Growth temperature dependence of channel size of the major outer-membrane protein (OprF) in psychrotrophic *Pseudomonas fluorescens* strains

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The outer-membrane (OM) permeability of the psychrotrophic bacterium *Pseudomonas fluorescens* strain MF0 for the β -lactam mezlocillin is increased at the optimum growth temperature (28 °C) compared to low growth temperatures (8 °C). In an attempt to explain this phenomenon, OM protein content was studied in cultures grown at both temperatures. No significant difference in proportion or composition was found, suggesting that a change in the structure and function of porins could be responsible for the differential permeability. The major OM protein OprF of two psychrotrophic *P. fluorescens* strains, MF0 and OE 28.3, was purified from cultures grown at 8 °C and 28 °C in order to reincorporate them in solvent-free lipid bilayers. From cultures grown at the same temperature, OprF displayed very similar channel-forming properties for both strains. Decreasing the growth temperature induced a threefold reduction of the major conductance values (250–270 pS in 1 M NaCl for 28 °C cultures and 80–90 pS in 1 M NaCl for 8 °C cultures). The trypsin digestion kinetics showed a very different reactivity for these porins between cultures grown at 8 °C and 28 °C. This may indicate that the pore structure of OprF is modified depending on the growth temperature, as suggested by its functional behaviour.

Keywords: *Pseudomonas fluorescens*, OprF, channel-forming properties, psychrotrophy, porins

INTRODUCTION

The intrinsic antibiotic resistance of Gram-negative bacteria may be explained in several ways. First, it is well known that the outer membrane (OM) of the cell acts as a permeability barrier for hydrophilic solutes (Nikaido & Vaara, 1985). The passive diffusion rate of molecules, mainly through the non-specific porins, depends on their hydrophobicity (Nikaido, 1985) and/or their size or physicochemical properties. Nevertheless the permeability of the *Pseudomonas aeruginosa* OM was shown to be 10- to 100-fold lower than that of the

OM of *Escherichia coli* (Yoshimura & Nikaido, 1982; Hancock, 1985). In the former genus, the size of the non-specific porin channels seems to be conserved, especially for the major OM protein. Indeed, this has been determined in planar lipid bilayers for the OprF of two *Pseudomonas fluorescens* strains, MF0 (isolated from raw milk) and OE 28.3 (isolated from soils) (Dé *et al.*, 1995) and also for the OprF of *P. aeruginosa* (Saint *et al.*, 1992). The resistance to antibiotics, mainly with regard to the β -lactam family, may also be the result of the induction of well-described enzymes, such as β -lactamases, which hydrolyse the drug (Davies, 1994). The resistance of the mesophilic bacterium *P. aeruginosa* to β -lactams is thought to result from a balance between the diffusion rate of the drug through the OM and the induction rate of the β -lactamases (Nikaido, 1989).

Studies on the antibiotic resistance of mesophilic bacteria are usually performed on strains that have been

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Abbreviations: DPhPC, diphytanoylphosphatidylcholine; octyl-POE, octyl-polyoxyethylene; OM, outer membrane; TLCK, *N*²-*p*-tosyl-L-lysine chloromethyl ketone.

cultured at the optimal growth temperature. However, recent studies have suggested that the growth temperature of psychrotrophic bacteria seems to be correlated with a modulation of OM permeability and drug resistance. In the antarctic psychrotroph *Pseudomonas syringae* Lz4W, for example, the lipopolysaccharide (LPS) is more phosphorylated at a higher growth temperature (22 °C) than at a lower growth temperature (0 °C), where the post-synthetic phosphorylation of LPS is low (Ray *et al.*, 1994). Under the latter culture conditions, the strain was also shown to be more sensitive to EDTA and to cationic antibiotics. Another study was performed with the psychrotrophic bacterium *P. fluorescens* (Orange, 1994). In this case, a minimum threshold of antibiotic penetration is required to cause induction of β -lactamase synthesis. At the optimum growth temperature (28 °C), this threshold is easily reached by OM permeation, whereas at a low growth temperature (8 °C) the penetration rate of the β -lactam is not high enough to reach this limit. In this condition the bacterium is found to be significantly less resistant. In these examples, the growth temperature seems to regulate the penetration of the drug through the OM independently of a modification of lipids as described by Russell (1990) or Bhakoo & Herbert (1980).

