Molecular signatures in protein sequences that are characteristic of cyanobacteria and plastid homologues

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Fourteen conserved indels (i.e. inserts or deletions) have been identified in 10 widely distributed proteins that appear to be characteristic of cyanobacterial species and are not found in any other group of bacteria. These signatures include three inserts of 6, 7 and 28 aa in the DNA helicase II (UvrD) protein, an 18–21 aa insert in DNA polymerase I, a 14 aa insert in the enzyme ADP-glucose pyrophosphorylase, a 3 aa insert in the FtsH protein, an 11–13 aa insert in phytoene synthase, a 5 aa insert in elongation factor-Tu, two deletions of 2 and 7 aa in ribosomal S1 protein, a 2 aa insert in the SecA protein, a 1 aa deletion and a 6 aa insert in the enzyme inosine-5'-monophosphate dehydrogenase and a 1 aa deletion in the major sigma factor. These signatures, which are flanked by conserved regions, provide molecular markers for distinguishing cyanobacterial taxa from all other bacteria and they should prove helpful in the identification of cyanobacterial species, simply on the basis of the presence or absence of these markers in the corresponding proteins. The signatures in six of these proteins (SecA, elongation factor-Tu, ADP-glucose pyrophosphorylase, phytoene synthase, FtsH and ribosomal S1 protein) are also commonly present in plastid homologues from plants and algae (chlorophytes, chromophytes and rhodophytes), indicating their specific relationship to cyanobacteria and supporting their endosymbiotic origin from these bacteria. In phylogenetic trees based on a number of these proteins (SecA, UvrD, DNA polymerase I, elongation factor-Tu) that were investigated, the available cyanobacterial homologues grouped together with high affinity (>95 % bootstrap value), supporting the view that the cyanobacterial phylum is monophyletic and that the identified signatures were introduced in a common ancestor of this group.

INTRODUCTION

The cyanobacteria constitute one of the main phyla within the *Bacteria*: more than 1500 species are known (Kondratieva *et al.*, 1992; Castenholz, 2001). They comprise the sole prokaryotic group capable of oxygenic photosynthesis, and their emergence was a major evolutionary event that changed the Earth's atmosphere from an anoxygenic one to an oxygenic one (Woese, 1987; Kondratieva *et al.*, 1992; Bryant, 1994; Castenholz, 2001). The cyanobacteria, through endosymbiosis, are also believed to be the

progenitors of plastids in eukaryotic cells (Giovannoni *et al.*, 1988; Gray, 1992; Morden *et al.*, 1992; Margulis, 1993; Whatley, 1993).

Cyanobacteria exhibit enormous diversity in terms of their morphology, physiology and differentiation characteristics, which has led to the proposal of their division into five subsections (Rippka *et al.*, 1979; Trüper, 1987; Kondratieva *et al.*, 1992; Bryant, 1994; Castenholz, 2001). However, phylogenetic trees based on 16S rRNA provide the primary means for the identification and assignment of novel species to this group (Woese, 1987; Honda *et al.*, 1999; Turner *et al.*, 1999; Wilmotte & Herdman, 2001). Although cyanobacteria form a monophyletic group in 16S rRNA trees, the branching of different species within this phylum is found to be highly variable, and different subsections identified on the basis of morphological characteristics are generally not distinguished (Honda *et al.*, 1999; Wilmotte & Herdman, 2001). Other than phylogenetic trees based on 16S rRNA or various proteins (Viale *et al.*, 1994; Eisen, 1995; Delwiche

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Abbreviations: ADP-Glc-PPase, ADP-glucose pyrophosphorylase; EF-Tu, elongation factor-Tu; Pol I, DNA polymerase I.

Partial sequence alignments of UvrD, EF-Tu, ribosomal S1 protein, inosine-5'-monophosphate dehydrogenase, σ^{70} and FtsH showing indels characteristic of cyanobacteria are available as supplementary data in IJSEM Online.

et al., 1995; Gupta *et al.*, 1997; Gruber & Bryant, 1997), no unique molecular signatures are presently known that can clearly distinguish cyanobacteria from all other bacteria (Castenholz, 2001).

We have recently described a new approach, based on conserved indels (i.e. inserts or deletions) found in various proteins, for identifying different groups of bacteria and for clarifying their interrelationships (Gupta, 1998). The signatures that have been identified are of two kinds. One type of signature, which we refer to as the 'main line' signatures, are shared by several major groups of bacteria but absent from the other bacterial phyla (Gupta, 1998). We have proposed that these signatures were introduced at critical branch points during the course of bacterial evolution, and that they provide useful information for deducing the branching order and interrelationships among different groups of bacteria (Gupta, 1998, 2001). On the basis of different main line signatures, the cyanobacterial phylum is indicated to have evolved after the divergence of various Gram-positive phyla (*Firmicutes*, *Actinobacteria*, clostridia and relatives), the *Deinococcus-Thermus* group and green non-sulfur bacteria, but before the emergence of spirochaetes, the chlamydiae-CFBG (*Cytophaga-Flavobacterium-Bacteroides* and green sulfur bacteria) group, *Aquifex* and different divisions of the *Proteobacteria* (Gupta, 1998, 2001, 2002, 2003; Gupta & Griffiths, 2002). The second type of signature is specific for particular groups of bacteria and was probably introduced when these groups or phyla evolved (Gupta, 1998; Griffiths & Gupta, 2002). Such signatures have been identified for the chlamydiae, *Proteobacteria*, spirochaetes, *Firmicutes* and *Actinobacteria* (Gupta, 1998; Griffiths & Gupta, 2002; Morse *et al.*, 2002; R. S. Gupta, unpublished results) and they should prove very useful in identifying different bacterial groups in clear molecular terms (Gupta, 2002; Griffiths & Gupta, 2002).

In this communication, we describe 14 conserved indels in 10 broadly distributed proteins that are specific for cyanobacteria (and, in many cases, also commonly shared by plastid homologues) but not found in any other bacteria. These signatures provide evidence that the cyanobacteria lineage is monophyletic and furnish further evidence for the derivation of plastids from this group of bacteria. Because of their observed specificity, these signatures should prove useful for the identification of cyanobacterial species and for studies on the origin of plastids.

