Development of a gene expression vector for *Thermus thermophilus* based on the promoter of the respiratory nitrate reductase

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Abstract

A specific expression system for *Thermus* spp. is described. Plasmid pMKE1 contains replicative origins for *Escherichia coli* and *Thermus* spp., a selection gene encoding a thermostable resistance to kanamycin, and a 720 bp DNA region containing the promoter (Pnar), and the regulatory sequences of the respiratory nitrate reductase operon of *Thermus thermophilus* HB8. Two genes, encoding a thermophilic β-galactosidase and an alkaline phosphatase were cloned in pMKE1 as cytoplasmic and periplasmic reporters, respectively. The expression of the reporters was specifically induced by the combined action of nitrate and anoxia in facultative anaerobic derivatives of *T. thermophilus* HB27 to which the gene cluster for nitrate respiration was transferred by conjugation. Overexpressions in the range of ~200-fold were obtained for the cytoplasmic reporter, whereas that of the periplasmic reporter was limited to ~20-fold, with respect to their intrinsic respective activities.

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1. Introduction

Production of enzymes from thermophilic organisms is most commonly achieved by heterologous expression on a mesophilic host (Coolbear et al., 1992; Ishida et al., 1997; Vieille et al., 1996). However, many complex enzymes, like heterooligomers or those requiring covalently bound co-

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competence of many *Thermus* spp. strains (Friedrich et al., 2001; Koyama et al., 1986), prompted the development of genetic tools for these organisms that presently allow the isolation of insertion mutants (Lasa et al., 1992a), the cloning of genes in plasmids (Koyama et al., 1990a; Koyama et al., 1990b; Lasa et al., 1992b; Mather and Fee, 1992) or integrative vectors (Tamakoshi et al., 1997; Tamakoshi et al., 1999) and the development of selection method for the thermostabilization of proteins from mesophiles (Kotsuka et al., 1996; Tamakoshi et al., 2001; Tamakoshi et al., 1995). However, the lack of knowledge on the regulation of potential transcription promoters (Maseda and Hoshino, 1995) precludes the development of genetic tools for the controlled expression of proteins. In a recent work, we have used the promoter region of a respiratory nitrate reductase operon from *Thermus thermophilus* HB8 (Ramirez-Arcos et al., 1998a) to express a thermostable eukaryotic ribozyme (Vazquez-Tello et al., 2002). In this article, we use an improved version of this promoter to develop a bifunctional *Escherichia coli–Thermus* plasmid, in which the expression of a cloned enzyme gene can be induced by adding nitrate to anoxic or microaerophilic cultures. To analyze such an expression, we use for the first time in *Thermus* two genes encoding thermophilic cytoplasmic and periplasmic reporters. Finally, we demonstrate that the presence of transcriptional activators encoded within the mobilizable element for the respiration of nitrate (*nar*) is necessary for such an induction.

2. Materials and methods

2.1. Bacterial strains

*Thermus thermophilus* HB8 was obtained from the American Type Culture Collection (Rockville, Maryland, USA). *T. thermophilus* HB27 was a gift of Dr. Koyama. The strain *T. thermophilus* HB27::*nar* is a derivative of HB27 that contains the *nar* cluster (Ramirez-Arcos et al., 1998b). The *E. coli* strain DH5α [supE44 flacU169 (p80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1] was used for genetic constructions.

2.2. Cell growth, transformation, and *Pnar* induction

*Escherichia coli* cells were grown in LB (Lennox, 1955) at 37°C. Kanamycin (30 mg/L) or/and ampicillin (100 mg/L) were used when needed. Aerobic growth of *T. thermophilus* was developed at 70°C under shaking (150 r.p.m.) in TB medium (Ramirez-Arcos et al., 1998a). For plasmid selection, kanamycin (30 mg/L) was added to TB plates. *T. thermophilus* transformation was achieved on naturally competent cells as described (de Grado et al., 1999). Transformation of *E. coli* was carried out as described (Hanahan, 1983). For the induction of the *Pnar* promoter, cells were grown aerobically in the absence of nitrate up to an OD550 of 0.2, and transcription was activated by the addition of KNO3 (40 mM) and the simultaneous arresting of the shaker (time 0 in induction experiments).

2.3. General methods and plasmid construction

Plasmid purification and restriction analysis were developed as described (Sambrook et al., 1989). Automatic methods (Applied Biosystems) were used for DNA sequencing. Plasmid pMK18r is a derivative of pMK18 (de Grado et al., 1999) in which the orientation of the *kat* gene and the *Thermus* replicon were reversed. Plasmid pKMPA was previously described (Castan et al., 2002). Plasmid pPSβgal is a derivative of pUC119 (Viera and Messing, 1987) that express a β-galactosidase from *Thermus* T2 (Koyama et al., 1990b) under the control of the promoter of the S-layer gene (*PslpA*) (Faraldo et al., 1992). Plasmid pNIT3 is a derivative of pUC119 that contains a 5.4 kbp DNA fragment encoding the first (*narC*), and part of the second (*narG*) genes of the respiratory nitrate reductase operon (Ramirez-Arcos et al., 1998a), and upstream sequences that includes the *Pnar* promoter region.

