The role of dor gene products in controlling the P2 promoter of the cytochrome c\textsubscript{2} gene, cycA, in \textit{Rhodobacter sphaeroides}

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This study explores the regulatory networks controlling anaerobic energy production by the facultative phototroph \textit{Rhodobacter sphaeroides}. The specific aim was to determine why activity of the P2 promoter for the gene (cycA) encoding the essential photosynthetic electron carrier, cytochrome c\textsubscript{2}, is decreased when the alternative electron acceptor DMSO is added to photosynthetically grown cells. The presence of DMSO is believed to activate the DorR response regulator, which controls expression of proteins required to reduce DMSO. A DorR\textsuperscript{−} strain showed no change in cycA P2 promoter activity when DMSO was added to photosynthetic cells, indicating that DorR was required for the decreased expression in wild-type cells. To test if DorR acted directly at this promoter to change gene expression, recombinant DorR was purified and studied \textit{in vitro}. Preparations of DorR that were active at other target promoters showed no detectable interaction with cycA P2, suggesting that this protein is not a direct regulator of this promoter. We also found that cycA P2 activity in a DorA\textsuperscript{−} strain was not decreased by the addition of DMSO to photosynthetic cells. A model is presented to explain why the presence of a functional DMSO reductase (DorA) is required for DMSO to decrease cycA P2 expression under photosynthetic conditions.

INTRODUCTION

Facultative bacteria are able to respire using oxygen or alternative electron acceptors such as nitrate, dimethyl sulfoxide (DMSO) or trimethylamine N-oxide (TMAO). Generally, electron acceptors other than oxygen are only used for energy generation when oxygen is lacking, due to tight regulation of alternative respiration pathways. Thus, the expression of the genes encoding factors necessary for the use of these alternative electron acceptors is often controlled by specific transcription factors that are part of larger regulatory networks.

We are studying transcriptional regulatory circuits that control respiratory pathways in the facultative phototroph \textit{Rhodobacter sphaeroides}. This \textit{x}–proteobacterium has a choice of anaerobic energetic lifestyles: it can grow via photosynthesis if light is present or it can use DMSO as an alternative electron acceptor in the absence or presence of light. The decision of which anaerobic energy production strategy to use adds complexity to the regulation of the metabolic lifestyle of this bacterium. In fact, there appears to be a hierarchy of regulation since the addition of alternative electron acceptors to photosynthetic cultures of \textit{R. sphaeroides} decreases the expression of genes encoding the photosynthetic apparatus (Karls \textit{et al.}, 1999; Oh & Kaplan, 1999). This work was aimed at providing a molecular explanation for how the alternative electron acceptor, DMSO, decreases expression of these photosynthesis genes under anaerobic conditions.

To analyse how DMSO decreases photosynthesis gene expression, we used the P2 promoter for the cytochrome c\textsubscript{2} gene, cycA, because its activity was previously found to be significantly lower under these conditions (Karls \textit{et al.}, 1999). Expression of cycA P2 has been shown to be directly activated by the response regulator PrrA, as are other genes encoding other components of the \textit{R. sphaeroides} photosynthetic apparatus (Eraso & Kaplan, 1994; Pemberton \textit{et al.}, 1998). The mechanism by which cycA P2 expression is decreased by DMSO under photosynthetic conditions was unknown, but one possibility was that electron transport to DMSO decreased activation of this promoter by PrrA. PrrA activity responds to the oxidation–reduction state of the electron-transport chain to increase expression of some photosynthesis genes under anaerobic conditions (Zeilstra-Ryalls \textit{et al.}, 1998). Alternatively, we previously hypothesized that the DorR response regulator, which is required for expression of genes in the DMSO respiratory...
pathway, could be acting to repress cycA P2 transcription in response to DMSO under anaerobic conditions (Karls et al., 1999).

Our results show that DorR is required for DMSO to decrease the expression of cycA P2 during photosynthetic growth in the presence of DMSO, even though active preparations of this protein failed to interact directly with cycA P2. In addition, we found that the DMSO reductase, DorA, is required for the decreased expression from cycA P2 during photosynthetic growth in the presence of DMSO. A model is presented to explain how DMSO reductase function can lead to a decrease in cycA P2 expression when DMSO is present under photosynthetic conditions.