METHODS

Signature sequences in protein alignments. Multiple sequence alignments for a large number of proteins were created in our earlier work (Gupta, 1998; Gupta, 2000; Griffiths & Gupta, 2002). To search for signature sequences that might be specific for cyanobacteria, these alignments were visually examined to identify those indels that were unique to the cyanobacterial homologues and were flanked by conserved sequences. The potential usefulness of any such indel was examined by carrying out additional BLAST searches

on short sequence segments (usually between 60 and 100 aa long) containing the indel and the flanking conserved regions. The purpose of these BLAST searches was to obtain sequence information from all available species. In most cases, two different BLAST searches were carried out, one with a sequence containing the indel and the other with a sequence that lacked the indel. Indels that were unique to particular cyanobacterial species were not investigated any further. However, for those indels that were commonly present in various cyanobacteria, sequence information from different species was compiled into signature files such as those shown here.

Phylogenetic analysis. Phylogenetic analyses of protein sequences were carried out as described previously (Gupta *et al.*, 1997). A multiple alignment of sequences from different groups of bacteria (and also plastid homologues, where found) was created using the ALIGN program. Any sequence region for which the alignment was deemed unreliable was omitted from phylogenetic analysis. The indel regions were also excluded from the alignment for this purpose. Neighbour-joining distance trees showing branch lengths were constructed using the programs PROTDIST, NEIGHBOR and DRAWTREE. The aligned sequences were also bootstrapped 100 times using the SEQBOOT program, and a consensus neighbour-joining tree based on these data was obtained using the programs PROTDIST, NEIGHBOR and CONSENSE. Bootstrap scores for different nodes that were >50 were noted on the trees. All phylogenetic programs used are part of PHYLIP version 3.5 (Felsenstein, 1994).

RESULTS

Description of indels that are specific to cyanobacteria and plastids

Conserved indels that are commonly shared by members of one or more taxon provide a powerful means for defining various taxa in molecular terms and for clarifying how they may be related. The indels that have proved useful for evolutionary studies are generally of defined size and present in the sequence at specific locations. They are also flanked by conserved regions, to ensure that they are not due to improper alignment or sequencing errors. We have identified many conserved indels in protein sequences that are characteristic of cyanobacterial species. The descriptions of these indels and their evolutionary significance are reported below.

The SecA protein is found in all sequenced bacterial genomes and is involved in the export of proteins to the periplasmic compartment (Valentin, 1997; Schmidt & Kiser, 1999). In this protein, we have identified a 2 aa insert in a highly conserved region that is specific to cyanobacteria (Fig. 1). Sequence information for SecA is available from a large number of cyanobacteria. The identified insert is present in all known cyanobacterial homologues, but not in any other bacteria. Interestingly, this insert is also present in SecA homologues from different plastids. Plastid lineages from which sequence information is available include the Chlorophyta (land plants), the Rhodophyta (red algae), the Chromophyta (brown algae) and the Cryptomonadida (*Guillardia theta*). These results indicate that this insert is a distinguishing characteristic of these groups and that they are specifically related to each other.

		476	513
		HNVLNAKFH	ANEAAIQAQGYPAVATATNMAGRGTDI
Proteobacteria	<i>E. coli</i>	AAC73209	-----E--K-G-----
	<i>Vib. cholerae</i>	AAF95537	-----E--K-G-----
	<i>Xyl. fastidiosa</i>	AAF83616	-E-----Q-ER--T--N--L-G-----
	<i>Ral. solanacearum</i>	CAD16541	-Q-----Q--R--E-----R-KM-----
	<i>Nei. meningitidis</i>	AAF41891	-----E-ER--L-----KVG-I-V-----
	<i>C. crescentus</i>	AA57279	-Q-----R-EQ--V-----D-V-G-----
	<i>Ri. conorii</i>	AAL03417	-K-----EQ--F-I-----RFG-----
	<i>B. melitensis</i>	AAL51303	-----RY-EQ--Y-I-----V-G-----
	<i>A. tumefaciens</i>	AAL44333	-----RY-EQ--Y--S-----V-G-----
	<i>Hel. pylori</i>	AAD06297	-T-----Q-TK--E-IKD--LKG-----V--
Chlamydia, Aquifex, Chlorobium	<i>Camp. jejuni</i>	CAB73199	-H-----N-EQ--L-IQD--KKG-----V--
	<i>Aqu. aeolicus</i>	AAC07677	-----H-ER--E-I-----RVG-----
	<i>Chlam. pneumoniae</i>	BAA99049	-T-----N-Q--E-I-G--KLG--V-----
	<i>Chl. muridarum</i>	AAF38956	-T-----N-Q--E-I-G--KVG--V-----
	<i>Chlorobium tepidum</i>	21674062	-----Q--R--E--M--QKG-----
Spirochaetes, Chloroflexus, Deinococcus	<i>Tre. pallidum</i>	AAC65365	-E-----N--R--L-I-E--AKGS-----
	<i>Bor. burgdorferi</i>	AAC66536	-E-----N-SR--F-I-E--AKH-----
	<i>Cfx. aurantiacus</i>	22971339	-----H-ER-----I--RKG--V-----
	<i>D. radiodurans</i>	AAF10155	-A-----E-Q--S-I-----RSGT-----
	<i>Synechocystis</i> PCC 6803	BAA10347	--L---RPE NV ER-SE-----RKG-----
Cyanobacteria	<i>Synechococcus</i> PCC 7942	CAA52669	--L---PE NV ER--E-----RRG--S-----
	<i>Phormidium laminosum</i>	CAA12257	--L---PE NV ER-SE-I--R-KGT-----
	<i>Nostoc</i> sp. PCC 7120	BAB76550	-EL---RPE NV ER--E-----RRG-----
	<i>Nostoc punctiforme</i>	23125247	-EL---RPE NV ER--E-----RKG-----
	<i>Pro. marinus</i> pastoris	23123440	--L---PE NV ER--E-----RAG-----
	<i>Pro. marinus</i> MIT 9313	23130807	--L---PE NV ER--E-----RAG-----
	<i>Pro. hollandica</i>	AJ006132	Y-L---PE NV ER--E-I--R-----*
	<i>Anabaena variabilis</i>	AJ006133	ELL---RPE NV ER--E-----RRG-----*
	<i>Prochloron didemni</i>	AJ224965	Y-L---PE NV ER-SE-----RQG-----*
	<i>Thermosyn. elongatus</i>	BAC09403	--L---PE NV ER--E-I--RKG--S-----
	<i>Synechococcus</i> WH 8102	23132780	--L---PE NV ER-SE-----RAG-----
	<i>Trich. erythraeum</i>	23040967	-QL---PE NV ER-SE-----RGG-----
	<i>Arabidopsis thaliana</i>	7268223	-E---PE NV ER--E--S--RLG-----
	<i>Pisum sativum</i>	1122325	-E---PE NV ER--E--S--RLG-----
	<i>Spinacia oleracea</i>	2500725	-E---PE NV ER--E--S--RLG-----
Plastids	<i>Oryza sativa</i>	12328570	-E---PE NV ER--E--S--RLG-----
	<i>Guillardia theta</i>	3602965	-L---PE NV QR--E-----RLG-----
	<i>Antithamnion</i> sp.	288998	Y-L--R-PE NI TR-SE-IT--RKYTI--S-----
	<i>Porphyra purpurea</i>	1276847	-SL---PE NV EK-SD-I--RQSS-----
	<i>Cyanidium caldarium</i>	2465749	--L---PQ NV KR-SE-I--RKY--S-----
	<i>Odontella sinensis</i>	1185178	-QI---PE NV RR-SE-----KKGSI-----
	<i>Pavlova lutheri</i>	12117	-SL---PE NA --SE-I--RKG-----
	<i>Heterosigma akashiwo</i>	527574	Y---RPE NL KY-SE--GE--CLN-I-----
	<i>T. maritima</i>	AAD36645	-Q---Y- EK--E--K--QKGM-----
	<i>Clo. perfringens</i>	BAB81877	-K---RY- EQ--E--SH--ELGNI-----
Gram-positive bacteria	<i>Cor. glutamicum</i>	BAA92789	-----H- EQ--Q-----L-G--V-----
	<i>Myc. tuberculosis</i>	AAK47680	-----Y- EQ--T-I-V--RRGG--V-----
	<i>Str. coelicolor</i>	CAB90916	-E-----Q- DR--T-----RKG--V-----
	<i>Bac. subtilis</i>	CAB15547	-Q-----N- ER--Q-IEE--QKG-----
	<i>Sta. aureus</i>	BAB56915	-D-----N- ER--E--G--QKG-----
	<i>L. lactis</i>	AAK04213	-E-----N- FR--Q-IMN--QQG-----
	<i>Lis. monocytogenes</i>	AA50286	-D-----Q- ER--D-IKH--ERG--V-----
	<i>Strep. pneumoniae</i>	AAK75780	-E-----N- YR--Q-IMN--QRG-----

