In vivo fragment complementation of a $(\beta/\alpha)_8$ barrel protein: generation of variability by recombination

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Abstract The high representation of the TIM barrel as a scaffold for enzymatic proteins makes it an interesting model for protein engineering. Based on previous reports of folding mechanisms of TIM barrels that suggest an independent folding unit formed by six (β/α) subunits, we interrupted the gene of phosphoribosylanthranilate isomerase (PRAI) from *Escherichia coli* at three different positions to yield fragments with different combinations of (β/α) subunits. When these constructions were expressed as polycistrons in a *TrpF-E. coli* strain, complementation of the function only occurred with fragments β1-α4 and β5-α8, demonstrating that $(\beta/\alpha)_4$ subunits are stable enough to survive in vivo conditions and to assemble to yield a functional enzyme. The expression of these fragments in a separated plasmid/phagemid system to complement the function gave a slower complementation in the *TrpF-E. coli* strain; this was overcome by introducing extra secondary elements to the structure that reinforce their interaction.

Key words: TIM barrel; Fragment complementation; Directed evolution; Phosphoribosylanthranilate isomerase; Protein engineering

1. Introduction

The $(\beta/\alpha)_8$ barrel (also called TIM barrel) protein architecture offers specially attractive opportunities for protein engineering. This protein fold is present in a significant fraction of the enzymes whose structure has been solved [3], thus providing a very large repertoire of catalytic activities implemented on the same basic scaffold. Notably, the active sites of TIM barrels are always located at the same face of the structure (the carboxy-termini of the β strands) making their analysis and manipulation more straightforward. These and other attributes have made TIM barrels favorite objects for protein engineering experiments over the past two decades [2,4–7].

The TIM barrel fold can be conceptualized as a repetition of eight basic units. Alternatively, it can be divided into four quarters or two halves, all of them having a pseudo-symmetric relationship. Indeed, it has recently been shown that some enzymes with TIM barrel architecture very likely evolved from two half-barrels [8]. Although it is unclear just how much of the current diversity of TIM barrel enzymes comes from a fundamentally divergent process, there is no doubt that it has played a significant role [9,10]. Within this evolutionary process, the role of half-barrels deserves further exploration.

Experiments aimed at fragmenting TIM barrels started many years ago, relying on proteolytic processing and exploring the folding properties of the pieces [1]. More recent experiments utilized recombinant DNA techniques to produce protein fragments in vivo. Results obtained with phosphoribosylanthranilate isomerase (PRAI) [2], chicken muscle triosephosphate isomerase (TIM) [6], and α-subunit of tryptophan synthase [7] suggest that fragment complementation could be achieved by two to five β/α units, but this likely varies with the specific protein.

We chose *Escherichia coli* PRAI as a scaffold for fragmentation studies. We set out to define positions on this TIM barrel protein that, when cut, generate fragments able to reassociate with each other and reconstitute activity. This enzyme, unlike many others with the same fold, comes naturally as a monomer. Further, the structure represents a 'classical' TIM barrel, in the sense that it is comprised of eight fully discernible β/α units and it does not contain large loops or additional domains. Finally, there are *E. coli* strains lacking PRAI activity that can be used for selection.

Having a hold on complementing fragments of a TIM barrel enzyme should facilitate the experimental exploration of their role in molecular evolution. It should also be possible to use these fragments in combinatorial systems for directed enzyme evolution (our results, manuscript in preparation).

The present work was aimed at identifying appropriate places to split the gene encoding *E. coli* PRAI that would afford fragments capable of reconstituting the active enzyme when expressed separately. We also explored the effects of expressing the fragments on the same and on separate cistrons.

2. Materials and methods

2.1. Enzymes

Restriction enzymes, high fidelity polymerase Expand™, T4 DNA ligase, and alkaline phosphatase were all purchased from Boehringer Mannheim. PACYC177 cloning vector was purchased from New England Biolabs.

2.2. Reagents

Buffers, reagents and agar noble were purchased from Sigma. Casamino acids were purchased from Difco Laboratories. Kits for plasmid and polymerase chain reaction (PCR) purification were purchased from Qiagene™.

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2.3. PRAI gene

The *E. coli* [ML256–452] PRAI gene, cloned into pUC18 through *Eco*RI/*Hin*dIII restriction sites, was generously provided by Dr. K. Kirshner's group of Basel University. This gene was engineered from the bifunctional gene expressing IGPS-PRAI, inserting a stop codon after residue 252, a Shine–Dalgarno sequence and codons for Met-Leu followed by the sequence coding for amino acids 256–452 that comprises PRAI function [11].