METHODS

Strains and growth conditions. Photosynthetic cultures of *R. sphaeroides* 2.4.1 were grown in Sistrom’s minimal medium A at 30 °C in filled, tightly capped 16 ml tubes at 10 W m⁻² light intensity (Sistrom, 1960). To maintain plasmid pRKK232 in *R. sphaeroides*, kanamycin was supplied at 25 μg ml⁻¹. Where indicated, the medium was supplemented with 0.625% DMSO. *Escherichia coli* DH5α and ER2566 (Table 1) were grown at 37 °C in Luria–Bertani medium with ampicillin (100 μg ml⁻¹), kanamycin (50 μg ml⁻¹) or tetracycline (10 μg ml⁻¹) as needed. DH5α was used for cloning and plasmid maintenance in *E. coli*. ER2566 was used as a source of recombiant DorR.

Expression and purification of DorR. The coding sequence of *dorR* was cloned into the pTYB1 intein/chitin-binding domain fusion system vector (New England Biolabs). A 727 bp PCR product containing the *dorR* coding sequence was amplified with *Pfu* (Stratagene) from pNMT16 using the DorR-1 (5'-CTCTCGCGCAT-ATGAAAGAAAAACTACACCATGC-3') and DorR-2 (5'-GGTCTGCTCTTCCGCAAGGCCCTGAAGCTAGCC-3') primers. DorR-1 adds an *NdeI* restriction site that overlaps the ATG start codon of *dorR*. DorR-2 adds one cysteine codon (GCA) to the end of *dorR*, followed by one extra base (C) and a *SapI* restriction site. The PCR product was digested with *NdeI* and *SapI* and ligated into *NdeI/SapI*-digested pTYB1, to make a plasmid (pC403) containing an in-frame fusion of DorR to the intein/chitin-binding domain.

To purify DorR, cultures of ER2566(pC403) were shaken for 6 h at 25 °C after addition of 0.3 mM IPTG. Cells were harvested by centrifugation, resuspended in 15 ml column buffer (20 mM Tris/HCl, pH 8-0, 0.1 mM EDTA, 0.5 mM KCl, 0.1 mM Triton X-100), and sonicated (eight times for 1 min each at 50% duty cycle) to lyse the cells. The cell lysate was centrifuged at 12 000 g for 30 min at 4 °C, and the supernatant loaded onto a 13 ml chitin column that was equilibrated with column buffer. Next, the column was washed with 10 volumes of column buffer, followed by a 2.5 volume wash with cleavage buffer (20 mM Tris/HCl, pH 8-0, 0.1 mM EDTA, 0.5 mM KCl, 100 mM DTT) before storage overnight at 4 °C to permit intein self-cleavage. DorR was eluted with 3 volumes of column buffer, and fractions were analysed by SDS-PAGE. Fractions containing DorR (~3 ml) were combined and dialysed against two changes of 1 l of 50 mM HEPES-Na, pH 7-8, 200 mM KCl, 10 mM MgCl₂, 1 mM DTT, 50 μM PMSE, followed by dialysis into this buffer with 50% (v/v) glycerol and storage at ~80 °C.

Phosphorylation of DorR. To assay autophosphorylation of DorR, the reaction was initiated by the addition of 30 mM ³²P-labelled acetyl phosphate to 10 μM DorR in 50 mM Tris/HCl, pH 7-0, 5 mM MgCl₂, 0.1 mM DTT, 0.1 mg BSA ml⁻¹ and incubated at 30 °C (McCleary & Stock, 1994). For the time-course experiment, the reaction volume was 90 μl; a 10 μl aliquot was removed at each time point and added to 5 μl of 3x SDS sample buffer (150 mM Tris/HCl, pH 6-8, 30 mM DTT, 6% SDS, 0-3%
bromophenol blue, 30% glycerol) on ice. Samples were analysed on a 12% SDS-polyacrylamide gel, followed by drying the gel and overnight exposure to a phosphorscreen.