Fig. 1. Partial sequence alignment for SecA protein, showing a 2 aa insert (boxed) in a highly conserved region that is unique to cyanobacterial and plastid homologues and is not found in any other bacteria. Dashes in the alignment indicate residues identical to those on the top line (*Escherichia coli* protein). The position of this sequence in the *E. coli* protein is indicated at the top. Accession numbers of proteins are given in the second column. The SecA protein is present in all bacterial genomes, and sequence information for only representative species from major bacterial and plastid groups is presented here. Sequences ending in an asterisk are partial. Genera are abbreviated as: A., *Agrobacterium*; Aqu., *Aquifex*; Bac., *Bacillus*; Bor., *Borrelia*; B., *Brucella*; C., *Caulobacter*; Camp., *Campylobacter*; Cfx., *Chloroflexus*; Chl., *Chlamydia*; Chlam., *Chlamydomonas*; Clo., *Clostridium*; Cor., *Corynebacterium*; D., *Deinococcus*; E., *Escherichia*; Hel., *Helicobacter*; L., *Lactococcus*; Lis., *Listeria*; Myc., *Mycobacterium*; Nei., *Neisseria*; Pro., *Prochlorococcus*; Ral., *Ralstonia*; Ri., *Rickettsia*; Sta., *Staphylococcus*; Str., *Streptomyces*; Strep., *Streptococcus*; T., *Thermotoga*; Thermosyn., *Thermosynechococcus*; Tre., *Treponema*; Trich., *Trichodesmium*; Vib., *Vibrio*; Xyl., *Xylella*.

We have also performed a phylogenetic analysis based on SecA sequences. Earlier phylogenetic studies on SecA have contained only a limited number of sequences from cyanobacteria and other bacteria (Valentin, 1997; Barbrook *et al.*, 1998). Fig. 2 shows a neighbour-joining tree with branch lengths for SecA sequences. The bootstrap scores for various nodes that were >50 are also noted. As seen from their bootstrap scores, most of the bacterial groups in this tree are well resolved from each other. However, their relative branching order is not resolved, which is a common

problem in phylogenetic trees (Gupta, 1998; Ludwig & Klenk, 2001). In the SecA tree, all cyanobacteria and plastid homologues formed a well-defined clade (100 % bootstrap score), supporting the inference of the identified signature that these groups shared a common ancestor exclusive of other bacteria. Within this clade, the sequences corresponding to cyanobacteria were clearly resolved from those of the Chlorophyta and the Rhodophyta–Chromophyta. An unusual aspect of this tree, which has also been noted in earlier studies (Valentin, 1997; Barbrook *et al.*, 1998), is that

Fig. 2. Neighbour-joining distance tree with branch lengths based on SecA protein sequences. The tree is based on 516 aa positions that could be aligned without any ambiguity. The insert region was not included in the alignment. Bootstrap scores (out of 100) of various nodes that were >50 are indicated. Genus name abbreviations are listed in the legend to Fig. 1. Additional abbreviations: *Arab.*, *Arabidopsis*; *Cy.*, *Cyanidium*; *Pav.*, *Pavlova*; *Po.*, *Porphyra*. The arrowhead marks the suggested stage at which the insert identified in this protein was introduced.

the plastid homologues branched more deeply than the cyanobacteria. This is an unexpected result if plastids have been derived from cyanobacteria. However, as seen in Fig. 2, in comparison with cyanobacteria, all of the plastid sequences have much longer branch lengths, indicating that they have been evolving at a faster rate. The deeper branching of the plastid lineages in SecA phylogenetic trees, therefore, is very probably a consequence of the long branch-length effect, which leads to a deeper and abnormal

branching of faster-evolving taxa (Felsenstein, 1978; Barbrook *et al.*, 1998).

