisely what properties of the Lpp-OmpA

fusion provide it with the ability to place the model soluble protein, β -lactamase, on the *E. coli* surface. The expression of recombinant proteins on the cell surface is expected to be of great interest in biotechnology. For example, the production of single-chain antibodies on the cell surface can offer several advantages over the recently developed phage expression systems and may form the basis for a bacterial analog of the immune system (33, 34). Another application is the expression of antigenic peptides on the cell surface for the development of live bacterial vaccines that confer immunity against infectious agents (35).

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