As the permeation of β -lactam through the OM of *P. fluorescens* strain MF0, and certainly through the non-specific porins, seems to determine the induction of β -lactamase production in the periplasmic space (Orange, 1994), we were interested to study the major OM protein of this bacterium (and of a related strain, OE 28.3) previously described as OprF-like porin (Dé *et al.*, 1995).

The results described here show that this OprF protein produced different channel sizes in single-channel conductance experiments when it was re-incorporated into planar lipid bilayers depending on whether it had been extracted from cultures grown at low or high temperatures.

METHODS

Bacterial strains and growth conditions. *Pseudomonas fluorescens* strains MF0 and OE 28.3 are psychrotrophic bacteria with an optimum growth temperature of 28 °C. The MF0 strain was grown in nutrient broth (Difco). Tryptic soy broth, a more convenient growth medium, was used for the *P. fluorescens* OE 28.3 strain because this strain secretes an exopolysaccharide which interferes with OM extraction in nutrient broth. After vigorous shaking at 28 °C overnight or at 8 °C for 48 h, cells were harvested in the late exponential phase by centrifugation at 8000 g for 10 min at 4 °C.

Isolation of bacterial membranes. OMs were extracted by the spheroplast procedure (Mizuno & Kageyama, 1978) for both strains at different growth temperatures. This procedure was modified by including a washing step with phosphate buffered saline, which allowed the elimination of exopolysaccharides surrounding the bacteria, to increase the extraction yield (unpublished data). Total protein content was determined by the bicinchoninic acid method (Pierce), and the protein

composition of the OM fractions was analysed by SDS-PAGE in a 7/15 % discontinuous gel system (Laemmli, 1970). The relative protein concentration of each band was estimated by densitometry at 590 nm after Coomassie blue staining (Shimadzu).

Purification of OprF proteins. Purification of the major OM protein OprF from 8 °C cultures was carried out by preparative native-like electrophoresis and electroelution in octyl-polyoxyethylene (octyl-POE) media (0.1 %, w/v, octyl-POE, 192 mM glycine, 25 mM Tris/HCl) as described by Dé *et al.* (1995). Electroelution of this protein requires particular care to preserve the protein integrity: the time of elution should be minimized (6 h) or the experiment should be carried out at low temperature (4 °C). Purity control was achieved by SDS-PAGE with silver staining. Proteins purified in octyl-POE were analysed by two-dimensional PAGE as described by De Mot & Vanderleyden (1989).

Reconstitution in planar lipid bilayers. Virtually solvent-free planar lipid bilayers were formed by the technique of Montal & Muller (1972) by the apposition of two monolayers on a 100 μ m diameter hole in a thin Teflon film (10 μ m) pretreated with hexadecane/hexane (1:40, v/v). Measurement compartments were glass cells. Membrane currents under applied voltage were measured using a BLM 120 amplifier (Biologic) and Ag/AgCl electrodes. The current fluctuations were stored on a DTR 1202 (Biologic) and were transferred to a computer where different measurements (current, amplitude histogram) were performed using software from Intracell. A diphytanoyl-phosphatidylcholine (DPhPC, Avanti) solution in hexane (0.5 %, w/v, 10 μ l) was used as lipid, the electrolyte solution was 1 M NaCl, 10 mM Tris/HCl (pH 7.4) and the bulk concentration of the re-incorporated proteins was about 10^{-9} M or 10^{-8} M for the selectivity experiments. These latter experiments needed installation of a KCl gradient through the lipid bilayer: from 0.1 M KCl on the *cis* side to 1 M KCl on the *trans* side. The zero-current potential was corrected by deducting the asymmetric potential due to the KCl gradient.

Determination of N-terminal amino acid sequence. OM fractions (MF0 strain) obtained from cultures grown at 8 °C were subjected to SDS-PAGE (7/17 % gel system) and then electrophoretically transferred onto a PVDF membrane (Immobilon, Millipore). The membrane was stained with 0.1 % (w/v) Amido Black in 45 % (v/v) methanol and 9 % (v/v) acetic acid and destained in water. The N-terminal sequence was determined by Sanofi-Winthrop Company (Notre Dame de Bondeville, France) using the Edman degradation (471A Protein Sequencer, Applied Biosystems) from the band corresponding to the major protein (M_r 32000).

