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Cloning and Functional Expression in *Escherichia coli* of the Polyhydroxyalkanoate Synthase (*phaC*) Gene from *Alcaligenes* sp. SH-69

LEE, IL, SUN-WOO NAM, YOUNG-HA RHEE, AND JEONG-YOON KIM*

Department of Microbiology, Chungnam National University, Taejon 305-764, Korea

Alcaligenes sp. SH-69 can synthesize poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from a single carbon source such as glucose. To clone the *phaC* gene from *Alcaligenes* sp. SH-69, a polymerase chain reaction was performed using the oligomers synthesized based on the conserved regions of the *phaC* genes from other bacteria. A PCR product (550 bp) was partially sequenced and the deduced amino acid sequence was found to be homologous to that of the *phaC* gene from *Alcaligenes eutrophus*. Using the PCR fragment Southern blotting of *Alcaligenes* sp. SH-69 genomic DNA digested with several restriction enzymes was carried out. To prepare a partial genomic library, about 5-Kb genomic DNA fragments digested with *Eco*RI, which showed a positive signal in the Southern blotting, were eluted from an agarose gel, ligated with pUC19 cleaved with *Eco*RI, and transformed into *Escherichia coli*. The partial library was screened using the PCR fragment as a probe and a plasmid, named pPHA11, showing a strong hybridization signal was selected. Restriction mapping of the insert DNA in pPHA11 was performed. Cotransformation into *E. coli* of the plasmid pPHA11 and the plasmid pPHA21 which has *phaA* and *phaB* from *A. eutrophus* resulted in turbid *E. coli* colonies which are indicative of PHA accumulation. This result tells us that the *Alcaligenes* sp. SH-69 *phaC* gene in the pPHA11 is functionally active in *E. coli* and can synthesize PHA in the presence of the *A. eutrophus phaA* and *phaB* genes.

To understand the mechanisms of polyhydroxyalkanoate (PHA) synthesis by a variety of bacteria, studies on the metabolic pathways for PHA synthesis and the molecular structures and functions of PHA biosynthetic genes have been conducted by many research groups over the last decade (1, 13, 16, 22, 24, 27, 29). In an attempt to cost-effectively produce PHAs, medium formulations and cultivation methods for PHA producing bacteria or recombinant *Escherichia coli* have been investigated and efficient fermentation processes for PHA production have been developed (2, 4, 8, 9, 17, 18, 26). Since three independent groups cloned the PHA synthetic genes, *phaA* (beta-ketothiolase), *phaB* (NADPH-dependent acetoacetyl-CoA reductase), and *phaC* (PHA synthase) from *Alcaligenes eutrophus* (14, 23, 25), many PHA biosynthetic genes from different bacteria have been cloned (3, 5, 6, 10-12, 15, 21, 27, 31-35).

While polyhydroxybutyrate (PHB) produced by *A. eutrophus* has been the most extensively studied, commercial interest in the production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [poly(3HB-*co*-3HV)] has increased considerably because the copolyester has better physical properties than PHB. The production of poly (3HB-*co*-3HV) is usually achieved by providing bacteria with a cosubstrate along with a main carbon source. Recently, it was reported that *Alcaligenes* sp. SH-69, *Rhodococcus ruber*, and a mutant strain of *A. eutrophus* could synthesize poly(3HB-*co*-3HV) from glucose without any secondary carbon sources such as propionate and valerate (9, 15, 27, 36). The synthesis of poly(3HB-*co*-3HV) from single unrelated carbon sources has advantages over poly(3HB-*co*-3HV) production from a carbon source and cosubstrates in that the former process will save substrate costs and facilitate fermentation processes (18, 28).