**Isolation of promoter fragments.** The 215 bp dorCBA promoter fragment was generated by PCR using the DorC-1 (5’-GGGAGA-GCTTGGGGCGTGGACCTAATGC-3’) and DorC-2 (5’-CCCGATCGCGAGATGCGGAGG-3’) primers, using pNMT16 as a template (Mouncey et al., 1997). The PCR product was purified from an agarose gel using a Qiagen Gel Extraction Kit. The cycA P2 reporter fragments were obtained by restriction digestion of cycA as a template (Mouncey et al., 1997). The promoter fragments were isolated by phenol extraction (Mouncey et al., 1997) in the presence of 50-fold excess non-specific competitor AGCTTGCGGCCGGTTGGACCTAATGC-3’ and analyzed on a 6% TBE polyacrylamide gel that had been pre-run for 30 min. Binding reactions were incubated at room temperature for 30 min and analysed at 250 V, 4°C without addition of acetyl phosphate. Binding reactions were incubated with 25 mM acetyl phosphate at 30°C for 1 h. In control reactions, DorR was also incubated at 30°C for 1 h in binding buffer, followed by the addition of DorR or DorR that had been previously incubated with 25 mM acetyl phosphate at 30°C for 1 h. In control reactions, DorR was also incubated at 30°C for 1 h without addition of acetyl phosphate. Binding reactions were incubated at room temperature for 30 min and analysed at 250 V, 4°C on a 6% TBE polyacrylamide gel that had been pre-run for 30 min.

**Gel shift assays.** For gel shift assays, DNA fragments were end-labelled with [γ-32P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (Promega), following the manufacturer’s instructions. Free [γ-32P]ATP was removed using a Qiagen Nucleotide Removal Kit (Qiagen).

DNA binding reactions were performed in binding buffer (50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.25 M sucrose, 0.025% bromophenol blue) in the presence of 50-fold excess non-specific competitor (λ DNA). Labelled promoter fragment DNA (8-8 nM) was added to binding buffer, followed by the addition of DorR or DorR that had been previously incubated with 25 mM acetyl phosphate at 30°C for 1 h. In control reactions, DorR was also incubated at 30°C for 1 h without addition of acetyl phosphate. Binding reactions were incubated at room temperature for 30 min and analysed at 250 V, 4°C on a 6% TBE polyacrylamide gel that had been pre-run for 30 min.

**Analysis of cycA P2 reporter activity.** Levels of β-galactosidase activity were measured from independent cultures of exponential-phase R. sphaeroides (pRKK232) cells using established protocols (Schilke & Donohue, 1995).

### RESULTS

**DorR is required for decreased cycA P2 expression in the presence of DMSO**

Previous experiments have shown that activity of cycA P2 is decreased when the alternative electron acceptor DMSO is added to photosynthetic cells (Karls et al., 1999). This negative effect of DMSO on cycA P2 activity is not due to an alteration in the generation time of cells under photosynthetic conditions (Fig. 1). We used strains containing a cycA P2 reporter gene to determine if this phenomenon was dependent on the presence of the response regulator, DorR. As expected, expression from a cycA P2::lacZ fusion decreased approximately twofold when DMSO was added to a photosynthetic culture of wild-type cells (Fig. 1). However, in the DorR" strain, expression from this reporter gene under photosynthetic conditions is independent of the presence of DorR (Fig. 1). Thus, it appears that DorR is required for the decrease in cycA P2 expression that is seen in wild-type cells in response to DMSO.

**Purification of recombinant DorR**

In order to determine if DorR acts directly at cycA P2, recombinant R. sphaeroides DorR was purified from E. coli as an intein fusion. This approach was successful for purification of other R. sphaeroides response regulators (Comolli et al., 2002), and DorR was obtained at >95% purity by SDS-PAGE analysis (data not shown).

DorR accepted a phosphoryl group from 32P-labelled acetyl phosphate to generate phosphorylated DorR (P~DorR). This incubation resulted in a time-dependent increase in the level of 32P-radioactivity associated with DorR (P~DorR). Under the tested conditions, maximal autophosphorylation of DorR occurred after between 20 and 30 min of incubation (Fig. 2b). These data suggest that purified R. sphaeroides DorR has biological activity typical of other response regulators and can be phosphorylated using acetyl phosphate as a phosphodonor in vitro.