Western blotting and immunodetection. After SDS-PAGE, the polypeptides were transferred to nitrocellulose (H. Bond, 0.22 μ m pore size; Amersham) as described by Towbin *et al.* (1979). Immunodetections were carried out using specific monoclonal antibody (MA28B9) directed against the *P. fluorescens* OE 28.3 OprF, as described elsewhere (De Mot *et al.*, 1994).

Trypsin digestion. Samples (100 μ g ml⁻¹) containing OprFs purified by the octyl-POE method, from cultures of *P. fluorescens* MF0 grown at 8 °C and 28 °C, were incubated with trypsin (Fluka) at 12 μ g ml⁻¹ at 37 °C for 3 h. Digestions were carried out simultaneously for both growth temperatures and stopped by addition of 0.1 mM N^ε-p-tosyl-L-lysine chloromethyl ketone (TLCK) at different times ranging from 0 to 3 h (Shaw *et al.*, 1965). The samples were analysed by SDS gel electrophoresis (7/15 %) and silver staining.

RESULTS

Comparison of OM proteins according to growth temperature

Previous studies (Orange, 1994) suggested that permeation of the β -lactam mezlocillin through the OM of the psychrotrophic bacterium *P. fluorescens* MF0 was higher at 28 °C than at 8 °C. In an attempt to address this question, the OM protein content was studied at the two growth temperatures.

OM proteins were extracted by the spheroplast method (Mizuno & Kageyama, 1978) from cultures grown at 28 °C or 8 °C and separated by SDS-PAGE after heating (Fig. 1a, lanes 2 and 4) or without prior heating (Fig. 1a, lanes 1 and 3) at 100 °C in SDS sample buffer. Heat-modified forms appeared, mainly for the major OM protein OprF, which was converted into a heat-modifiable form of M_r 37 000 (Dé *et al.*, 1995). This behaviour is characteristic of monomeric porins like *P. aeruginosa* OprF and the OprFs of *P. fluorescens* MF0 and *P. fluorescens* OE 28.3. However, no significant quantitative differences were found after densitometric analysis of bands obtained from the two temperatures.

Influence of growth temperature on *P. fluorescens* OprF

Fluctuations of OM permeability depending on growth temperature in *P. fluorescens* can be explained neither by the appearance of a particular porin at 28 °C nor by a change in the relative proportion of proteins. This led us to study the possible change of function and/or structure of the major OM porin, i.e. OprF protein with M_r 32 000, depending on growth temperature.

Purification. OprF was extracted from cultures at 28 °C or 8 °C and purified in octyl-POE as described elsewhere (Dé *et al.*, 1995). However, the purification of the OprF produced at 8 °C needed particular attention (see Methods) to avoid proteolytic degradation. The electrophoretic analysis of the purified proteins showed that the growth temperature did not alter the mobility of the OprFs in the SDS-polyacrylamide gels (Fig. 1b) and that the proteins had a very similar behaviour after heating. Moreover two-dimensional electrophoresis of these OprFs allowed us to check the high purity of the samples and clearly showed the same isoelectric point for the purified proteins regardless of the growth temperature of *P. fluorescens* MF0 (data not shown).

Function. The purified proteins from *P. fluorescens* MF0 were re-incorporated in planar lipid bilayers at a concentration of 10^{-9} M in the *cis* compartment of the measurement cell. Several single-channel experiments were carried out for the 28 °C and 8 °C OprFs and allowed us to detect small increments of current after application of constant potential in DPhPC membranes (Figs 2a and 3a). OprF protein from cultures grown at 28 °C gave a mean conductance value of 250 pS in 1 M NaCl (Fig. 2b, associated amplitude histogram, see Dé *et al.*, 1995), whereas the same experiments per-