2.4. Complementation strain

In order to evaluate the fragment complementation we used *E. coli* strain JMB9 r^- m^+ *TrpF*-, generously provided by Dr. K. Kirschner. This strain has a substitution of the IGPS-PRAI gene by the monofunctional version of the IGPS gene in its chromosome [2].

2.5. Construction of the bicistronic PRAI fragments

The PRAI gene was interrupted at the bottom loops of the barrel between α2 and β3, α4 and β5 and finally between α6 and β7 using the following oligonucleotides: oligo Aα2/β3, with the sequence 5′-TCAACACCCCTCCTGA**CTATCATTA**TGCCGCAGCCATCACT-TC-3′, oligo Bα2/β3, 5′-AGTCAGGA GGGGTGTTGA**TGC**TCGA-GCCGTTGCAGTATGTTGGC-3′; oligo Aα4/β5, 5′-TCAACACC-CCTCCTGA**CTATCATTA**TGGCAGAGCTTCACGCAG-3′; oligo Bα4/β5, 5′-AGTCAGGAGGGGTGTTGA**TG**CTCGAGGCACAT-GTTGCCATCTGG-3′; oligo Aα6/β7, 5′-TCAACACCCCTCCTG-A**CTATCATTA**CGTTTGACCATTTAATAGTGAC-3′; oligo Bα6/β7, 5′-AGTCAGGAGGGGTGTTGATGCTCGAGCTTGGCAAC-GTTCTGCTG-3′. Oligonucleotides A hybridize with the non-coding sequence, while Oligonucleotides B prime the coding sequence of the PRAI gene. Oligonucleotides A were used in combination with reverse cap primer (5′-GGAATAGCCGATCGAGATCTAGGAAACAGC-TATGACCATG-3′) to amplify the N-fragment of the gene by PCR, introducing three different stop codons consecutively (bold) at the desired positions to avoid read-through translation. Oligonucleotides B introduce a ribosomal binding site (underlined), a 9 bp separation between the Shine–Dalgarno sequence and the starting codon ATG followed by codons for Leu and Glu and continuing with the corresponding sequence of PRAI (residue 51 in the first couple, residue 98 in the second, and 146 for the third one). These oligonucleotides were used in combination with a forward universal primer (5′-GTTGTAAAACGACGGCCAGT-3′) to amplify the C-fragment of PRAI by PCR. The products obtained from these reactions were used to construct the bicistronic operons by overlap-extension PCR, and cloned through the *Eco*RI and *Hin*dIII restriction sites in pUC18 plasmid, as shown in Fig. 1. The correct DNA sequence of the constructs was confirmed using a Perkin Elmer ABI Prism sequencer.

2.6. Cloning of fragments β1-α4 and β5-α8 in different plasmids

Once complementation was demonstrated in the construction with the bicistronic operon formed by fragments β1-α4 and β5-α8, these fragments were subcloned in different plasmids to check for complementation when independently synthesized. For that purpose, the N-fragment was amplified with oligonucleotides P-II (5′-ATCCT-G**AAGCTT**ATCATTATGGCAGAGCTTCA-3′) and the reverse cap primer, and cloned into the *Eco*RI and *Hin*dIII sites on plasmid pBGS18, which is a derivative of plasmid pUC18, with a *ColE1* replication origin, and a kanamycin resistance gene [12]. On the other hand, the C-fragment β5-α8 was amplified by PCR from the bicistron construction with oligonucleotides BamXhoβ5 (5′-ATAG**GGAT-CCTCGAG**AGGAGGGGTGTTGATGC-3′) and forward universal primer and subcloned into the *Bam*HI and *Hin*dIII restriction sites on plasmid pACYC177, which is a low copy number plasmid with a P15a replication origin and carries a β-lactamase gene for ampicillin resistance and a kanamycin resistance gene [13,14]. Cloning of the fragment β5-α8 into the *Xho*I/*Hin*dIII restriction sites removed most of the kanamycin resistance cassette. The DNA of three independent clones of these constructions was sequenced to avoid errors introduced by Taq polymerase. As a control, the bicistronic fragments were also cloned into pBGS18.

