Homing endonuclease and mobile intron in the novel hyperthermophilic archaeon *Aeropyrum pernix* isolated from marine hydrothermal vent

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SUMMARY: Marine hyperthermophilic microorganisms are valuable resources for thermostable enzymes which are well suited for biotechnological applications. A novel DNA endonuclease I-*Ape* I was found from the hyperthermophilic archaeon *Aeropyrum pernix* and characterized. Of particular interest is that I-*Ape* I is encoded in the optional intron I α within the 16S rRNA gene. Probably, I-*Ape* I confer mobility to the host intron I α , like group I intron homing endonucleases from eukaryotes and bacteriophages. Also, this rare-cutting enzyme is a useful tool for genome mapping.

KEY WORDS: archaea, hyperthermophile, ribosomal RNA gene, intron homing, DNA endonuclease

INTRODUCTION

The myriad of microorganisms inhabiting marine hydrothermal ecosystems represent a vast repository of valuable thermostable enzymes for biotechnological applications. In the past two decades, a number of new hyperthermophiles have been isolated from deep-sea submarine vents or coastal hot springs.¹⁾ Among them, organisms with the highest growth temperatures (between 103 and 113 °C) are anaerobic members of the archaeal genera *Methanopyrus*, *Pyrococcus*, *Pyrodictium*, *Pyrobaculum* and *Pyrolobus*.

In previous studies, we succeeded in discovery of a strictly aerobic archaeon *Aeropyrum pernix*, which can grow at temperatures up to 100 °C^{2), 3)} and have completed its whole genome sequencing (1.67 Mbp).⁴⁾ Our current project is to extract useful genetic resources from the dataset. DNA polymerases,⁵⁾ a variety of proteases^{6), 7)} and restriction DNA endonucleases (Nomura N et al., unpubl. data, 2001) have so far been obtained from this organism.

During the work described above, we unexpectedly found an intron (I α) interrupting the 16S rRNA gene of this prokaryote. Most striking was that I α is "optional," i.e. is present in some but not all strains of the natural *A. pernix* populations. This observation suggests that I α is a mobile genetic element which could propagate itself through the population. Here, we demonstrate that I α contains an open reading frame whose product (I-*Ape* I) can act as a site-specific DNA endonuclease. In addition, we propose a hypothetical invasion strategy of I α , in which I-*Ape* I could play a pivotal role as a trigger.

MATERIALS AND METHODS

Comparative sequence analysis of the 16S rRNA gene from natural isolates of *A. pernix*

The *A. pernix* strains used in this study, OH1, TB1 and TB7, were isolated as described previously.²⁾ Near-complete length of the 16S rRNA genes were amplified by polymerase chain reaction (PCR) with the following primers: sense, 5'- ACTCCGGTTGATCCTGCCGGA and antisense, 5'- AGGTGATCCAGCCGCAGGTT. The resulting PCR product were cloned into pCR2.1 vector (Invitrogen). DNA sequencing was performed by the dideoxy-chain termination method on both strands of overlapping fragments.

Overexpression and purification of the intron I α -encoded protein, I-*Ape* I

To overexpress the presumptive I-*Ape* I coding sequence independently of the rest of the 16S rRNA gene of *A. pernix* K1, codons 1 to 222 (according to the numbering in ref. 8) were inserted into a bacterial expression vector, as follows. To place an initiator methionine codon (ATG) and an *Nde* I site at the 5' end of the I-*Ape* I coding segment, two oligonucleotide primers (sense, 5'- ACAT ATGAATGGTTGTG ATA TA and antisense, 5'- GGATC CTTAGCTGGGCCCCG GGGAG -3') and *A. pernix* K1 chromosomal DNA as a template, a corresponding double-stranded DNA fragment was generated by PCR. One end of this PCR product has the sequence corresponding to the carboxy-terminal end of the presumptive I-*Ape* I polypeptide followed immediately thereafter by a translational stop codon and an *Bam* HI site. Therefore, a 677-bp *Nde* I-*Bam* HI fragment of the PCR product that contains this region was subcloned into pET-15b to yield pHA1-01. Nucleotide sequence analysis confirmed that the

inserted segment did not contain any unwanted PCR-generated mutations.