**DorR binds to the dorR–dorCBA promoter region**

It has been proposed that activation of the dorCBA operon by DorR occurs via its interaction with four putative DorR binding sites (consensus sequence C[T/G]GT[T/A]CJACJC) between the divergently transcribed dorR and dorCBA promoters (Mouncey & Kaplan, 1998). To determine if DorR interacts with the dorR–dorCBA intergenic region, we tested the ability of DorR to bind a 215 bp DNA fragment containing both of these promoters in a gel shift assay (Fig. 3a). This fragment includes all four putative DorR binding sites and the start of transcription (Fig. 1). Analysis of the fragments for each condition.

[Fig. 1. β-Galactosidase activity (Miller units) from a cycA P2::lacZ reporter gene in wild-type, DorR"-, DorCBA"- or DorA"- cultures grown photosynthetically in the absence (PS, black bars) or presence (PS+DMSO, grey bars) of 0.625% DMSO. Values are the mean of three independent cultures. Bars indicate standard deviation. Generation time (h) is shown below the strains for each condition.]

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Both purified DorR and DorR that had been incubated with acetyl phosphate altered the mobility of the dorR–dorCBA intergenic fragment. In addition, multiple shifted species of DNA fragment were seen, a result consistent with DorR binding to multiple DorR binding sites in this fragment. Concentrations of DorR as low as 0.05 μM caused significant retardation (I) of this promoter fragment (Fig. 3a; lane 2). At 0.5 μM DorR, a second, slower-migrating species (II) was visible, suggesting that the protein may be binding the DNA at multiple sites (Fig. 3a, lane 5). DorR incubated with acetyl phosphate led to the formation of similar species (I, II) as untreated DorR (Fig. 3a, lanes 9–15), but reproducibly less protein (0.2 μM as compared to 0.5 μM DorR) was required to completely shift the promoter fragment. Thus, it appears that treatment of DorR with acetyl phosphate increased its affinity for this DNA (Fig. 3a, lanes 11 and 5, respectively).

**DorR does not interact with the cycA P2 promoter**

Since the cycA P2 promoter contains a sequence similar to a DorR binding site (sequence CTAGTCACATC) centred around 88 nt upstream of the start of transcription (Karls et al., 1999), we also tested if the negative effect of DMSO on this promoter resulted from a direct interaction with this protein. Two cycA P2 promoter fragments were tested: one including the sequence from −73 to +22 (data not shown) and the other from −228 to +22 relative to the start of transcription, both of which had demonstrated a DMSO-dependent decrease in promoter activity (Karls et al., 1999). At concentrations of up to 2 μM protein (∼40 times higher than the amount necessary to detect binding

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**Fig. 2.** (a) Time-course of in vitro phosphorylation of DorR by 32P-labelled acetyl phosphate. (b) Quantified band intensities with background subtracted for each time point.

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**Fig. 3.** (a) Gel shift assay demonstrating the interaction of DorR with the dorR–dorCBA promoter fragment. Two shifted species are designated (I and II). Concentrations of DorR are as follows: lane 1, 0 μM; lanes 2 and 9, 0.05 μM; lanes 3 and 10, 0.1 μM; lanes 4 and 11, 0.2 μM; lanes 5 and 12, 0.5 μM; lanes 6 and 13, 0.7 μM; lanes 7 and 14, 1.0 μM; lanes 8 and 15, 1.5 μM. For lanes 9–15, DorR was incubated with 25 mM acetyl phosphate for 1 h before use in the gel shift assay. (b) Gel shift assay of DorR and the cycA P2 promoter fragment (see Methods). Concentrations of DorR are as follows: lane 1, 0 μM; lane 2, 0.25 μM; lanes 3 and 6, 0.5 μM; lanes 4 and 7, 0.75 μM; lanes 5 and 8, 1.0 μM; lane 9, 2.0 μM. For lanes 2–8, DorR was incubated with 25 mM acetyl phosphate for 1 h before use in the gel shift assay.
to *dorCBA*), neither DorR nor DorR treated with acetyl phosphate caused a shift in the mobility of either *cycA* P2 promoter fragment (Fig. 3b and data not shown). From this analysis, we conclude that neither DorR nor its phosphorylated counterpart interacts with *cycA* P2 under conditions where this protein can bind to the *dorR–dorCBA* intergenic region.