Oligonucleotides O-citCNde (5′-CGCATATG CACCTCCGGCCCCA-3′) and pUCreverse (5′-AGGGAACAGCTATGAC-3′) were used for the amplification by PCR from plasmid pNIT3 of a 1.8 kbp DNA region containing the *Pnar* promoter and upstream sequences. A 720 KpnI–NdeI DNA fragment from the PCR product was inserted into a derivative of pMK18r which contained a modi-
fied multicloning site sequence (Fig. 1A). The sequence of the \( P_{\text{nar}} \) promoter was submitted to the EMBL GenBank (Accession No. AJ504993).

2.4. Enzyme activities

The nitrate reductase (NR) activity was measured at 80°C (Ramirez-Arcos et al., 1998a) with methyl viologen as the electron donor and potassium nitrate (40 mM) as the electron acceptor on entire cells permeabilized with (0.1% w/v) tetradecyl-trimethyl ammonium bromide (Snell and Snell, 1949). The activities corresponding to the \( \beta \)-galactosidase and the alkaline phosphatase were assayed at 70°C on soluble fractions corresponding to samples of equivalent cell mass obtained.

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**Fig. 1.** The pMKE1 plasmid. (A) Restriction map of pMKE1. The genes encoding a thermostable resistance to kanamycin (\( kat \)) and the *Thermus* replication protein (\( repA \)) are indicated. Sequences derived from pUC18 are represented as a thick line, in which the replicative origin for *E. coli* (oriE) is labeled. The promoter of the nitrate reductase operon (\( P_{\text{nar}} \)) is indicated as a plasmid insert. Symbols for restriction enzymes: (E) EcoRI; (H) HindIII; (K) KpnI; (N) NdeI; (Nc) NcoI; (Nt) NotI; (P) PstI; (S) SalI; (Sp) SphI; (Xb) XbaI. (B) Sequence of the \( P_{\text{nar}} \) promoter. The transcription start is indicated with an arrow. Restriction sites for BamHI, KpnI, and NdeI are labeled over the sequence. The putative-35 promoter sequence is labeled by a gray frame, the ribosome binding site is in bold, and the ATG start codon is underlined. There is no obvious -10 promoter sequence.
after disruption of the cells by sonication. The β-galactosidase activity was assayed with ortho-nitrophenyl-galactopyranoside (ONPG) as chromogenic substrate in buffer 20 mM Tris–ClH, pH 8. The phosphatase activity was assayed with para-nitrophenyl-phosphate as chromogenic substrate, in a 100 mM Tris–ClH buffer, pH 8. For both activities, enzyme units were normalized with respect to the cell mass as described by Miller (Miller, 1972).

3. Results and discussion

3.1. Cloning of reporter genes in pMKE1

The main features of plasmid pMKE1 are depicted in Fig. 1A. The plasmid includes a minimal replicon for *Thermus* spp. (de Grado et al., 1998) that encodes the replication protein (RepA). It also has a replicative origin from the high copy number plasmid pUC18 that allows its amplification and the development of constructions in *E. coli*, and the *kat* gene, encoding a thermostable kanamycin nucleotidil transferase transcribed from a bifunctional *E. coli–Thermus* promoter, that can be selected both at 37 and 70 °C (Lasa et al., 1992a). For the expression in *Thermus* spp., a 720 bp DNA region, that contains the *Pnar* promoter and downstream sequences encoding the leader mRNA from the first gene (*narC*) of the operon encoding for the respiratory nitrate reductase from *T. thermophilus*, was included (Fig. 1B). Single restriction sites for NdeI, NcoI, EcoRI, SalI, HindIII, and NotI are located downstream *Pnar*, in such a way that the NdeI site overlaps the translation start codon (CATATG) located at the adequate distance from a *Thermus* ribosome binding site (GGAGG).

To evaluate the ability of pMKE1 to direct the controlled expression of desired proteins, we chose two genes, one encoding a β-galactosidase from a *Thermus* spp. (Koyama et al., 1990b) as cytoplasmic reporter, and the other encoding a periplasmic alkaline phosphatase (AP) from *T. thermophilus* (Castan et al., 2002), respectively. In both instances, we used NdeI (5′) and HindIII (3′) restriction sites to isolate the coding region of these genes from plasmids pPSβgal and pKMPA. The resulting plasmids were named pMKEβgal and pMKEPA, respectively.