2.7. Construction of the fragments with a Leu zipper

Six oligonucleotides with overlapping sequences were synthesized to facilitate the synthesis by overlap-extension PCR of two amphipathic helices, each one appended to one of the $(\beta/\alpha)_4$ subunits. The construction was designed as a bicistron with *Bam*HI and *Xho*I restriction sites between the two fragment genes to facilitate their later subcloning in different plasmids. The oligonucleotides were: oligo α4CZ, which contains the non-coding strand sequence at the end of α-helix 4 of PRAI and the connector to the C-helix zipper, used in combination with reverse cap primer to form Megaprimer I; oligo CZ, which contains the coding sequence for the C-helix of the Leu zipper and has a 22 bp overlap with oligo α4CZ on one end and a 19 bp overlap with

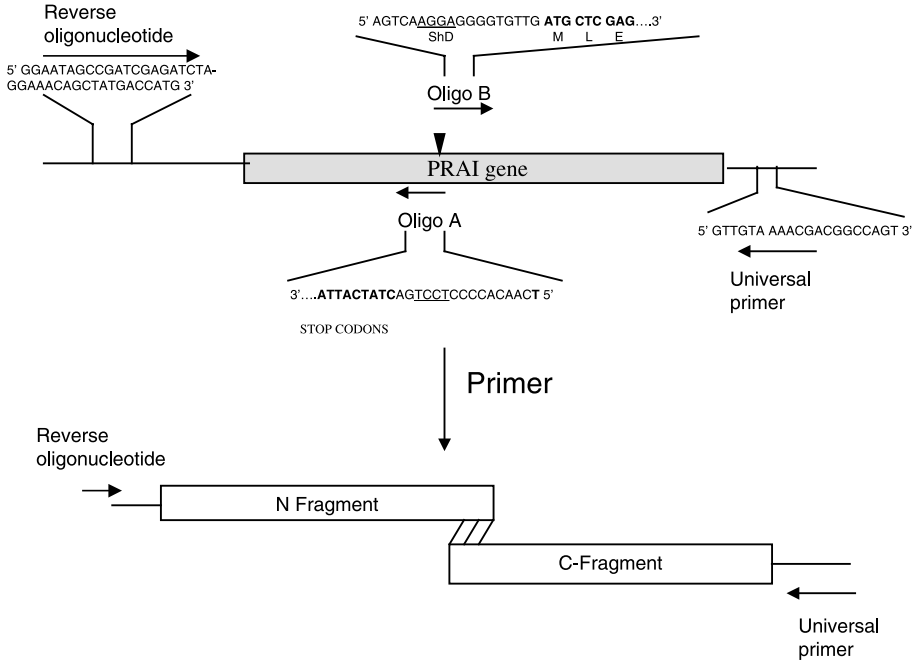

Fig. 1. Fragmentation of the *E. coli* PRAI gene. The N-fragment is amplified with the reverse oligonucleotide and any of the oligonucleotides A (depending on the desired fragmentation site) (▼). These oligonucleotides introduce the consecutive stop codons indicated in bold. In another PCR reaction, the C-fragment is amplified with the universal primer in conjunction with any of the oligonucleotides B, which introduce the ribosomal binding site (underlined) and the inserted Met, Leu and Glu codons (in bold). The two PCR products were annealed and primer extended with the reverse and universal primers.

oligo *Bam*HI/*Xho*I at the other end; oligo *Bam*HI/*Xho*I contains basically the sequence of the non-coding strand at the end of the C-helix, restriction sites for *Bam*HI and *Xho*I, a ribosomal binding site and the start of helix NZ; it overlaps with oligo NZ1. Oligo NZ1 contains the coding sequence of part of the N-helix of the Leu zipper and overlaps with oligo NZ2, which continues the N-helix sequence and overlaps with oligo NZβ5, which is the connector between the N-helix and the start of the second fragment of PRAI (see Fig. 2). The sequences of the oligonucleotides are as follows (underlined are the ribosomal binding site and ATG starting codon for the second fragment, in bold are the restriction sites):