In the DNA helicase II (or UvrD) protein, which plays an essential role in the nucleotide excision repair and methyl-directed mismatch repair pathways (Eisen & Hanawalt, 1999), we have identified three cyanobacteria-specific indels. These signatures include a large, 28 aa insert (Fig. 3) as well as two inserts of 6 and 7 aa (see

		62	104	
Proteobacteria	<i>E. coli</i>	43297	TFTNKAAMRHRIGQLM	GTSQGMVWGTFHGLAHLRLR
	<i>Xyl. fastidiosa</i>	15836655	-----G-IQQ--DLQL	RHGKR---I---S-----
	<i>Vib. cholerae</i>	15640220	-----G--EE--	HGTAS--C-----IC--I--
	<i>Nei. meningitidis</i>	15793060	-----K--QT-L-AMI	PVNTRA--L-----C--F--
	<i>Nit. europaea</i>	22955362	-----R-VA--AML	PVNTRA--L-----C--V--
	<i>A. tumefaciens</i>	5889343	-----R-KE--V-V	G-AVE-MP-L-----SIGVK--
	<i>B. melitensis</i>	17986879	-----R-KE--H-V	G-AVE-MP-L-----SIGVK--
	<i>C. crescentus</i>	16125775	-----R--E--THII	GPEAE-LR-L-----SV-AQI--
	<i>Ri. conorii</i>	15619709	-----K--AE-VNS-I	NCY-LNI-----SM-A-I--
	<i>Hel. pylori</i>	15612436	-----SK--QE-ALK-L	KNQALIPPLC--RFGLLP--
Chlamydia, Aquifex, Chlorobium	<i>Camp. jejuni</i>	15792115	-----SK--IG-L-KFF	DKNITSKILA--ST-YT--
	<i>Aqu. aeolicus</i>	15606167	-----K-IKE--KNTF	GLELE-S-----SV-LKI-K
	<i>Chl. muridarum</i>	15835512	-----K-LNE-VLTQC	NFPDYR-IPM-S-----S-GVYI--
	<i>Chlam. pneumoniae</i>	7190013	-----R-LKE--VNQC	ASTNEFDVPM-C-----S-GVFI--
	<i>Chlorobium tepidum</i>	21673182	-----G--Q--DSIL	EYG-ASSL-I-----SVFA---
	<i>Tre. pallidum</i>	7436418	-----R--T-AC AL	ESAAQ-ATIC--A-GVWI--
	<i>Bor. burgdorferi</i>	15594689	-----N--ND--ND-L	KFDKHLHIQ---SFGSW---
	<i>Cfx. aurantiacus</i>	22971001	-----R-I-E-LNL-V	GE-VAHEITI-----AICT-W--
	<i>D. radiodurans</i>	15806776	-----E-A-H-V	PGA-DL-MS-----SAGV-I--
	<i>Thermus thermophilus</i>	2618592	-----E--E-LRG-V	PGA-EV--S-----AA-L-I--
Spirochaetes, Chloroflexus, Deinococcus	<i>Nostoc sp. PCC 7120</i>	17231987	-----R-KE--QR-F	K-TIKEL-C-----S-FS-I--
	<i>Nostoc punctiforme</i>	23126742	-----R-KE--QR-F	KSTIKDL-C-----S-FS-I--
	<i>Synechococcus WH 8102</i>	23132865	-----R-KE-LEV-L	REVSKEL-I-----A-FA-M--
	<i>Trich. erythraeum IMS101</i>	23043549	-----Q-KD--EKVF	KNITKHL-----N-CA-I--
	<i>Thermosyn. elongatus BP-1</i>	22300001	-----R-KE--ET-F	H-YTQPL-I-----S-CA---
	<i>Synechocystis PCC 6803</i>	16330097	-----K-KE-LEKIF	K-VTKPL-I-----S-CS-I--
	<i>Pro. marinus MIT 9313</i>	23132357	-----R-KE-LEL-L	REVTKEL-I-----A-FA-M--
	<i>Pro. marinus CCM 1375</i>	5734491	-----R-KD-LEI-L	REVIKEI-I-----A-FA---
	<i>Pro. sp. PAC-1</i>	7007365	-----R-KD-LEL-L	REISREL-I-----A-FS---
	<i>Pro. marinus pastoris</i>	23122171	-----K-KA-LQV-L	QERLRDL-I-----A-FS---
Cyanobacteria	<i>T. maritima</i>	4981792	--RA--R--VE-AKAVT	--RELSE-LA---HVCNHF--
	<i>Clo. perfringens</i>	18311242	-----G--E-VKS-V	--EVANN--IS---SSCV-I--
	<i>Myc. tuberculosis</i>	15608089	-----E--VVG-V	--EKARY--S---STCV-I--
	<i>Cor. glutamicum</i>	19552081	-----E-VS-V	--PVAER--A---SVCV-I--
	<i>Str. coelicolor</i>	21223175	-----G-KE-VE--V	--PRANA--M---SACV-I--
	<i>Sta. aureus</i>	15924895	-----R-KE-VQK-V	--DQAEVI-MS---SMCV-I--
	<i>L. lactis</i>	12724080	-----K--E-ALS-T	--PRAQDTLIA---SMCV-I--
	<i>Strep. pneumoniae</i>	14972562	-----R-KE-AYS-N	--PATQDCLIA---SMCV-I--
	<i>Lis. monocytogenes</i>	16803799	-----R-KS--N--	--GEAESI-IS---SMCV-I--
	<i>Bac. subtilis</i>	16077729	-----R-KE-VESIL	--PGADDI-IS---SMCV-I--

Fig. 3. Excerpt from a sequence alignment of DNA helicase II (UvrD) protein showing a 28 aa insert (boxed) that is characteristic of cyanobacteria. The UvrD protein is present in all bacterial genomes, and sequence information for only representative species is presented. Abbreviations of genus names are given in the legend to Fig. 1.

Supplementary Fig. A in IJSEM Online) in conserved regions. UvrD is present in all bacterial genomes, but not in the *Archaea* or plastids. Since the inserts identified are not found in any other bacteria, they are cyanobacteria-specific. In a phylogenetic tree based on UvrD sequences (excluding the indel regions), all cyanobacterial homologues grouped together with a 100 % bootstrap score (Fig. 4). This result supports the monophyletic nature of the cyanobacterial taxa and provides evidence that the indels identified in this protein were introduced in a common ancestor of this group (see the arrowhead in Fig. 4).