**DMSO reductase activity is required for DMSO to decrease *cycA* P2 expression**

Given the apparent inability of DorR to interact with *cycA* P2, it was likely that the effect of DMSO on *cycA* expression was indirect. One possibility is that this effect depends on the function of a protein that requires DorR for its synthesis or activity; for instance, one of the *dorCBA* gene products (Mouncey & Kaplan, 1998). To determine if any *dorCBA* gene products are required for the DMSO-dependent decrease in expression from *cycA* P2::*lacZ* under photosynthetic conditions, we tested expression of this promoter in cells containing an insertion in *dorC* that has been shown to be polar onto *dorBA* (Mouncey *et al.*, 1997). In the DorR− strain, the presence of DMSO did not decrease activity of *cycA* P2 under photosynthetic conditions (Fig. 1), suggesting that one or more *dorCBA* gene products were required for this effect. DMSO also did not alter the activity of the *cycA* P2 reporter in DorR− cultures under photosynthetic conditions. In addition, *cycA* P2 expression levels in the DorR− strain were comparable to those of the DorR+ strain and to those seen when wild-type cells are grown photosynthetically without DMSO (Fig. 1). Thus, the negative effect of DMSO during photosynthetic growth on *cycA* P2::*lacZ* appeared to require the presence of DorA. Considering these results together with the lack of DorR binding to this promoter, we conclude that the loss of DorR indirectly affects this promoter through the inability to activate *dorCBA* expression. A model to explain the role of DorA in this response is presented below.

**DISCUSSION**

This study sought to gain insight into how DMSO diminishes *cycA* P2 expression under photosynthetic conditions. Unlike other well-studied facultative anaerobes that can use DMSO as an electron acceptor, *R. sphaeroides* has the option of growing anaerobically by photosynthesis. Thus, the anaerobic energy lifestyles of this *α*-proteobacterium are likely to be governed by unique regulatory hierarchies.

**Is DorR necessary for DMSO to decrease *cycA* P2 expression in *R. sphaeroides*?**

Consistent with the hypothesis that DorR was both an activator of *dorCBA* and a repressor of *cycA* P2 expression, a DorR− strain does not show a DMSO-dependent decrease in *cycA* P2 expression during photosynthetic growth. Also, DorR expression is low during aerobic growth (Mouncey & Kaplan, 1998), when *cycA* P2 expression has been seen to be unaffected by DMSO (J. C. Comolli & T. J. Donohue, unpublished). However, DorR failed to interact with *cycA* P2, though it did interact with another putative target sequence, the *dorR–dorCBA* intergenic region, suggesting that DorR is not repressing transcription at this photosynthesis promoter.

If DorR does not act directly at *cycA* P2, why is the DorR− strain unable to decrease expression of this promoter in response to DMSO? Cells lacking the DMSO reductase (because of a mutation in *dorA*) also do not show a detectable DMSO-dependent decrease in *cycA* P2 expression, which is not significantly different from the results seen in the DorR− mutant. Thus, the inability of the DorR mutant to activate *dorCBA* transcription in the presence of DMSO is probably the reason why *cycA* P2 activity is not decreased in this strain (Mouncey & Kaplan, 1998).

**How might the function of DorA, the DMSO reductase, alter *cycA* P2 expression anaerobically in the presence of DMSO?**

When an alternative electron acceptor, such as DMSO, is present, electron flow through the photosynthetic apparatus should be altered, according to what is known about anaerobic electron transport in this bacterium. During photosynthesis in *R. sphaeroides*, electrons are carried by quinones from the photochemical reaction centre to the cytochrome *bc* complex to generate a proton gradient. These electrons are then used by cytochrome *c* to reduce the photo-oxidized bacteriochlorophyll molecules in the reaction centre. It is likely that the reduced quinone generated during photosynthetic growth can be oxidized either by the cytochrome *bc* complex or by DorC, a membrane-bound *c*-type cytochrome that is proposed to act as an electron donor to DMSO reductase, DorA (Shaw *et al.*, 1999). Then, shuttling of reductant to DorA under photosynthetic growth conditions could alter the activity of PrrA, a global response regulator known to increase *cycA* P2 activity under these conditions (Karls *et al.*, 1999). PrrA is required for expression from *cycA* P2 and for photosynthetic growth (Karls *et al.*, 1999). It is believed that the PrrBA two-component system (homologous to the RegRA, RegSR and RoxRS systems in other bacteria) monitors changes in the oxidation–reduction state of the electron-transport chain, to control photosynthesis gene expression (Comolli & Donohue, 2002; Emmerich *et al.*, 2000; Eraso & Kaplan, 1994; Oh & Kaplan, 2000). Consequently, our model predicts that when electrons are diverted from the electron-transport chain by the reduction of alternative electron acceptors like DMSO, PrrB/PrrA activity may be modulated to decrease the expression of target genes like *cycA* P2.