Oligo α4CZ, 5′-TTTTTATTAGCCTGCAGTTCCTTTTTCAGTGCACCTGAGCCTGAGCCACCTGCTGGCAGAGCTTC-3′

Oligo CZ, 5′-GGAACTGCAGGCTAATAAAAAAGAATTAGCGCAGCTGAAATGGGAACTGCAGGCACTGAAAAAAG-3′

Oligo *Bam*HI/*Xho*I, 5′-CAACACCCCTCCT**GGATCCTCGAG**CTATCATTACTGCGCCAGTTCTTTTTTCAGTGCCTGCAG-3′

Oligo NZ1, 5′-**CGAGGATCC**AGGAGGGGTGTTGATGGCATCGGAACAGCTGGAAAAGAAACTGCAGGCGCTGGAAAAGAAATT-3′

Oligo NZ2, 5′-GCCTGATTTTTCCATTCCAGTTGTGCCAGCTTTTTTTCCAGTGCCTGTAATTTCTTTTCCAGCGCC-3′

Oligo NZβ5, 5′-GGAATGGAAAAATCAGGCACTGGAAAAGAAACTGGCACAGGGCGGTTCGGGCCACGTTGCCATCTGGAAAG-3′

After overlap-extension PCR, the resulting fragment was digested with *Eco*RI and *Hin*dIII and cloned into both pUC18 and pBGS18 (as a control) and transformed in electrocompetent JM101 cells. Plasmid DNA from each construction was purified and the gene sequenced to make sure that the Taq polymerase did not introduce more changes than the desired ones.

2.8. Construction of the fragments with a Leu zipper in isolated plasmids

Each of the fragments containing an amphipathic helix was subcloned in a different plasmid. Fragment 1-CZ-helix was subcloned into the *Eco*RI/*Bam*HI restriction sites in plasmid pBGS18 while fragment 2-NZ-helix was subcloned into the *Xho*I/*Hin*dIII sites of vector pACYC177.

2.9. In vivo complementation of PRAI function

All constructions were electrotransformed into competent cells of *E. coli* strain JMB9 r^- m^+ *TrpF*-. After the recovery period, the cells were spun down at 2000 rpm in a microcentrifuge and washed with Vogel–Bonner (VB) minimal medium [15] three times before plating on Luria–Bertani (LB) and VB minimal medium prepared with noble agar and supplemented with the corresponding antibiotic. The constructions of fragments in different plasmids were simultaneously transformed with a mixture of the two plasmids and equal volumes of the washed cells were plated on LB plates supplemented with kanamycin, ampicillin or kanamycin/ampicillin, as well as in VB minimal medium prepared with noble agar and supplemented with both antibiotics to compare the yield of the double transformation and to check for complementation.

2.10. Growth curves

To compare the growth rate (taken as indicator of the degree of complementation), colonies were picked out from the corresponding plate of each construction and inoculated in 3 ml of LB medium. They were incubated at 37°C overnight to reach saturation. The cells from 1 ml of culture were pelletted at 2000 rpm for 3 min, resuspended in VB minimal medium to wash them. This operation was repeated three times to remove any trace of tryptophan and the OD_{600} was measured to inoculate 3 ml of VB minimal medium with the necessary amount of cells to achieve an initial OD_{600} of approximately 0.02. Cells were incubated at 37°C and their OD_{600} was measured every 1 or 2 h depending on their growth rate.

3. Results

3.1. Choice of cleavage site

The sites for the gene interruption were picked out based on the 3-D structure reported for the *E. coli* IGPS-PRAI heterodimer single chain protein [16]. The chosen sites lay at the bottom loops of the barrel and the fragments formed contained $(\beta/\alpha)_2$ subunits or multiples of them. Three constructs were made: at the loop formed between helix α2 and strand β3, at Pro 51; at the loop between helix α4 and strand β5, at Ala 98; and at the loop between helix α6 and strand β7, at Ser 145.

3.2. Construction of bicistrons

The PRAI gene was interrupted at the three different positions by inserting a 24 bp linker containing the three different stop codons, a ribosomal binding site separated 9 bp from the start codon ATG as well as codons for Leu and Glu. The introduction of codons for Met, Leu and Glu was decided based on the previous construction of the isolated PRAI gene from the IGPS:PRAI complex [11]. These constructions were checked by DNA sequencing to make sure that there were no further mutations or frame shifts and transformed