Another prominent cyanobacterial signature consisting of an 18–21 aa insert has been identified in the DNA polymerase I (Pol I) protein (Fig. 5). Similarly to UvrD and SecA, Pol I is found in all sequenced bacterial genomes. The insert identified is present in all known cyanobacterial homologues, but not in any other bacteria (Fig. 5). The length of this conserved insert shows some variation in different species, possibly due to further mutations/changes that have occurred in individual species. In a phylogenetic tree based on Pol I sequences, all cyanobacterial species again formed a well-defined clade (100 % bootstrap score; results not shown), supporting their monophyly as suggested by the shared signature. The Pol I homologues of eukaryotic species do not contain this insert. However, most of the eukaryotic sequences are for polymerase θ ,

except possibly that from *Oryza sativa*, which Kimura *et al.* (2002) have reported corresponds to a Pol I-like protein and is localized in the plastids.

Two additional cyanobacteria- and plastid-specific signatures are given in Fig. 6. The first of these signatures is in the enzyme ADP-glucose pyrophosphorylase (ADP-Glc-PPase), which is involved in the synthesis of glycogen in bacteria and starch in plants (Preiss, 1996). The cyanobacterial homologues of this protein contain a 14 aa conserved insert that is not found in any other bacteria (Fig. 6a). In bacteria, the ADP-Glc-PPase is a homotetramer, whereas the plastid enzyme is made up of two small (catalytic) and two large (regulatory) subunits (Preiss, 1996). Interestingly, the signature identified is present in both the large and small subunits of the plastid enzyme, providing evidence that both originated from cyanobacteria. The other cyanobacteria-plastid signature, shown in Fig. 6(b), is in the enzyme phytoene synthase, which plays a key role in the biosynthesis of carotenoids (Armstrong, 1997). The carotenoid pigments play important roles as light-harvesting molecules in photosynthetic reaction centres. The phytoene synthase from various cyanobacteria contains an 11–13 aa insert that is not found in any other bacteria. With the exception of *Deinococcus-Thermus*, all of the bacterial phyla in which this protein is found contain at least some photosynthetic members (Woese, 1987). The plastid homologues of this

Fig. 4. Neighbour-joining tree with branch lengths based on UvrD protein sequences. The tree is based on 280 aa positions and bootstrap values >50 are shown on nodes. The arrowhead marks the position at which the insert in this protein was introduced. Abbreviations of genus names are given in the legend to Fig. 1. Additional abbreviations: *Nit.*, *Nitrosomonas*; *The.*, *Thermus*.

protein also contain an insert in the same position (Fig. 6b). However, this insert is shorter than that in cyanobacteria (7 aa versus 11 aa), indicating either that it arose independently or that a further deletion occurred in this gene during the evolution of plastids.

We have identified seven other conserved indels that are characteristic of cyanobacteria. Sequence alignments of these signatures are provided as Supplementary Figs B–F in IJSEM Online, and a brief description of them follows. Delwiche *et al.* (1995) have previously described a 5 aa conserved insert in elongation factor-Tu (EF-Tu) that was present in cyanobacterial and plastid homologues. We have

updated the sequence information for this signature (Supplementary Fig. B) and have confirmed its specificity for these groups. Phylogenetic trees based on EF-Tu sequences again support the monophyletic nature of the cyanobacterial phylum and the derivation of plastids from this group of bacteria (Delwiche *et al.*, 1995; R. S. Gupta, unpublished results). Ribosomal S1 protein, which is widely distributed among bacteria (Subramanian, 1983), also contains two signatures (2 and 7 aa deletions) that are characteristic of cyanobacteria (Supplementary Fig. C). In contrast to other bacteria, cyanobacteria possess two different S1 protein homologues, both of which contain these signatures, suggesting that they arose from a gene-duplication event