In addition to *cycA*, other photosynthesis genes have been shown to be negatively affected by the presence of alternative electron acceptors. For example, the levels of light-harvesting complexes and reaction centres are decreased when electron acceptors like DMSO, nitrate or carbon dioxide are present during photosynthetic growth of cultures...
that are supplied with a fixed carbon source (Michalski et al., 1985; Oh & Kaplan, 1999). As expected, this decrease in the levels of light-harvesting complex was not detected in DorA− or DorR− strains under photosynthetic conditions in the presence of DMSO (data not shown). Since these external electron acceptors share the ability to siphon reducing power from the electron-transport chain, it is possible that they act by a common mechanism to decrease the activation of photosynthesis genes, potentially via PrrA.

**Interactions of DorR with dorCBA**

In *Rhodobacter sphaeroides*, DorR is a member of the DorSR two-component regulatory system, whose activity is thought to be stimulated by the presence of DMSO (Mouncey & Kaplan, 1998). The properties of DorR− mutants predict that DorR is required for activation of the dorCBA operon, which encodes a c-type cytochrome (DorC), a putative membrane protein of unknown function (DorB), and the DMSO reductase enzyme (DorA) (Mouncey & Kaplan, 1998; Mouncey et al., 1997). The ~100 bp of DNA between the divergent *dorCBA* and *dor* open reading frames contains four proposed DorR binding sites (Mouncey & Kaplan, 1998) that share sequence similarity with elements bound by the related response regulator, TorR, in *E. coli* (Simon et al., 1994). TorR directly controls expression of the genes for TMAO reductase (Ansaldi et al., 2000). A similar DMSO-responsive system has been found in *R. sphaeroides* f. sp. *denitrificans*, which includes the DorR homologue, DmsR, and the dmsCBA operon, which encodes a DMSO reductase (Ujiyi et al., 1997). Additionally, extracts of *E. coli* cells expressing DmsR contain a polypeptide that binds a dmsCBA promoter fragment in vitro, suggesting that this protein may be a DNA-binding protein (Ujiyi et al., 1997).

In the course of these experiments, we purified DorR, phosphorylated it using the low-molecular-mass phosphodonor acetyl phosphate, and showed that it formed a stable complex with DorR–dorCBA intergenic DNA. If DorR is binding to a series of elements in this DNA that are similar to sites recognized by the homologous *E. coli* TorR (Simon et al., 1995), it is possible that the different complexes observed with the dorR–dorCBA fragment reflect the formation of higher-order complexes between DorR and the target DNA, as proposed for TorR (Simon et al., 1995). While the treatment of DorR with acetyl phosphate may have increased its relative affinity for target DNA, additional experiments are needed to assess the functional significance of this observation.

**Concluding remarks**

In summary, we have demonstrated that both DorR and DMSO reductase (DorA) are required for the negative effect of DMSO on cycA P2 expression under photosynthetic conditions. Our data indicate that DMSO reductase activity is needed for the decline in expression of cycA P2 and possibly other photosynthesis genes under anaerobic conditions with DMSO. This effect may be due to changes in the activation state of the photosynthesis response regulator, PrrA, in response to alteration of electron flow through the electron-transport chain caused by the presence of DMSO. Further experiments are needed to determine if this sensing occurs via PrrA, and, if so, exactly how changes in electron flow are monitored in the absence of oxygen and what other genes involved in anaerobic energy generation in *R. sphaeroides* are regulated by the presence of alternative electron acceptors like DMSO.

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