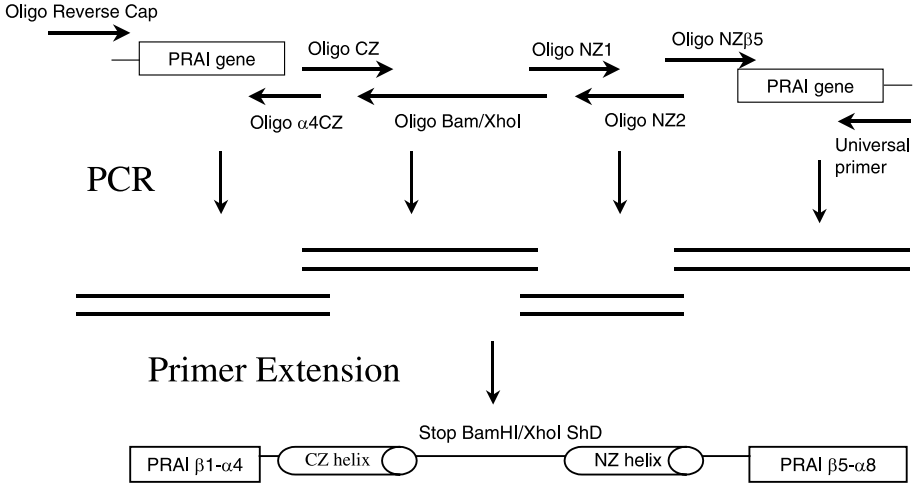

Fig. 2. Construction of the fragments tied by a Leu zipper. An amphipathic helix was built downstream of the α-4 coding sequence from the PRAI gene, followed by *Bam*HI and *Xho*I restriction sites, a ribosomal binding site separated 9 bp from the start codon for an antiparallel amphipathic helix upstream of the β5 coding sequence from the PRAI gene. The arrows represent each of the oligonucleotides used for this construction. In the lower part is schematized the structural parts of the encoded resulting peptides.

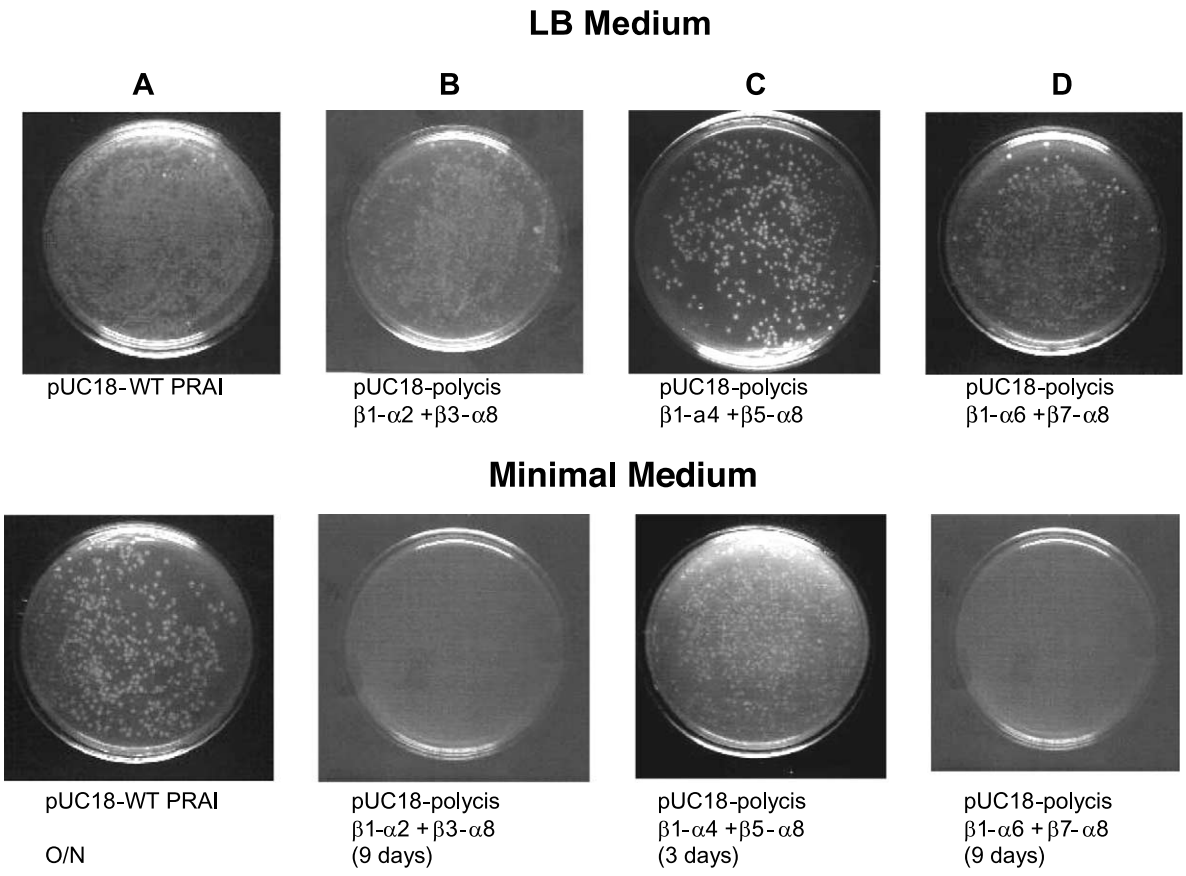

Fig. 3. In vivo complementation of strain JMB9 *TrpF-* by the constructs of the PRAI gene fragments expressed as bicistrons. In the upper part are shown the transformant cells plated on LB medium and in the lower part on VB minimal medium. A: Pseudo-WT PRAI gene [ML256–452]. B: PRAI gene fragmented between α2 helix and β3 strand. C: PRAI gene fragmented between helix α4 and strand β5. D: PRAI gene fragmented between helix α6 and strand β7.