The metabolic pathways for poly(3HB-*co*-3HV) synthesis in *Alcaligenes* sp. SH-69 have not been investigated, but the result that amino acid addition to the culture medium increases the molar fraction of 3HV in the poly(3HB-*co*-3HV) synthesized by *Alcaligenes* sp. SH-69 (36) implies that the ability of *Alcaligenes* sp. SH-69 to synthesize poly(3HB-*co*-3HV) from a single unrelated carbon source may be due to efficient for-

*Corresponding author
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mation of propionyl-CoA or 3-hydroxyvaleryl-CoA. However, the possibility of different substrate specificities or distinct charateristics of the PHA synthesizing enzymes from *Alcaligenes* sp. SH-69 cannot be excluded. In order to investigate the molecular structures of the PHA synthetic genes and properties of the PHA synthesizing enzymes of *Alcaligenes* sp. SH-69, we cloned the *phaC* gene from *Alcaligenes* sp. SH-69 and functionally expressed the gene in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

Alcaligenes sp. SH-69, which was isolated from an activated sludge sample, and *E. coli* DH5α [F^- Φ80d*lacZ* DM15 Δ(*lacZYA-argF*) *U*169 *hsdR*17 ($r_k^-m_k^+$) *deoR recA*1 *supE*44 λ-*thi*-1 *gyrA*96 *relA*1] were used. The plasmids used in this study are listed in Table 1. *Alcaligenes* sp. SH-69 was grown at 37°C in a rich medium containing yeast extract 1%, peptone 1%, and meat extract 0.5%. *E. coli* was cultivated at 37°C in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml), tetracycline (10 μg/ml) or glucose (20-30 g/l) when needed. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were used at concentrations of 20 and 40 μg/ml, respectively.

DNA Manipulation

Total genomic DNA of *Alcaligenes* sp. SH-69 was isolated according to the method described in ref. 30. Plasmid DNA was isolated by using the alkaline lysis method and DNA restriction digestions and ligations were performed as described by Sambrook *et al.* (19). Transformations into *E. coli* were carried out using the method of Inoue *et al.* (7).

Polymerase Chain Reaction

Total genomic DNA of *Alcaligenes* sp. SH-69 was used as the template for PCR with the primers synthesized based on the conserved regions of the *phaC* genes from other bacteria. The primers were 5'-CCGCCG(C)TGG(C)ATCAACAAGTAG-3' and 5'-GT(C)TG(C)GTGG(C)T(C)GTCC(T)C(T)CGTTCC-3' which correspond to 254-260 and 435-440 amino acids of the *A. eutrophus* PHA synthase, respectively. PCR was carried out by using the Taq DNA polymerase (Perkin-Elmer Cetus, USA) in a PCR machine (Perkin-Elmer Cetus, USA). The amplification was carried out by running 30 cycles of denaturation (94°C, 1 min), annealing (53°C, 1 min), and extension (72°C, 1.5 min).

Southern Hybridization

Alcaligenes sp. SH-69 chromosomal DNA was digested to completion with appropriate restriction enzymes and electrophoresed on 0.8% agarose gels. DNA was blotted to Hybond-nylon membrane (Amersham, UK) by the alkaline transfer procedure (19). The PCR clone isolated and purified by Gene Clean II Kit (Bio101, USA) was used as a probe. Hybridization and washing of the membrane was carried out according to the instructions for ECL-DNA labelling, detection system kit (Amersham, UK).

Partial Library Construction and Screening

To prepare a partial genomic library, about 5-kb chromosomal DNA fragments digested with *Eco*RI, which showed a positive signal in Southern hybridization, were eluted from an agarose gel and ligated with pUC19 cleaved with *Eco*RI. The ligated DNAs were transformed into *E. coli*. The partial genomic library was screened by Southern hybridization using the PCR fragment as a probe. The insert DNA of the selected plasmid was analyzed by various restriction endonucleases and a restriction map of the insert DNA was prepared.

DNA Sequence Analysis

Sequencing of the DNA fragment cloned into pBluescript II KS+ was performed by the dideoxy chain termination method of Sanger *et al.* (20) with [α-^{35}S]dATP using a Sequenase Kit (U.S. Biochemical Corp., Cleveland, Ohio) according to the manufacturer's protocol. The nucleotide and deduced amino acid sequences of the PCR clone were compared with those of other bacteria listed in the National Center for Biotechnology Information (NCBI) database with the BLASTN or BLASTX program.

Table 1. Characteristics of plasmids used in this study.