		855		919
Cyanobacteria	<i>Synechocystis</i> PCC 6803	16331949	GYVTITVGRRRYFNFVTEALRLRGKTV	TELDLVDVK MNYNDAQLL RSAANAPIQGSSADIKIA
	<i>Nostoc</i> sp. PCC 7120	17228749	---E--L-----D-TNNS--K-K-SKP	EDI--SKL- NLGPY--G--
	<i>Trich. erythraeum</i>	23040910	---E--L-----ELESKTI-D-KD-EP	E-IN-QEL- FSQ---I-
	<i>Nostoc punctiforme</i>	23125316	---E--L-----D-TNNS--R-K-SNP	EDI--SKL- NLGPY--G--
	<i>Thermosyn. elongatus</i>	22298882	---E--L-----A-ESRE-QS---	PL-DV-AD--PSKLL-SNYERG--
	<i>Synechococcus</i> WH 8102	23133301	---E--L-----P-H-DRNG-GR-L-EP	L-I--DVAR RGGME--Q-
	<i>Pro. marinus pastoris</i>	23122969	---E--F--K-E-K-DKNG-GR-I--DP	Y-I--QTAR KAGME--S-
	<i>Pro. marinus</i> MIT 9313	23131695	---E--LL---P-H-DRNG-GR-L--DP	MDI--DVAR RGGME--Q-
	<i>E. coli</i> K12	16131704	---E--LD---L-LPDIKSSNGAR-AAAE	---A-I---M--TA---R
	<i>Vib. cholerae</i>	15640140	---E--F---LHLPEI-SRNAMR-KAAE	---A-I---M--TA---K
Proteobacteria	<i>Xyl. fastidiosa</i>	15837705	---E--VF---L-L-SIASGNQQT-AGAE	---A-I---M--TA---R
	<i>Ral. solanacearum</i>	17546949	---E--VF---LWLPDINGGNGPR-QAAE	---A-I---M--TA---L--LS
	<i>Nei. meningitidis</i>	15793464	-F-E-LF---L-LPDINKNTNA-AGAE	---A-I---M--TAS-L--R
	<i>B. melitensis</i>	17988108	---E--F---AHYPDIRASNP-V-AFNE	---A-I---M--AA---RR
	<i>A. tumefaciens</i>	15887465	---E--F---AHYPEIRSSNPVSKAFNE	---A-I---M--AA---RR
	<i>C. crescentus</i>	16127694	---F--KINIPDIEAKSAH-QFAE	---A-I---M--AA---VMRR
	<i>Ri. conorii</i>	15893129	---NCF--KCFVPLI HDKK-KQFAE	---A-I---M--TNT---
	<i>Hel. pylori</i>	2314647	TSKA-LL--YRV-D FTGANDYVKNYL	EGV--IF--AS-LL-L
	<i>Camp. jejuni</i>	15791706	-FIV-LS--K--D FEN-KPMQIAMYE	ESI-SIL--A-V-L-
	<i>Chl. trachomatis</i>	15605221	---ML--E-ILSDWESS PGA-AASG	-L-V-TR---A-EL--L-
Chlamydia, Chlorobium	<i>Chl. muridarum</i>	15835394	---ML--E-ILSDWESS PGA-AASG	-L-V-TR---A-EL--L-
	<i>Chlam. pneumoniae</i>	15618522	---ML--E-IIDSWN-F PGS-AASG	-F-V-TR---A-EL--L-
	<i>Chlorobium tepidum</i>	21674485	---LM---VPLDLS-NSNI-KAAE	-VTM-T---TA---F-
	<i>Bor. burgdorferi</i>	15594893	-SE-LK---IKEINSNNYLE-SAAE	-I-I-SI---A--M---
	<i>Tre. pallidum</i>	15639099	---SLA---IRTIDSRNTLE-ARAE	-M-L-TQ--S-A--V---
	<i>Lep. interrogans</i>	24216324	---Q-LT---PVTIDINTHKSAKAAK	-I-I-S---T---M---
	<i>Cfx. aurantiacus</i>	6015002	---Q-LF---VMDLIRAS GAR-AAAE	-E-I---M--TA--LM-M-
	<i>D. radiodurans</i>	15806710	---E-LY---VPGLSRN-VQ-EAEE	-L-Y-M---TA--M-L-
	<i>Thermus thermophilus</i>	1097211	---E-LF---VPLDNARVKS-V-EAAE	-M-F-M-V--TA--M-L-
	<i>T. maritima</i>	15644367	---R-LF---K-DIPQLMARD-NTQAEGE	-I-I-T---TA---L-
Gram-positive bacteria	<i>Clo. acetobutylicum</i>	15894383	---N-MN---IPEIASSNKIVK-FGE	-L-M-T---A--L--LS
	<i>Fuso. nucleatum</i>	19714234	---K-LF--K--ISGIDSKNTIKSQAE	-M-V-TV---TA-EVL-
	<i>Myc. tuberculosis</i>	15608767	---TS-VL---LPELDSN--V-EAAE	-A-L---M--TA---V-
	<i>Str. coelicolor</i>	21220485	---TA-LF---LPLDLSN--R-EAAE	-M-L---M--TA---V-
	<i>Cor. glutamicum</i>	19552567	---TE-LF---LPEL--SDN-VA-ENAE	-A-L---M--TA---V-
	<i>Sta. aureus</i>	21283362	---E-LLH---IPDI-SRNFN--FAE	-T-M-T---A---L--
	<i>Bac. subtilis</i>	16079961	---LMH---IPEL-SRNFN--FAE	-T-M-T---A---K-
	<i>L. lactis</i>	15674124	-F-E-MSH---KIPDINARFN--FAE	-T-I-S---A---L--
	<i>Li. monocytogenes</i>	16803605	---E-LH---IPEIVSRNFN--FAE	-T-M-T---A---K-
	<i>Strep. pneumoniae</i>	15899978	---E-LFK---ELPDINSRNFN--FAE	-T-I-S---A---L--
Eukaryotes	<i>Homo sapiens</i>	27666712	-F-E-L--L---LPGIKDNNPYHKAHAE	-Q-I-TTV--A--V-V-
	<i>Mus musculus</i>	28493899	-F-E-L--L---LPGIKDNNPYHKAHAE	-Q-I-TTV--A--V-V-
	<i>Dros. melanogaster</i>	2340084	-F-E-T--L---LENINDSVEH-KNQAE	-Q-V-ST---A--A-N-
	<i>Arabidopsis thaliana</i>	3063700	-IQ-LK---FLSKIKFGNAKEKS-AQ	-Q-V-SMC--A-----

Fig. 5. Sequence alignment of Pol I showing a large conserved insert (boxed) that is specific for cyanobacteria. Dashes in the alignment indicate identity to the amino acid on the top line. Pol I is found in all bacterial genomes and sequence information for only representative species is presented. Sequences from animal species are for polymerase θ . The nature of the plant homologues is uncertain at present. Abbreviations of genus names are given in the legend to Fig. 1. Additional abbreviations: *Lep.*, *Leptospira*; *Fuso.*, *Fusobacterium*; *Dros.*, *Drosophila*.

that occurred after these signatures were introduced. These deletions are also present in the plastid homologues, supporting their specific relationship to cyanobacteria. Two cyanobacterial signatures (a 5 aa insert and a 1 aa deletion) have been identified in the enzyme inosine-5'-monophosphate dehydrogenase, which is involved in the synthesis of guanine nucleotides (Supplementary Fig. D). These signatures are not found in any of the eukaryotic homologues, indicating that they are probably of either mitochondrial or nuclear-cytosolic origin (Gupta, 1998). A 1 aa cyanobacteria-specific deletion is present in the major sigma factor (σ^{70}), which is part of the RNA polymerase holoenzyme (Supplementary Fig. E). A divergent form of σ^{70} -like protein is found in plastids (Isono *et al.*, 1997); however, the insert is not present in such homologues. The last of the cyanobacterial signatures described here is a 3 aa insert in the enzyme FtsH protease, involved in the degradation of membrane proteins (Akiyama, 2002) (Supplementary Fig. F). Unlike those of other bacteria, the genomes of cyanobacteria have two different homologues of FtsH, only one of which contains this insert. This insert is also present in a number of plastid homologues.