Fig. 1. (a) Comparison of OM proteins of *P. fluorescens* MF0 grown at different temperatures by SDS-PAGE (7/15% discontinuous gel system) with silver staining. OMs were isolated by the spheroplast method and solubilized in SDS buffer before loading. Lanes 1 and 2, OM from 28 °C culture; lanes 3 and 4, OM from 8 °C culture. Lanes 1 and 3, OM without heating; lanes 2 and 4, OM heated at 100 °C for 10 min. (b) SDS-PAGE (with silver staining) of OprF of the *P. fluorescens* MF0 extracted from 28 °C and 8 °C cultures purified by the octyl-POE method. The protein was heated at 100 °C for 10 min to show the heat-modified form (HM). Lanes 1 and 2, OprF protein from 28 °C culture; lanes 3 and 4, OprF protein from 8 °C culture. Lanes 1 and 3, purified protein without heating; lanes 2 and 4, OprF heated.

formed for the 8 °C OprF protein showed a considerably smaller major conductance value, 80 pS in 1 M NaCl (Fig. 3b).

In order to verify whether this behaviour was specific to this particular *P. fluorescens* strain, the OprF was also purified from the psychrotrophic *P. fluorescens* strain OE 28.3 grown at different temperatures (De Mot & Vanderleyden, 1991; De Mot *et al.*, 1992, 1994; Dé *et al.*, 1995). The re-incorporation of these OprFs was performed under the same experimental conditions. Discrete fluctuations of current were observed corresponding to a conductance value of 270 pS for the 28 °C OprF protein and 90 pS for the 8 °C OprF in 1 M NaCl (Table 1). These results are in good agreement with the values for the MF0 strain described above.

The selectivity of these proteins was also investigated by using a gradient of KCl concentration (0.1 M:1 M, *cis:trans*). For these experiments, protein concentration in the baths was tenfold higher than in single-channel measurements. After establishing the electrolyte gradient, a zero-current potential of +20 mV and +18 mV was found (after deducting the asymmetric potential) for the 8 °C protein from, respectively, strains MF0 and OE 28.3. According to the Goldman-Hodgkin-Katz

Fig. 2. Conductance of re-incorporated OprF protein purified from *P. fluorescens* MF0 grown at 28 °C. (a) Trace of current fluctuations of the OprF protein (10^{-9} M) in a DPhPC membrane when the applied voltage was 70 mV. Electrolyte, 1 M NaCl; digitization rate, 3000 Hz; filter, 300 Hz. o, open state; c, closed state. (b) Associated amplitude histogram with a Gaussian fitting which shows a conductance level of 250 pS.

Fig. 3. Conductance of re-incorporated OprF protein purified from *P. fluorescens* MF0 grown at 8 °C. (a) Single-channel current records of the OprF protein (10^{-9} M) in 1 M NaCl, under a -110 mV potential. Digitization rate, 3000 Hz; filter, 300 Hz. o, open state; c, closed state. (b) Associated amplitude histogram with a Gaussian fitting which shows a conductance level of 80 pS.

Table 1. Single-channel conductance and selectivity values for the OprF proteins purified from *P. fluorescens* strains MF0 and OE 28.3 grown at 28 °C and 8 °C

Values of conductance are given for OprF reincorporation in a DPhPC membrane bathed by 1 M NaCl. Selectivity ratios are calculated for an electrolyte gradient of KCl of 0.1 M:1 M (*cis:trans*).

Strain	Culture at 28 °C		Culture at 8 °C	
	Single-channel conductance (pS)	Selectivity P_K/P_{Cl}	Single-channel conductance (pS)	Selectivity P_K/P_{Cl}
<i>P. fluorescens</i> MF0	250 ± 7	1.7	80 ± 3	2.8
<i>P. fluorescens</i> OE 28.3	270 ± 7	1.4	90 ± 3	2.5

equation (Hille, 1984), the P_K/P_{Cl} ratio values 2.8 and 2.5 indicate a cationic selectivity. These values show a higher cationic selectivity than those determined by Dé *et al.* (1995) for the 28 °C protein (Table 1). It is noteworthy that these values (from 28 °C or 8 °C OprF proteins) are close to the selectivity ratio $P_K/P_{Cl} = 2$ found by Hancock (1986) for OprF from *P. aeruginosa* grown at 37 °C.

These single-channel results show a functional change in the channel size of the major OM porin depending on the growth temperature. Several hypotheses can be proposed to explain this behaviour and in particular

temperature-dependent modifications of the structure of OprF porins.