Plasmid	Relevant characteristics	Source
pBluescript II KS+	cloning vector, Ap^r	Stratagene
pBluescript-tac	pBluescript II KS+ with tac promoter	This study
pUC19	cloning vector, Ap^r	NEB
pSYL101	pGEM-7Zf+ with *phaC*, *phaA*, and *phaB* of *A. eutrophus*, Ap^r	S.Y. Lee
pPHAAB	pBluescript II KS+ with *phaA* and *phaB* of *A. eutrophus* and tac promoter	This study
pLG339	low copy plasmid with replication origin of pSC101, Km^r, Tc^r	Lab collection
pPHA11	pUC19 with 5 kb *Eco*RI fragment of *Alcaligenes* sp. SH-69, Ap^r	This study
pPHA12	pBluescript II KS+ with 3 kb *Eco*RI-*Stu*I fragment of *Alcaligenes* sp. SH-69, Ap^r	This study
pPHA21	pLG339 with *phaA* and *phaB* of *A. eutrophus* and tac promoter. Tc^r	This study

RESULTS

PCR Cloning of the *phaC* Gene Fragment from *Alcaligenes* sp. SH-69

In order to clone the *phaC* gene fragment from *Alcaligenes* sp. SH-69, polymerase chain reaction was performed using the primers synthesized based on the conserved region of the *phaC* genes of other bacteria. The expected size of the PCR-amplified DNA fragment was about 560 bp and a DNA fragment of between 500 and 600 bp in size was amplified under optimum PCR conditions. The PCR clone was inserted into pBluescript II KS+ and sequenced. The comparison of the deduced amino acid sequence of the PCR clone with those of the PHA synthases from other bacteria is shown in Fig. 1. About 70% identity in amino acid sequence was found between the PCR clone of *Alcaligenes* sp. SH-69 and the *phaC* gene of *A. eutrophus*, and the amino acids which are conserved in all the sequences from other bacteria are also present in the PCR clone of *Alcaligenes* sp. SH-69. From this result we can confirm that the PCR clone is a part of the *Alcaligenes* sp. SH-69 *phaC* gene.

Isolation and Restriction Mapping of the *phaC* Gene from Genomic DNA

In an attempt to clone the whole *phaC* gene from *Alcaligenes* sp. SH-69, the chromosomal DNAs cleaved with *Eco*RI, *Sma*I, *Pvu*II, *Pst*I, *Eco*RI/*Sma*I, and *Eco*RI/*Pvu*II were hybridized with the PCR clone. As shown in Fig. 2, single hybridization signals appeared from each restriction enzyme digested chromosomal DNA. Of the DNA bands showing hybridization signals, the *Eco*RI-cut 5 kb DNA fragments which were expected to contain the *phaC* gene were excised from an agarose gel and inserted into pUC19 cut with *Eco*RI. Ten individual transformants that had an insert DNA were pooled and plasmid DNAs were isolated from each pool. After Southern hybridization of the isolated plasmid DNAs with the PCR clone as a probe, one pool which showed a strong hybridization signal was selected. Plasmid DNAs were isolated from each transformant belonging to the pool and Southern hybridization was again carried out using the same PCR clone as a probe. Finally, a recombinant plasmid (pPHA11) that contained the *phaC* gene fragment was selected and the insert DNA was further analyzed by restriction mapping. Fig. 3A shows

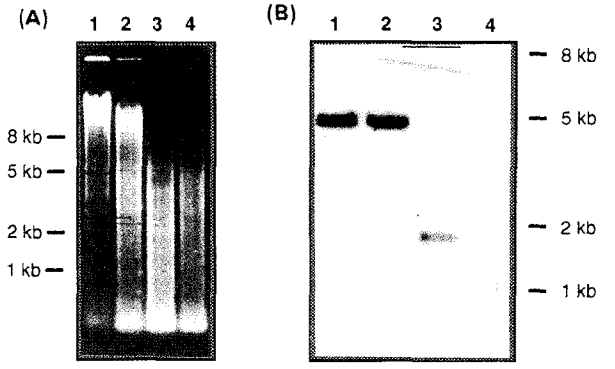

Fig. 2. Southern hybridization of *Alcaligenes* sp. SH-69 chromosomal DNA.
Chromosomal DNAs were completely digested with various restriction enzymes (A) and hybridized with the PCR clone (B). Lane 1, *Eco*RI; lane 2, *Sma*I; lane 3, *Pst*I; lane 4, *Pvu*II; lane 5, *Eco*RI/*Sma*I; lane 6, *Eco*RI/*Pvu*II.