3.2. Expression of cytoplasmic and periplasmic reporters in *T. thermophilus*

Preliminary work from our laboratory, had shown that the *narCGHJK1K2* operon, encoding the respiratory nitrate reductase of *T. thermophilus*, was located within a DNA region (“nar element”) that could be transferred by conjugation from its natural host, a facultative anaerobe strain of *T. thermophilus*, to the aerobic strain *T. thermophilus* HB27, allowing the exconjugant (HB27::nar) to grow anaerobically (Ramirez-Arcos et al., 1998b). It was also shown in that work that in both, the natural host and the exconjugant strains, the expression of the nitrate reductase required the combined action of anoxia and nitrate (Ramirez-Arcos et al., 1998a). Thus, these data suggested the existence of two regulatory proteins, one signaling anoxia and the other nitrate, in a way similar to that of the expression of the nitrate reductase A in *E. coli*. In this system, the reduced FNR protein (anoxia detection) and the phosphorilated NarL component of the NarX/L two-components system (nitrate signaling), bind to specific DNA sequences upstream the -35 promoter box, leading to the recruitment of the RNA polymerase and the subsequent transcriptional activation of the promoter (Unden and Bongaerts, 1997).

In order to check if such a regulatory circuitry was associated with the presence of the “nar element,” the natural host of the element, and the aerobic (HB27) and its facultative anaerobic derivative (HB27::nar), were transformed with pMKE1, pMKEβgal, and pMKEPA, and inductions experiments were developed as described in Section 2.

In Fig. 2A we show the expression of the β-galactosidase activity along the time in the three strains. As it can be seen, whereas in the aerobic HB27 strain the β-galactosidase activity remained constant along the time (30–40 units), a clear induction was detected after 2 h of static incubation with nitrate in its HB27::nar derivative, reaching its maximum (6800 units) after 20 h of incubation.
Therefore, the expression from the \textit{Pnar} promoter responds to positive controls encoded by the "\textit{nar} element". It is also interesting to note that the presence of plasmid pMKE\textit{b}gal does not increase the intrinsic \(\beta\)-galactosidase activity of the HB27::\textit{nar} strain in uninduced cultures, which remains at the same level as when the plasmid pMKE1 was used for transformation, whereas in the natural host of the "\textit{nar} element" a 3-4 fold expression of the \(\beta\)-galactosidase activity (~130 units) was evident even before the induction. This suggests a lower threshold level for the expression from \textit{Pnar} in the natural host than in the exconjugant strain. Whether the difference in sensitivity was at the level of nitrate or oxygen detection can not be deduced at present, and merits future investigations, but we can conclude that it is not an effect related to the construction of the plasmid pMKE1. In any case, and from an applicative point of view, the fact that \textit{Pnar} remained completely silent in uninduced cultures of the HB27::\textit{nar} strain, makes this promoter/host system a good candidate for the expression in \textit{T. thermophilus} of putatively toxic proteins.

The expression of the periplasmic reporter confirmed the above conclusions about the requirement of transcriptional activators encoded by the "\textit{nar} element" for the induction of \textit{Pnar} (Fig. 2B). However, the overexpression level observed with this periplasmic reporter was much lower (10- to 20-fold the basal level) than with the \(\beta\)-galactosidase, a limitation that could be the additive consequence of two factors. On one hand, the basal phosphatase activity of this strain represents probably an overestimation, as to it can contribute up to four different phosphatases (Pantazaki et al., 1998). On the other hand, the AP expression seems to be saturated after 3 h, probably as consequence of the existence of a "bottle neck" at the secretion level. In this sense, the signal peptide of the alkaline phosphatase overexpressed contains two arginines at its N-terminus, which allow its secretion through the Tat (twin arginine transport) system of \textit{E. coli} (Angelini et al., 2001). Therefore, it could be possible that a similar system was a limiting step for enzyme overproduction in \textit{Thermus}.

3.3. Concluding remarks

In conclusion, we have described a plasmid/host system that can be used for the overexpression of selected proteins directly in \textit{T. thermophilus} up to a
level ~200-fold higher for a cytoplasmic enzyme, and, at least, 10-20 for periplasmic ones. This system is based on a complex promoter which responds to the simultaneous effects of two inexpensive input signals (nitrate and anoxia). It is likely that these signals interact with specific receptors that, either directly as in the case of FNR, or through a signal transduction system, like NarX/L, bind to the promoter and form a nucleoprotein complex that is competent for recruiting the RNA polymerase to start the transcription. It is also clear from our experiments that these activators are coded by the “nar element.” In fact, we have identified recently an homologue of the FNR family encoded upstream of the narCGHJK1K2 operon which could be one of the regulators proposed (unpublished results). Nevertheless, the Pnar promoter is not active in E. coli (not shown), as it could be expected from the absence of any of the sequence binding motifs so far described for FNR and NarL factors, making necessary to identify and purify such specific factors from Thermus spp. to analyze their interactions with the Pnar promoter.

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References


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