Structure. The primary structure of the OprFs extracted from *P. fluorescens* MF0 grown at high or low temperature was compared after the analysis of the N-terminal amino acid sequence. This sequence, QGQGAVERGFYKKQYNDTV, was identical for both proteins. Using PCR amplification and Southern hybridization, a single *oprF* gene was identified in *P. fluorescens* strains MF0 and OE 28.3. This result rules out the possibility that two *oprF* genes could be present and expressed differentially according to the growth

Fig. 4. Reactivity of monoclonal antibody MA28B9 with OprF from *P. fluorescens* MF0 depending on the growth temperature. Some samples of OM extracts and purified OprF proteins were heated at 100 °C for 10 min (HM, heat-modified form). Lanes 1–4, OM extracted from cultures at 28 °C (lanes 1 and 2) and 8 °C (lanes 3 and 4); lanes 5–8, purified OprF proteins from cultures at 28 °C (lanes 5 and 6) and 8 °C (lanes 7 and 8). Lanes 1 and 3, OMs non-heated; lanes 2 and 4, OMs heated; lanes 5 and 7, OprFs non-heated; lanes 6 and 8, OprFs heated.

temperature. The *oprF* gene of both strains has been sequenced previously (De Mot *et al.*, 1994; Dé *et al.*, 1995) and showed a strong homology (94% identity) between the deduced amino acid sequences of the two OprF proteins. The high homology between the two OprF proteins allowed us to use a monoclonal antibody (MA28B9) against the OprF of *P. fluorescens* OE 28.3 (De Mot *et al.*, 1994) and to compare its reactivity with the OprF of strain MF0 produced at the two temperatures. Fig. 4 shows the reactivity of the *P. fluorescens* MF0 OM extracted from 28 °C and 8 °C cultures (lanes 1–4) after SDS-PAGE and electrotransfer onto nitrocellulose membrane and the reactivity of the purified proteins (lanes 5–8). After immunodetection, no difference was found between these samples, showing that the epitope was conserved.

Progress of proteolytic degradation by trypsin of OprFs purified from 8 °C and 28 °C cultures of *P. fluorescens* MF0 was followed by SDS-PAGE (Fig. 5a, b). Time-dependent sampling (3 min, 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h) was performed simultaneously for both proteins. After 3 min incubation (Fig. 5, lane 4) trypsin digestion seemed to generate the same proteolytic fragment of M_r 28000 for both porins. It is noteworthy that the disappearance of OprF purified from the 8 °C culture occurred more rapidly than for the 28 °C preparation: after 1 h (Fig. 5, lane 6) the 8 °C porin had completely disappeared, whereas the 28 °C one still remained after 2.5 h incubation (Fig. 5, lane 9). Cleavage sites for trypsin seem to be less accessible for the 28 °C OprF, suggesting a difference of tertiary structure between the 8 °C and 28 °C major OM proteins.

Fig. 5. Trypsin digestion kinetics of OprF from *P. fluorescens* MF0 depending on the growth temperature. SDS-PAGE (7/15%) of samples was carried out after the addition of TLCK to stop the digestion. (a) Trypsin digestion of OprF purified from a 28 °C culture. (b) Trypsin digestion of OprF purified from an 8 °C culture. Lane 1, purified OprF; lane 2, trypsin; lane 3, after incubation for 3 min; lane 4, after 0.5 h; lane 5, after 1 h; lane 6, after 1.5 h; lane 7, after 2 h; lane 8, after 2.5 h; lane 9, after 3 h.

DISCUSSION

Previous studies have shown that the psychrotrophic bacterium *P. fluorescens* MF0 is more sensitive to the β -lactam mezlocillin at 8 °C than at higher growth temperatures. This difference seems to originate from a modification of the OM permeability depending on the growth temperature and in particular an increasing permeability allowing induction of β -lactamase synthesis at high growth temperatures (Orange, 1994).

Some authors have described the effects of temperature on bacterial OM proteins. An effect of the growth temperature on *E. coli* OM proteins was studied by Lugtenberg *et al.* (1976). They showed that the quantity of osmoregulated OmpC porin increased with increasing growth temperature concomitant with a decrease in the amount of the OmpF porin. Few studies are available on the effect of growth temperature on *Pseudomonas aeruginosa* OM proteins. Kropinski *et al.* (1987) observed some modifications of the OM protein profiles: at low growth temperatures there was an increase in a polypeptide with M_r 43000 and a decrease in the content of M_r 21000 (protein H_1) and M_r 27500 proteins.