in the *TrpF-E. coli* strain. Fig. 3 shows plates of the *TrpF-E. coli* strain transformed with the different constructs on LB and on VB minimal medium. The plates were incubated at 37°C for several days. It can be observed that only the construct of the bicistron formed by $(\beta/\alpha)_4$ subunits was able to complement the function with growth of colonies after 3 days of incubation. The other two constructs did not show any growth even after 9 days of incubation Our results are in agreement with those of Kirschner et al. [2] who observed no in vivo complementation of similar fragments of the yeast enzyme. They did, however, observe complementation in vitro, suggesting that a degradation process within the cell may be operating in vivo.

3.3. Expression of fragments on separate plasmids

The expression of fragments on separated plasmids was carried out only with the $(\beta/\alpha)_4$ subunits, since this was the only combination capable of function complementation in vivo. The first and second cistronic genes were amplified by PCR and cloned in pBGS18 plasmid (high copy number) and in pACYC177 (low copy number), respectively. Growth was observed only after 8 days (data not shown). The slower growth rate observed when the fragments are expressed on different plasmids may result from the difference in copy number of the plasmids, the strength of the promoter in each case, or the fact that the fragments are not produced near one another for an opportune assembly. We cloned the second half of the gene in a modified version of pACYC177, in which we introduced a pBTAC promoter, as well as in the phagemid pM846, which contains the *lac* promoter [17], and observed faster growth rates as can be seen in Fig. 4, suggesting that an uneven expression level of the fragments could be the major cause of the less efficient complementation.

3.4. Design of Leu zipper as polycistron and in separate plasmid

In order to reinforce the interaction between the fragments synthesized independently, an amphipathic helix with heptad Leu repeats was appended to the C-terminal of fragment 1, β1-α4, and an amphipathic antiparallel-running helix was appended to the N-terminal of fragment 2, β5-α8, based upon Ghosh et al.'s design [18]. This construct was designed to contain a sequence for recognition of *Bam*HI and *Xho*I restriction endonucleases in the intergenic zone to facilitate the subsequent cloning of the fragments in separated plasmids. Complementation of the function by the bicistron occurred overnight, although to get a better contrast of the colonies, Fig. 4 shows the growth after 40 h. Interestingly, complementation of the fragments expressed in different plasmids occurred even faster. In order to account for this unexpected result, the bicistron was constructed in the same high copy number plasmid used for the first fragment of the gene (pBGS18). With this construction, colonies of similar size were obtained for the PRAI [ML256–452] gene [11] (pseudo-