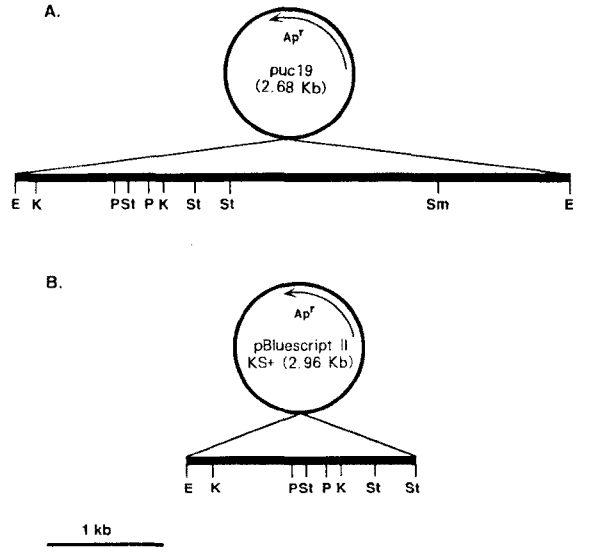

Fig. 3. Restriction map of the 5 kb (A) and the 2 kb (B) cloned fragments containing the *phaC* gene of *Alcaligenes* sp. SH-69.
E, K, P, Sm, and St are *Eco*RI, *Kpn*I, *Pst*I, *Sma*I, and *Stu*I, respectively.

Alcaligenes sp. SH-69	Y I L D L Q P E N S L I R Y A V E Q G H R T F V V S W R N P D D S L A H K T W D
A. eutrophus	Y I L D L Q P E S S L V R H V V E Q G H T V F L V S W R N P D A S M A G S T W D
M. extorquens	Y I L D L N P Q K S L I G W M V S Q G I T V F V I S W V N P D E R H R D K D F E
R. ruber	Y I L D L A P G R S L A E W A V Q H G R T V F M I S Y R N P D E S M R H I T M D
R. sphaeroides	Y I L D L K P Q N S L L K W L V D Q G F T V F V V S W V N P D K S Y A G I G M D
P. denitrificans	Y I L D L K P Q N S L I K W I V D Q G H T L F V V A W K N P D P S Y G D T G M D
Acinetobacter sp.	Y V L D L R E Q N S L V N W L R Q Q G H T V F L M S W R N P N A E Q K E L T F A
P. oleovorans (phaC1)	Y V F D L S P E K S L A R Y C L R S Q Q Q T F I I S W R N P T K A Q R E W G L S
P. aeruginosa (phaC1)	Y V F D L S P D K S L A R F C L R N G V Q T F I V S W R N P T K S Q R E W G L T

Fig. 1. Alignment of the deduced amino acid sequence of the PHA synthase from *Alcaligenes* sp. SH-69 with those of other bacteria.
Box indicates identical sequences present in all the sequences.