However, these data concern mesophilic bacteria and only few data are given for psychrotrophic bacteria. Our experiments show that these variations in the composition and the relative proportion of OM proteins are not observed for *P. fluorescens*.

The major OM porin, the OprF-like protein (Dé *et al.*, 1995), was also studied. It could indeed be responsible, as in *P. aeruginosa*, for most of the non-specific OM permeability properties (Nicas & Hancock, 1983). We demonstrated here that the conductance values of the channels formed by OprF extracted from cultures at the higher growth temperature (250–270 pS at 28 °C) are about threefold higher than these obtained with a porin produced at low temperature (80–90 pS at 8 °C). These results indicate that the pore size is larger for the 28 °C OprF, which is compatible with previous studies on OM permeability (Orange, 1994). In other respects, the highest conductance values (for the 28 °C OprF) determined in our results are in good agreement with the main value of 340–380 pS (in 1 M KCl) found for OprF from *P. aeruginosa* and also with the value clearly defined (280 pS in 1 M KCl) for *P. syringae* OprF (Woodruff *et al.*, 1986; Saint *et al.*, 1992; Ullstrom *et al.*, 1991). These low conductance values are not surprising in pseudomonads. Porins of *P. aeruginosa*, OprE₁, OprC, OprD₂, have also been shown (Yoshihara & Nakae, 1989) to form small pores. OprE₁ shows single channel conductance of 230 pS in 1 M KCl (from a culture of *P. aeruginosa* at 37 °C) and has no known substrate specificity (Obara & Nakae, 1992). Conductance values for OprC are still not known, but it seems to form small non-specific channels (Nakae, 1995). Among these porins, only OprD₂ is specific and is involved in the transport of basic amino acids (Trias & Nikaido, 1990). This protein is known to display two types of behaviour. Indeed it exhibits in DPhPC lipid bilayers a major closed state of the channel with conductivity of 30 pS in 1 M NaCl and in DPhPC-LPS bilayers a major open state with conductance value of 400 pS in 1 M NaCl. This suggests that OprD₂ behaves differently according to the presence or absence of LPS in bilayers. LPS could thus play a role in the regulation of the two states of the channel (Ishii & Nakae, 1993). This particular variation in behaviour is observed for the optimal growth temperature in a mesophilic strain. On the other hand, in *P. fluorescens* psychrotrophic strains, the size of channels in OprF fluctuates with growth temperature, just as the phosphorylation state of LPS fluctuates in the psychrotrophic *P. syringae* (Ray *et al.*, 1994). Structural modifications can be proposed to explain this functional change in the size of channels. Indeed the tertiary structure of these porins varies with the growth temperature, which is demonstrated here by a modification of accessibility of the cleavage sites to trypsin.

Such different states for the major OM porin OprF could explain the decreasing OM permeability at low temperatures already described (Orange, 1994). This particular behaviour of *P. fluorescens* MF0 can be related to the psychrotrophic nature of this bacterium, although there

are similarities with the mesophilic bacterium *E. coli*. In *E. coli* the OM permeability is reduced when the environmental temperature increases, after human infection for example. Indeed the proportion of OmpC (smaller channel size) in the OM increases in relation to OmpF (larger channel size). This reaction protects *E. coli* against toxins (e.g. bile salts) which are found in the human digestive tract. The response of *P. fluorescens* MF0 to a temperature that falls under its optimum growth temperature could have a similar explanation. When *P. fluorescens* colonizes soil, water or plants it could decrease the OM permeability to avoid the entry of toxic molecules such as xenobiotics or toxins from plants. This effect is counterbalanced by the overproduction, at low temperature, of exocellular enzymes (lipases, proteases for example; Gügi *et al.*, 1991) for degrading macromolecules into small units which provide *P. fluorescens* MF0 with carbon and energy sources.

The adaptation to environmental conditions which is described here for two major OM porins extracted from strains found in habitats as different as soil and milk could be a general feature of the OprFs from psychrotrophic *P. fluorescens*. While the behaviour of the minor porins in response to the growth temperature is still not known, their contribution to the phenomenon cannot be ruled out.

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