Overexpression of I-*Ape* I was obtained by transforming pHAI-01 into *E. coli* strain BL21 (DE3)pLysS. A culture (6 liter) of transformed BL21 (DE3) pLysS cells was grown at 37 °C in LB broth (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl, 1 mM NaOH) supplemented with 50 µg/ml ampicillin to an OD₆₀₀ = 0.4. Induction of the T7 RNA polymerase, and subsequently the I-*Ape* I gene under control of the T7 RNA polymerase-dependent phage promoter, was achieved by the addition of isopropyl-1-thio-β-D-galacto- pyranoside (IPTG) to a concentration of 1.0 mM followed by further growth for 6 h at 37 °C. The culture was then chilled on ice, and the cells (15 g, wet weight) were harvested by centrifugation at 4 °C. Unless otherwise stated, all subsequent operations were performed at 4 °C. The cell pellet was washed and resuspended in 1.5 volumes of buffer A [50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 10% (v/v) glycerol, 0.5 mM dithiothreitol] containing 0.2 M NaCl. The cell suspension was sonically irradiated with cooling on ice. The crude lysate was subjected to centrifugation for 20 min at 20,000 ×g to remove unbroken cells and cellular debris. The resulting supernatant fraction was heated at 60 °C for 30 min, and the thermal denatured proteins were removed by the sedimentation at 20,000 ×g for 20 min. The supernatant solution was adjusted slowly to a final concentration of ammonium sulfate at 20 % of saturation by the addition of a sufficient amount of the solid salt with stirring over 30 min on ice before centrifugation for 20 min at 20,000 ×g. Ammonium sulfate was then added to the supernatant to a final concentration of 50 %. After incubation on ice and centrifugation, the pellet was dissolved in 20 ml of buffer A containing 0.2 M NaCl, and dialyzed extensively against the same buffer twice. The dialysate was subjected to cation exchange chromatography (HiTrap SP; Amersham Pharmacia) followed by affinity chromatography (HiTrap Heparin; Amersham Pharmacia) and gel filtration chromatography (Superdex 75; Amersham Pharmacia). Fractions (1 ml each) were collected and assayed for endonuclease activity. Active fractions containing the bulk of the I-*Ape* I activity were pooled, and aliquots were stored at -20 °C. The purity of the recombinant I-*Ape* I protein in each fraction was verified by SDS-PAGE.

Cleavage assay

The partial portion of the 16S rRNA gene from *A. pernix* strain OH1 (position 6 to 1290 according to the *E. coli* numbering system) was amplified by PCR and cloned into pCR2.1 vector to construct a plasmid

pO2A2. Target DNA (0.5 µg of *Eco* RI-linearized pO2A2 plasmid; Fig.1) was incubated with 0.5 µg of the purified I-*Ape* I protein in a total volume of 10 µl containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1mM dithiothreitol, and 50 mM NaCl at 90 °C for 30 min. During the incubation, the mixture was overlaid with 25 µl of mineral oil. The reactions were stopped on ice with 2 µl of dye mixture [0.25%(w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 1mM EDTA, 30% (v/v) glycerol]. Cleavage products were separated by electrophoresis in 1.5 % agarose gels and visualized by staining with ethidium bromide.

RESULTS

Polymorphic occurrence of optional introns in the 16S rRNA genes of natural populations of *A. pernix*

The sequence analysis of the single rRNA operon in the three *A. pernix* isolates, OH1, TB1 and TB7, showed polymorphism in intron insertion (Fig.1). The 16S rRNA gene of OH1 was intron-free. The 16S rRNA gene of TB1 possessed the following two introns: Iδ (62 bp after position 908 according to the *Escherichia coli* numbering of the 16S rRNA) and Iε (122 bp after position 1068 of the 16S rRNA). The introns in the 16S rRNA gene of TB7 were identical to those of TB1. Striking was that Iδ was inserted into a position which coincided with the Iα in the 16S rRNA gene of K1. Despite a remarkable difference in size among Iα (699 bp) and Iδ (62 bp), they shared similar terminal sequences, implying that these two introns derive from the same origin. The gap in the internal region could be attributed to a deletion. Direct sequencing of the mature 16S and 23S rRNAs confirmed that all of the newly found introns were spliced out of the RNAs (data not shown). The RNA sequences of Iδ and Iε at the exon-intron junctions could be folded into a secondary structure with the well-established features of the 'bulge-helix-bulge (BHB)' core structure, 3-base loops on opposite strands separated by a 4-bp helix, of archaeal introns.

Fig. 1 Intron occurrence in the 16S rRNA gene of the *A. pernix* isolates. Filled and open triangles indicate ORF-possessing and ORF-less introns, respectively. Numbers in parentheses denote the intron length (in bp).