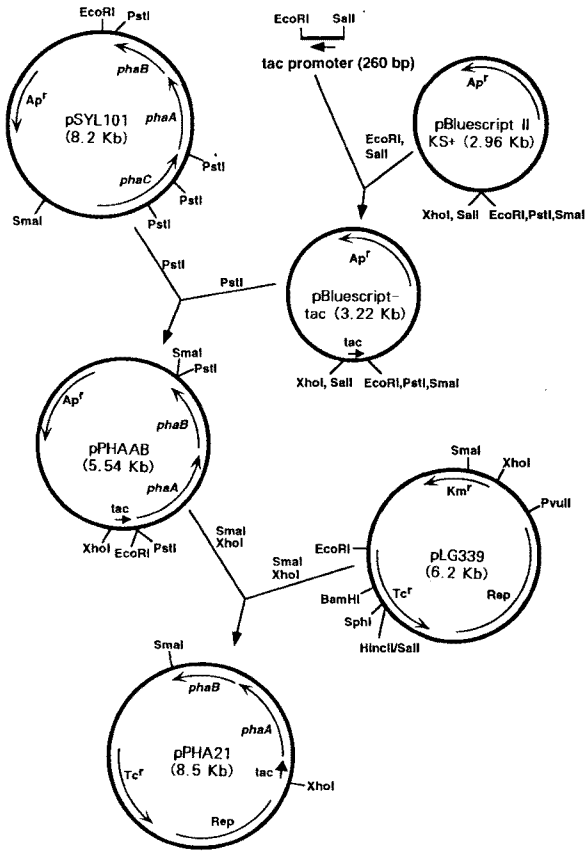

Fig. 4. Schematic representation of pPHA21 construction.
pPHA21 contains the *phaA* and *phaB* genes from *A. eutrophus* which are controlled by the tac promoter.

the restriction map of the insert DNA in pPHA11.

Functional Expression of the *phaC* Gene

Whether or not the cloned 5-kb fragment in pPHA11 contained the whole *phaC* gene was tested by the functional complementation between the cloned fragment of the pPHA11 plasmid and the *phaA* and the *phaB* genes of *A. eutrophus*. If pPHA11 carried the intact *phaC* gene with a promoter functional in *E. coli*, the pPHA11-bearing *E. coli* would synthesize the PHA synthase of *Alcaligenes* sp. SH-69. If a recombinant plasmid with the *phaA* and the *phaB* genes of *A. eutrophus* were cotransformed into the pPHA11-bearing *E. coli* strain, PHAs would be produced in the recombinant *E. coli* with the help of the β-ketothiolase and NADPH-dependent acetoacetyl-CoA reductase of *A. eutrophus*. A recombinant plasmid, pPHA21, that carries the *phaA* and the *phaB* genes of *A. eutrophus* downstream of the tac promoter was constructed (Fig. 4). The pPHA21 plasmid is a derivative of the low copy plasmid pLG339 and imparts tetracycline resistancy to a host cell. The plasmids, pPHA11 and pPHA21, were cotransformed into *E. coli* DH5α and plated onto a selection medium composed of LB, 2% glucose, 1 mM IPTG, 100 μg/ml ampicillin, and 25 μg/ml tetracycline. Fig. 5 shows that the coexistence of the plasmids, pPHA11 and pPHA21, conferred a turbid colony phenotype of PHA-producing cells on the recombinant *E. coli*. This result suggests that pPHA11 has the intact *phaC* gene of *Alcaligenes* sp. SH-69 and that the promoter of the *phaC* gene is transcriptionally active in *E. coli* just as the *phaC* promoter of *A. eutrophus* is. By contrast, the recombinant *E. coli* that has pPHA11