DISCUSSION

In the present work, we have identified 14 conserved indels in 10 widely distributed proteins; they appear to be distinctive characteristics of cyanobacteria, as they are not found in any other bacteria. Although sequence information for cyanobacterial proteins is limited at present, the available species are quite divergent and belong to several clusters in the 16S rRNA tree (*Nostoc punctiforme* and *Nostoc* PCC 7120, cluster I; *Prochlorococcus marinus* and *Synechococcus* PCC 7942, cluster II; *Thermosynechococcus elongatus*, cluster III; *Synechocystis* PCC 6803, cluster XI; *Trichodesmium erythraeum*, cluster XII) (Wilmotte & Herdman, 2001). Therefore, it is likely that many of these signatures will prove distinctive for the entire cyanobacterial phylum and will provide molecular markers for distinguishing cyanobacteria from all other bacteria. The availability of such markers should facilitate the identification of novel cyanobacterial species simply on the basis of the presence or absence of these markers in corresponding proteins. All of the identified signatures are flanked on both sides by highly conserved sequence regions. This should facilitate the design

(a)		185	225
Cyanobacteria	<i>Synechocystis</i> PCC 6803	16332282	FSEKPKQGEALRAMQ VDTSVLGLSLAEKAK LNPYIASMGIYVF
	<i>Nostoc punctiforme</i>	23126438	-----K-----TQ-----TK-Q-Q KQ-----
	<i>Trich. erythraeum</i>	23040615	-----K-N-K-A-----I-V-P-I-T KQ-----
	<i>Pro. marinus pastoris</i>	23122620	-----T-K-KS-A-----KF-TK-S-S EK-L-----
	<i>Synechococcus</i> WH 8102	23133659	-R--K-DS-LE-A-----RF--NS-- ER-L-----
	<i>Pro. marinus</i> MIT 9313	23131224	-R--K-S-K-A-----RF-----S-R NK-L-----
	<i>Thermosyn. elongatus</i>	22298830	-R--T-D--D-R-----TRY--TI-E-H RK-----
	<i>Nostoc</i> sp. PCC 7120	17232137	-----K-----TK-R-----T-----TP-Q-A SQ-----
	<i>Arabidopsis thaliana</i>	15239684	-----K-DD-K-A-----TI-----K-E-E KK-----V---
	<i>Zea mays</i>	14582768	-A--K-DQ-K-M-----TI--DD-R-- EM-----
Plastids (small subunit)	<i>Pisum sativum</i>	1237082	-A--K-Q-K-K-----TI--DD-R-- EM-F-----
	<i>Solanum tuberosum</i>	21403	-A-----Q-Q-K-----TI--DDKR-- EM-F-----
Plastids (large subunit)	<i>Triticum aestivum</i>	82597	-----K-DD-E-K-----F-NFAIDDDPA KY-----V---
	<i>Oryza sativa</i>	22831238	-L--KD-S-KS--L-MGTF--RP-V-D TCK-M-----
Proteobacteria	<i>Arabidopsis thaliana</i>	25288667	-----K-DD-K-----I--PPKE-A ES-----V---
	<i>Solanum tuberosum</i>	82265	-A--K-FD-K-----TLV--PQD-- KS-----V---
	<i>Pisum sativum</i>	1619325	-----K-ADPK--T-----KDE-L EK-----V---
	<i>Triticum aestivum</i>	21677	-----R-AD-KE-E E-E KKP-----V-I
	<i>E. coli</i>	299130	-V--ANPPSPMND PSKSL-----
	<i>Vib. cholerae</i>	15601455	-V--ADPPCIPNR PDHSL-----I-
	<i>Pasteurella multocida</i>	15602408	-V--KDPAMVGK P-TSL-----
	<i>Haemophilus influenzae</i>	16273269	-V--KDPAMVGK PDVSL-----
	<i>Nitrosomonas europaea</i>	22955853	-A--EHP-PIPGK PGHALI-----
	<i>A. tumefaciens</i>	729581	-I--ADPPGIPGN EGFAL-----
Other bacteria	<i>Mesorhizobium loti</i>	14279431	-V--ADPPGIPGN PEFAL-----
	<i>Rhodobacter sphaeroides</i>	5834406	-L--ADPPGIPGN EANAL-----
	<i>Rhizobium tropici</i>	11493198	-V--ADPPGIPGN PDFAL-----
	<i>Rhodospirillum rubrum</i>	19554293	-L--ANPPGIPGR PDRAF-----I-
	<i>Magnetococcus</i> sp. MC-1	22998476	-L--TPPELPNR PGWAL-----I-
	<i>Geobacter metallireducens</i>	23055090	-E--ANPVTIPGR ETCF-----N-I-
	<i>Thermus caldophilus</i>	2731770	-Q--EEPKEIPGR PDMAL-----N-I-
	<i>D. radiodurans</i>	15806692	-H--VPDPPTIPG ADLSLT--N-I-
	<i>Myc. leprae</i>	15827523	-T--LKPPGTPDD PDKTFV--N-I-
	<i>Myc. tuberculosis</i> H37Rv	15608353	-V--LEPPGTPDD PDDTFV--N-I-
Other bacteria	<i>Bifidobacterium longum</i>	23465440	-Q--PTTGLPDN P-QIL-----N-
	<i>Str. coelicolor</i>	21219479	-L--ADPPGL-DD PGCVF--N--