Fig. 2 DNA substrate and assay for recombinant I-Ape I activity. (A) Schematic representation of plasmid pO2A2. The unique *EcoRI* sites in the vector (solid circular line) and the DNA fragment containing intronless 16S rRNA gene that was inserted (closed arc) are indicated. The nucleotide sequence of the I-Ape I recognition/cleavage site, the staggered double-stranded break generated by I-Ape I reaction, and the predicted sizes of the two DNA fragments produced by I-Ape I cleavage from plasmid that was linearized by digestion with *EcoRI* are also shown. I α insertion site is indicated by an asterisk. (B) Substrate DNA (pO2A2 linearized with *EcoRI*; 0.5 μ g) was incubated for 30 min at 90 °C in the absence (-) or in the presence (+) of 0.5 μ g of purified recombinant I-Ape I from *E. coli*. M, λ Hind III.

I-Ape I cleaves cognate intronless alleles in the vicinity of the I α insertion site

The recombinant I-Ape I overexpressed in *E. coli* cells were tested in cleavage assays with the intronless 16S rRNA gene form *A. pernix* OH1 as the DNA substrate. It showed high levels of cleavage activity (Fig. 2). Consistent with the hyperthermophilic nature of *A. pernix*, which grow optimally at 90 to 95 °C, I-Ape I was thermostable. The enzyme required Mg^{2+} , was virtually inactive at temperatures up to 37 °C, reached peak activity in the 85 to 90 °C range, and lost activity at higher temperatures (data not shown). It is unclear whether loss of activity above 90 °C reflects denaturation of the enzyme and/ or the substrate DNA.

The single cleavage site, which was observed exclusively for the intronless and not the intron-containing allele, was mapped by bidirectional primer extension analysis (data not shown). The cleavages on the two strands generated 4-nt 3' overhangs, which overlap the site at which the intron is inserted in the intron-containing allele (Fig. 2). I-Ape I appears to have an asymmetric recognition sequence.

DISCUSSION

The intron I α contain an open reading frame (ORF), and the following circumstantial evidences suggest that it is expressed *in vivo*: (i) only one ORF is possible for the intron, (ii) putative ribosome-binding sites, GGGAGGG, complementary to the 3' end of mature 16S rRNA occur 10 to 15 nt upstream of the start codons, (iii) free intronic RNA I α which could function as mRNA are stably accumulated in the cells, although their relative quantity could fluctuate in dependence on the physiological conditions.⁸⁾ Comparative sequence analyses against DDBJ, EMBL, GenBank nucleotide sequence databases and SWISS-PROT, PIR protein sequence databases reveal no genes or proteins with any overall similarity. However, a search made with the sequence, ¹²⁸VKGFVDAEG¹³⁶ in I α ORF revealed a similar motif (LAGLIDADG) characteristic of proteins encoded by many mobile group I introns from eukaryotes and bacteriophages.⁹⁾ In *Saccharomyces cerevisiae*, the LAGLIDADG protein encoded by the intronic ORF of the mitochondrial 21S rRNA gene possesses endonuclease activity and causes a site-specific double strand break of intronless variants of the

Fig. 3 A model for intron mobility. Intron mobility or homing is initiated and targeted by a site-specific DNA double strand break made in an intron-less allele by homing endonuclease I-Ape I encoded by the mobile intron ORF (HEG). Double strand break repair (DSBR) transfers the mobile intron containing HEG to cleaved recipient allele. Resolution generates two alleles that contain the mobile intron. Intron insertion disrupts the homing site, and renders intron (+) alleles resistant to endonuclease cleavage.

gene, promoting efficient conversion to the intron-containing form.¹⁰ In this context, we have developed the overexpression system of the $I\alpha$ ORF (I-Ape I) in *E. coli* and verified the intron-homing endonucleolytic activity.

Active I-Ape I is expressed from excised forms of the intron $I\alpha$, suggesting that splicing is a prerequisite for synthesis of the active enzyme. Like the phage and eukaryotic enzymes, I-Ape I cleaves an intronless allele by making a staggered double-strand cut at an asymmetric target site.

Although homing of the intron $I\alpha$ has not been demonstrated in *A. pernix*, mobility is implied by the existence of intron-containing and intronless variants (Fig. 1) and by cleavage of the intronless allele by I-Ape I encoded in the intron-containing variant (Fig. 2). Coexistence of intron-containing and intronless alleles is implicit in homing, and intron mobility would therefore suggest that intercellular genetic exchange takes place in the population of *A. pernix*. A model for intron mobility is given in Fig. 3. Probably, the ORF-lacking introns, $I\delta$ and $I\epsilon$, are deprived of mobility.

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