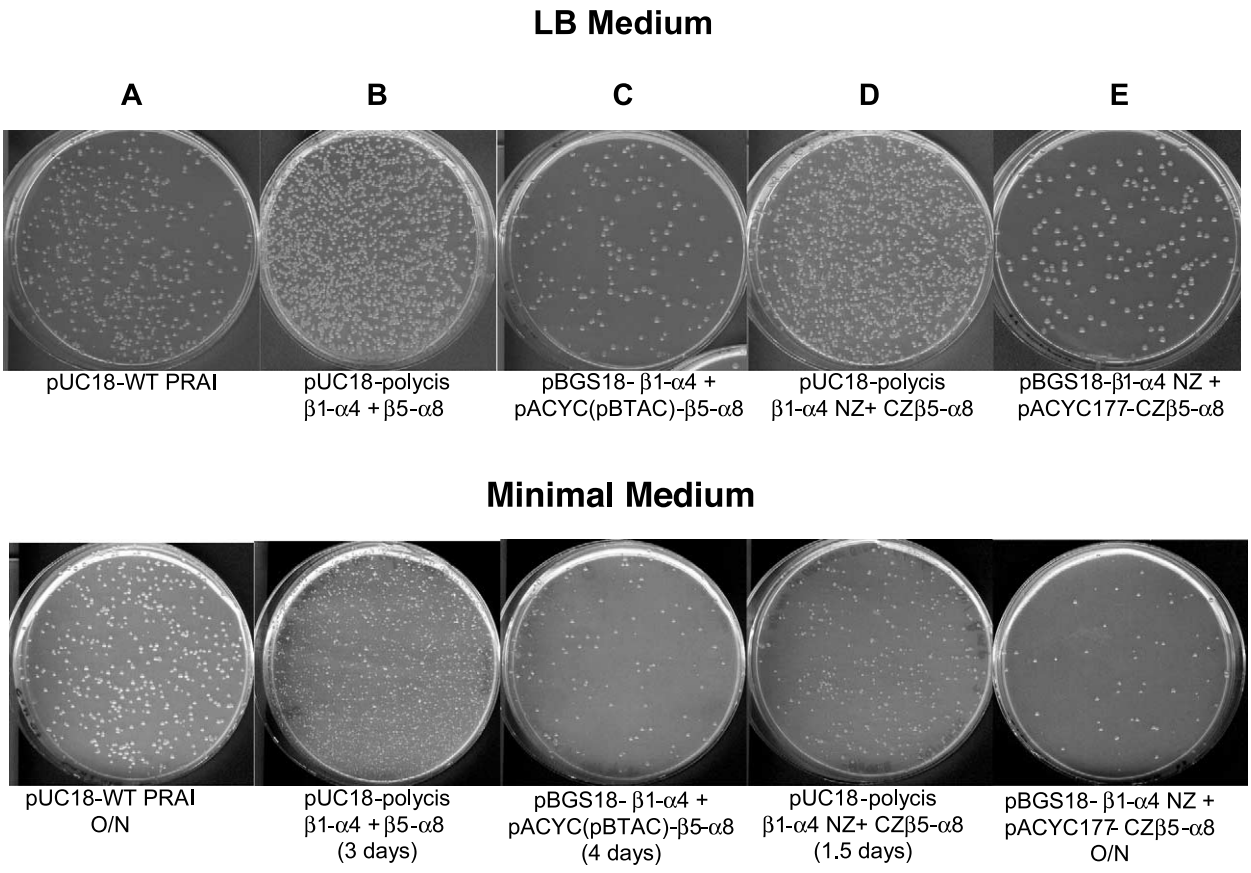

Fig. 4. In vivo complementation of the strain JMB9 *TrpF*- by the $(\beta/\alpha)_4$ fragments of PRAI. In the upper part are shown the transformant cells plated on LB medium and in the lower part in VB minimal medium. A: Pseudo-WT PRAI gene [ML256–452]. B: PRAI gene fragments expressed as bicistron in pUC18. C: PRAI gene fragments expressed in different plasmids: pBGS18-β1-α4, and pACYC177 with a pBTAC promoter-β5-α8. D: Fragments with amphipathic helices expressed as a bicistron in pUC18 (pUC18-polycys β1-α4 NZ+CZ β5-α8). E: Fragments with amphipathic helices expressed in different plasmids pBGS18-β1-α4 NZ and pACYC177-CZ β5-α8.

WT gene for our purposes), the bicistronic gene tied by a Leu zipper and the Leu zipper fragments expressed in different plasmids. Thus, the difference in expression level due to the low copy number vector used for expression of the second fragment did not have any effect in the complementation. Therefore, the lack of complementation observed when the fragments of PRAI were expressed in different plasmids must be due not only to a lower production of the fragment but also to a faster degradation than assembling of the fragments.

3.5. Growth curves

The growth rate was used as a quantitative measure of the degree of complementation. Three isolated clones were picked out of each construct and their growth in liquid VB minimal medium was monitored as OD_{600} as a function of time. The results shown in Fig. 5 illustrate the dramatic effect that the plasmid has on the growth rate. When the growth is compared in the same plasmid background, it can be observed that it slows down considerably upon fragmentation of the gene and is practically undetectable when the fragments are expressed by different plasmids. In contrast, the presence of a Leu zipper to reinforce the association of the fragments reconstituted practically full activity. The expression of these fragments in different vectors had only a minor effect in the growth rate even though one of them was expressed by a low copy number vector.