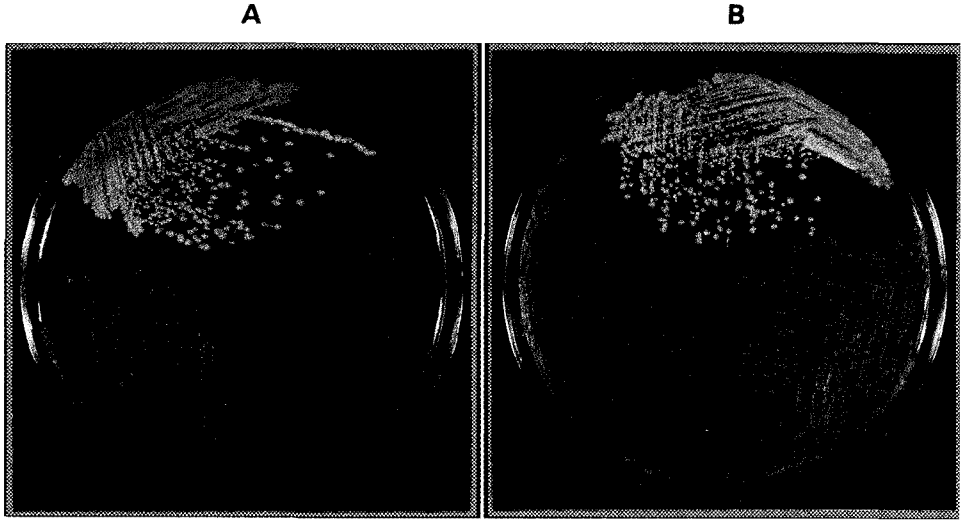

Fig. 5. Coexpression in *E. coli* of the *A. eutrophus phaA* and *phaB* genes and the *Alcaligenes* sp. SH-69 *phaC* gene.
A-1, A-2, and A-3 show *E. coli* cells cotransformed with pPHA11/pPHA21, pPHA11/pLG339, and pUC19/pPHA21, respectively. B-1, B-2, and B-3 show *E. coli* cells cotransformed with pPHA21/pPHA21, pPHA12/pLG339, and pBluescript II KS+/pPHA21, respectively. They were grown in LB plus 2% glucose media supplemented with ampicillin (100 μg/ml) and tetracyclin (25 μg/ml).

only did not show a turbid colony phenotype, which implies that the 5 kb insert DNA does not cover all the three genes, *phaC*, *phaA*, and *phaB*, required for the synthesis of PHAs. To further analyze which region of the insert DNA includes the *phaC* gene, the *Eco*RI/*Stu*I (about 2 kb) fragment was subcloned into pBluescript II KS+, resulting in a new plasmid, pPHA12 (Fig. 3B). The same turbid colony phenotype was found when pPHA12 and pPHA21 were introduced into the same *E. coli* (Fig. 5), which indicates that the *phaC* gene is included in the *Eco*RI/*Stu*I fragment.

DISCUSSION

The *phaC* gene of *Alcaligenes* sp. SH-69 was cloned and functionally expressed in *E. coli*. The coexpression of the *phaC* gene with the *A. eutrophus phaA* and *phaB* genes in *E. coli* resulted in turbid colonies in a glucose-containing LB medium, which demonstrates the accumulation of PHAs in *E. coli*. The PHA biosynthetic genes are organized in a single operon in many bacteria synthesizing PHAs or at least clustered even if they are not organized in an operon (27). The *phaC* gene of *Alcaligenes* sp. SH-69 was found to be present in the 2 kb 5' portion of the 5 kb cloned fragment (Fig. 3B). The sizes of *phaA* and *phaB* are generally less than 1.2 kb and 800 bp respectively in other bacteria and, therefore, the remaining 3 kb of the 5 kb cloned fragment is long enough to accomodate *phaA* and *phaB* if the two genes are clustered and organized like those of *A. eutrophus* (27). Since the introduction of the plasmid pPHA11 into *E. coli* did not lead to the synthesis of PHAs (Fig. 5), it is thought that the 5 kb *Eco*RI fragment in pPHA11 does not contain the *phaA* and/or the *phaB* genes. When the 3 kb 3' portion of the 5 kb fragment was partially sequenced, homologous sequences to the *phaA* genes of other bacteria were found (data not shown) but no homologous sequences to the *phaB* genes were observed, which suggests that the *phaA*, *phaB*, and *phaC* genes are not organized in a single operon in *Alcaligenes* sp. SH-69. We are now analyzing sequences of the 5 kb cloned fragment to investigate the molecular structures of the *phaC* and the *phaA* genes and sequences downstream and upstream of the cloned fragment to find the *phaB* gene.

Alcaligenes sp. SH-69 is able to synthesize poly(3HB-*co*-3HV) from glucose as a sole carbon source. This ability of *Alcaligenes* sp. SH-69 may be due to a novel biosynthetic pathway leading to the efficient formation of propionyl-CoA or 3-hydroxyvaleryl-CoA and thus to the synthesis of poly(3HB-*co*-3HV). Alternatively, distinct substrate specificities or characteristics of the PHA synthesizing enzymes from *Alcaligenes* sp. SH-69 may play an important role in the synthesis of poly(3HB-*co*-3HV). To investigate which of the two possibilities is true, hybrid operons consisting of various combinations of the *phaA*, *phaB*, and *phaC* genes from *Alcaligenes* sp. SH-69 and *A. eutrophus* should be constructed and expressed in *E. coli*. Analyses of the PHAs produced by the recombinant *E. coli* cells carrying the hybrid operons will reveal whether or not the enzymes of *Alcaligenes* sp. SH-69 are responsible for the synthesis of poly(3HB-*co*-3HV).

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