(b)		158	209
Cyanobacteria	<i>Trich. erythraeum</i>	23040328	VAGTVGLMTMPVMGIETSNV AAPQKSHQQFQYP ASEAIALGIANQLTNILRD
	<i>Nostoc</i> sp. PCC 7120	17229325	-----STA--EVDNTTN T--WHQSKPLYI TQ-----
	<i>Nostoc punctiforme</i>	23127300	-----STE--VDSTLY T--WHQNK-PYL TE-----
	<i>Pro. marinus pastoris</i>	23122018	-----QN--DSAYT S--WS NMPDS SES-----
	<i>Pro. marinus</i> MIT 9313	23132434	-----QH--LDPAYT T--WS SSPET SDA-----
	<i>Synechococcus</i> WH 8102	23134622	-----QG--VDGAYT S--WS DRPDT SDA-V-----
	<i>Synechococcus</i> PCC 7942	585009	-----SQ--ST-S R--WDPT TPD TQ-L-----
	<i>Synechocystis</i> PCC 6803	585010	-----SSA-L-VD-G-G Q--WQPD AVYI QE-----V---
	<i>Spirulina platensis</i>	2189955	-----SLA--TAEPLD SV-WNRD-SIY KE-----
	<i>Thermosyn. elongatus</i>	22299103	-----SLE--GDPYSD RL-WDPY-AGTLL KQD-----
Plastids	<i>Zea mays</i>	1346883	-----SV--A-ESK -TTESVY -A-L-----
	<i>Oryza sativa</i>	18476089	-----V--APDSK -STESVY NA-L-----
Proteobacteria	<i>Arabidopsis thaliana</i>	15810497	-----SV--DPKSK -TTESVY NA-L-----
	<i>Lycopersicon esculentum</i>	19347	-----SV-I--APESK -TTESVY NA-L-----
	<i>Erwinia uredovora</i>	117496	--V--MAQI--VRDNAT LDR-CD--L-F--A--
	<i>Burkholderia fungorum</i>	22985834	--SAA-RLSVRF-MQDEPG RLL-HH--R-L-----
	<i>Nei. meningitidis</i>	15677007	--V--CLIAFIL-FSDDQT LEY-DKM-L-L-----I--
	<i>Ral. metallidurans</i>	22975141	--SA--RLAVR-F-LDEAPG IAL-HH--R-L-----
	<i>Zymomonas mobilis</i>	4388701	--SA--RLSVY-F-MDPNIG E-L-YH--R-L-----
	<i>Brad. japonicum</i>	2113821	--SA--RLSVR-F-LPEEDG IQL-YH--R-L-----
	<i>Rhodobacter sphaeroides</i>	1075575	--AA--A-MCVL-RVRDPD LAR-CD--L-M--A--
	<i>Rhodopseud. palustris</i>	22963486	-----V-MTL--QRKEDI VAR-CD--C-M--A--
Other bacteria	<i>Paracoccus</i> sp. MBIC1143	1706137	--I--V-MAR--VRDDP LDR-CD--L-F--A--
	<i>Rhizobium</i> sp. NGR234	16519643	--SA--R-SVR-F-LSEEDG IAL-HH--R-L-----
	<i>Rhodospirillum rubrum</i>	22968927	-----A-MTVL--VREPA LAR-CD--V-M-F--A--
	<i>Magnetococcus</i> sp. MC-1	23000914	-SVA--AAL--F-VTGERA QQF-HH--M-L-----
	<i>Myxococcus xanthus</i>	321671	--V--LT--L-CSDAAA VEP-AD--R-M-----
	<i>Chlorobium tepidum</i>	21674208	--SV--VEIF-YSNKEA LQH--D--M-----
	<i>Cfx. aurantiacus</i>	22974245	--SV--LVIGIT-AAPGAQ PY--K--V-L-----
	<i>D. radiodurans</i>	15805888	--V--F-VA-IS-YRGGAA TLND-LQ--Q-M-----
	<i>Thermus aquaticus</i>	585011	-----R-MA-IA-GGKEAE AR-VK--Q-M-----
	<i>Str. coelicolor</i>	21225053	-----AI-RLSLG-F-T-PGAR GAERAPEY-DT--L-L-----
Other bacteria	<i>Myc. tuberculosis</i>	1449390	G---I-KLCLSIF-PVSTAT SRY-EQ--L-Q-----
	<i>Sta. aureus</i>	15925552	-----EVLTI-ILSPHETHQ TYDV-RR--ESL--I-----

Fig. 6. Partial sequence alignments of ADP-Glc-PPase (a) and phytoene synthase (b) showing signatures (boxed) that are characteristic of cyanobacterial and plastid homologues. Plastids have two homologues of ADP-Glc-PPase, both of which contain the insert. The insert in phytoene synthase in plastids is smaller and could have involved a further deletion. Additional abbreviations: *Brad.*, *Bradyrhizobium*; *Rhodopseud.*, *Rhodopseudomonas*.

of PCR primers to amplify the sequence region from other bacteria, which will be carried out in future work.

Cyanobacteria are a very diverse group, exhibiting enormous variation in their cell division and morphological

characteristics. However, the taxonomic relationships within this phylum are not understood (Rippka *et al.*, 1979; Trüper, 1987; Kondratieva *et al.*, 1992; Castenholz, 2001; Wilmotte & Herdman, 2001). In phylogenetic trees based on 16S rRNA, 14 major clusters have been identified

within this phylum; however, it is unclear whether the placement of species within these clusters is evolutionarily meaningful. Also, how these different clusters relate to the major morphological differences within the cyanobacteria is unclear (Wilmutte & Herdman, 2001). In this context, signature sequences could provide a new and useful means of clarifying taxonomic relationships within this group. In the present work, we have focused mainly on indels that are shared by different cyanobacteria; other indels that were unique to particular cyanobacterial species were not studied further. However, such indels could provide signatures for identifying intermediate-level taxa within the cyanobacterial phylum.

The identification, in the present work, of 14 cyanobacteria-specific indels in 10 essential and widely distributed proteins provides evidence that the cyanobacterial phylum is monophyletic and that these signatures were probably introduced in a common ancestor of this group. The phylogenetic trees based on various genes/proteins also strongly support this inference. These results also provide evidence that the genes for these proteins have not been laterally exchanged between cyanobacteria and other bacteria, as has been suggested for many other genes (Gogarten *et al.*, 2002; Raymond *et al.*, 2002). If these genes were subjects of lateral gene transfers, the presence of these indels would be expected to be more random, which is clearly not the case here. However, we have previously described a few other signatures that are shared by cyanobacteria and the *Deinococcus-Thermus* group, and these could be a consequence of lateral gene transfers (Gupta & Johari, 1998; Gupta, 1998).

The signatures described here are also of value in clarifying the evolutionary relationship between cyanobacteria and plastids (Whatley, 1993; Delwiche *et al.*, 1995; Stiller & Hall, 1997; Valentin, 1997). For six of these proteins that are also present in plastids (SecA, ADP-Glc-PPase, phytoene synthase, EF-Tu, FtsH and ribosomal S1 protein), the signatures identified are commonly shared by the plastid homologues. Phylogenetic trees based on some of these proteins show a specific grouping of plastid homologues with cyanobacteria. These results provide additional evidence in support of the endosymbiotic origin of plastids from cyanobacteria (Gray, 1992; Margulis, 1993; Delwiche *et al.*, 1995). However, for three of these proteins, Pol I, inosine-5'-monophosphate dehydrogenase and σ^{70} , the cyanobacteria-specific indels were not found in eukaryotic homologues. It is possible that these homologues are of either mitochondrial or nuclear-cytosolic origin and would therefore not be expected to contain cyanobacteria-specific signatures (Gupta, 1998). Additional studies are required to clarify this aspect. With the availability of new signatures that allow discrimination between different groups of cyanobacteria, the signature approach could provide important insights into which group of cyanobacteria are the closest relatives of plastid lineages. The functional significance of the identified signatures is not known.

Because these indels have not been lost from any cyanobacteria, they are expected to be functionally important. Hence, studies examining their functional effects should be of much interest.

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