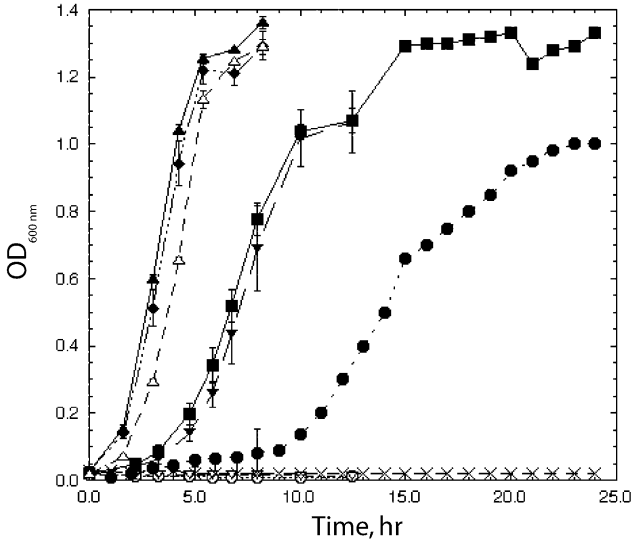

Fig. 5. Growth curves of the complemented strain JMB9 *TrpF*-. (X), pUC18; (■) pUC18-pseudo-WT PRAI [ML256–452]; (●) PRAI fragments expressed as bicistron; (○) PRAI fragments expressed in different plasmids: pBGS18-β1-α4 and pACYC177-β5-α8; (◆) pBGS18-pseudo WT PRAI [ML256–452]; (▲) pBGS18 fragments of PRAI with appended amphipathic helices expressed as bicistron; (▼) pUC18 fragments of PRAI with appended amphipathic helices expressed as bicistron; (△) fragments of PRAI with appended amphipathic helices expressed in different plasmids: pBGS18-β1-α4 NZ and pACYC177-CZ β5-α8; (▽) pBGS18-β1-α4 NZ.

4. Discussion

Using PRAI as scaffold, we were able to express fragments yielding a functional ensemble. However, contrary to previous experience with PRAI [2] and the α-subunit of tryptophan synthase [1] in which fragment complementation was achieved in vitro with modules of $(\beta/\alpha)_6+(\beta/\alpha)_2$, we found that only $(\beta/\alpha)_4$ subunits were capable of proper folding and association to withstand in vivo proteolysis. Our results are consistent with recent reports about the high internal similarity between halves of the *HisA* and *HisF* genes, other TIM barrels [19]. In the case of *HisF*, fragment complementation of halves has been demonstrated in vitro yielding a 1:1 complex with wild-type catalytic activity, suggesting that the elementary building blocks could be half-barrels, at least in these closely related proteins [19].

The different complementation pattern of the $(\beta/\alpha)_4$ fragments observed when expressed as polycistron and in separated plasmids could be explained in different ways. Since the linker that separates the two genes was 24 bp long, a translational reading through could account for it. However, the use of three different stop codons consecutively makes this highly unlikely to occur. Furthermore, the same linker was used in the three designed constructs, and only the one between α4 and β5 yielded an active polycistron. A plasmid–plasmid compatibility problem can be ruled out, since the pair pBGS18/pACYC177 was successful when the fragments had the amphipathic helices appended. Another possibility that accounts at least partially for the result is the low expression level of one of the fragments. The introduction of a pBTAC promoter in the low copy number vector reduced the time for colony appearance (Fig. 4). Finally, a fourth possibility is that assembling occurs during folding, when the peptide is still joined or just leaving the ribosome, in which case, having the synthesis of the fragments in the same ribosome makes assembling more successful than when they are produced in different ribosomes. Since neither expression levels nor assembly were a limitation when a Leu zipper tied the interaction of the fragments, it seems that a combination of the last two possibilities is a plausible explanation for the differences observed between the two expression systems.

The application of the system to generate variability has without doubt a great potential. The limited library sizes obtained by conventional methods (10^5–10^6 in most of the cases) can be exponentially expanded through fragment recombination. By expressing one of the fragments on a phagemid, DNA can be introduced with high efficiency into more than 10^{10} cells harboring the other fragment, thereby achieving an effective library size of 10^{10} or more.

The dynamic range of the system will depend on the genetic selection method. For the PRAI function specifically, one can observe a large growth rate decrease upon fragmentation, which obviously would limit its sensitivity and bias the selection towards a reinforcement of fragment association. However, the addition of a Leu zipper overcame this limitation increasing considerably the sensitivity of the method to wild-type levels.

Further, the impressive differences observed in growth rate of the fragments alone compared to the Leu zipper-appended fragments show the potential of the system as an in vivo sensor for protein–protein interactions, promising a larger dynamic range than obtained with DHFR [20] or β-lactamase [21] fragments.

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