Conditional Silencing of the *Escherichia coli* *pykF* Gene Results from Artificial Convergent Transcription Protected from Rho-Dependent Termination

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Abstract

PykF is one of two pyruvate kinases in *Escherichia coli* K-12. λP_L was convergently integrated into the chromosome of the MG1655 strain, downstream of *pykF*, face-to-face with its native promoter. In the presence of $\lambda cts857$, efficient *pykF* ts-silencing was achieved when the 5'-terminus of the P_L -originated antisense RNA (asRNA), consisting of the *rrnG-AT* sequence, converted elongation complexes of RNA polymerase to a form resistant to Rho-dependent transcription termination. *pykF* silencing was detected by the following features: (a) impaired growth of the strain when *pykA* was also disrupted and when using ribose as a non-phosphotransferase system-transporting carbon source; (b) a pattern of reduced synthesis of the full-sized *pykF* mRNA, mediated by reverse transcription PCR, and (c) a significant decrease in PykF activity. The advantages of anti-terminated convergent transcription were clearly manifested in the strains where the *rho_a*-terminator was inserted specifically to interrupt asRNA synthesis. Most likely, the target gene was silenced by transcriptional interference due to collisions between converging RNA polymerases, although, strictly, the

role of *cis*-asRNA effects could not be excluded. While details of the mechanisms have yet to be determined, anti-terminated convergent transcription is a promising new technique for silencing other target genes.

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Introduction

The regulated redistribution of metabolic fluxes into branched pathways is extremely important for applied molecular microbiology and biotechnology when efficient biosynthesis of a desired product leads to growth retardation and decreased activity of the producing strain. Switching from the bacterial growth stage to bioconversion of substrates into desired products could be based on metabolically inducible transcription of recombinant pathway genes [Farmer and Liao, 2000; Imaizumi et al., 2007]. On the other hand, the silencing of some key genes could lead to a significant redistribution of the precursors into a desired metabolic branch as the result of a compensatory strategy [Kim et al., 2007], thus enabling the cell to maintain a stable state when exposed to various types of perturbations. At the very least, the deletions of some *Escherichia coli* genes involved in central metabolism [Mascarenhas et al., 1991] and/or global regulation

[Tatarko and Romeo, 2001] of highly engineered strains lead to a greater increase in the performance of the strain than the more obvious genetic manipulations that amplify genes directly involved in a recombinant pathway.

Conditional silencing is a good alternative to chromosomal gene disruption, especially for genes that are essential for cell viability and active growth when knockout mutants cannot be successfully cultured [Nakashima et al., 2006]. Until recently, at least two general¹ approaches appeared to be suitable for silencing genes in bacterial cells: first, the synthesis of antisense RNA (asRNA) [Ji et al., 2004; Kemmer and Neubauer, 2006; Kim and Cha, 2003]² to downregulate its target gene by either blocking the binding of the ribosome or reducing the stability of the mRNA [Daugherty et al., 1989; Desai and Papoutsakis, 1999]; second, the interruption of mRNA formation of the target gene through transcriptional interference (TI) as a result of the presence of two, face-to-face aligned, convergent promoters [Adhya and Gottesman, 1982; Ward and Murray, 1979]. TI defined as 'the suppressive influence of one transcriptional process, directly and in cis, on a second transcriptional process', in general, can arise as a result of several different promoter arrangements [reviewed by Shearwin et al., 2005]. In case of convergent promoters, TI can interrupt mRNA synthesis through the following molecular mechanisms [Sneppen et al., 2005]: (a) 'promoter occlusion', in which passing *E. coli* RNA polymerase molecules (RNAPs) from convergent promoter block the access to the direct promoter [Adhya and Gottesman, 1982]; (b) 'sitting duck collision,' in which RNAP complexes that are waiting to fire at the promoter are removed by passing RNAPs [Calen et al., 2004], or (c) head-on 'elongation complex collision' (EC collision) between RNAPs as they try to transcribe from convergently aligned promoters, each thereby impeding the progress of the other [Ward and Murray, 1979].

Artificial asRNA genes, placed in trans in multi-copy-number plasmids [Kemmer and Neubauer, 2006; Srivastava et al., 2000], are usually constructed for bacterial

gene silencing. The efficacy of silencing depends appreciably on the stability of the asRNA and the features of its hybridization with mRNA [Engdahl et al., 1997, 2001]. For these reasons, improvements in silencing induced by artificial asRNAs in *E. coli* have been based on the systematic alteration of parameters and the targeting of multiple reporter and essential genes [Cheng et al., 2009; Nakashima et al., 2006]. Nevertheless, even in the best man-made constructions of asRNA genes located in trans, the expression level of the silenced target seldom decreases to lower than 30–40% of the wild type [Kemmer and Neubauer, 2006].

Gene silencing based on transcription from a promoter inserted convergently downstream of the target gene has been convincingly demonstrated [Elledge and Davis, 1989; Ward and Murray, 1979]. Nevertheless, the practical application of the TI-based approach has been limited; even when the corresponding man-made constructs were used, the formation of *cis*-encoded asRNA was considered to be the main factor in the observed gene silencing [O'Connor and Timmis, 1987].

In *E. coli*, two genes, *pykF* and *pykA*, have been found to encode pyruvate (PYR) kinases [Ponce et al., 1995]. When glucose is the sole carbon source, both isoenzymes have an active role in PYR biosynthesis, but the specific activity of PykF is 15-fold higher than that of PykA. Inactivation of only one of the *pyk* genes under phosphotransferase system-inducing (PTS⁺) conditions leads to a slight decrease in the bacterial growth rate on glucose, but disruption of both PYR kinase genes results in a more significant decrease [Ponce et al., 1995] and altered flux ratios for reactions connecting the phosphoenol-pyruvate (PEP) and PYR pools [Gosset et al., 1996; Ponce et al., 1998; Sauer et al., 1999; Siddiquee et al., 2004]. Ponce et al. [1995] showed that when ribose, a non-PTS sugar, was used as the sole carbon source, single *pyk* mutants were capable of growth; however, the strain bearing the double *pykA-pykF* mutation was unable to grow on ribose because it lacked the ability to produce PYR (fig. 1).

The $\lambda P_L/O_L$ regulatory region (P_L) has one of the strongest promoters recognized by *E. coli* σ^{70} -RNAP [Deuschle et al., 1986; Liang et al., 1999], is governed by the λ CIts857 repressor in a temperature-dependent fashion [Ptashne, 1992], and is stimulated by integration host factor (IHF) [Giladi et al., 1992; 1996]. The *nutL* sequence essential for N-dependent anti-termination of transcription of the λ genes [Greenblatt et al., 1993; Ptashne, 1992] is located immediately downstream of P_L in the native phage DNA.

¹ In some cases, an effect that looks like gene silencing may be achieved by a controllable degradation of protein product [see, for instance, McGinness et al., 2006], but this approach is based on protein-specific modifications including possible infringement of enzymatic activity; therefore, this approach cannot be considered as a general solution.

² Gene-specific silencing induced by the synthesis of artificial parallel complementary RNA (pRNA) has been described as well [Liu et al., 2009; Tchurikov et al., 2000]. But the mechanisms of the parallel RNA-RNA duplex formation in vivo and its influence on mRNA expression are not clear.

To construct strains with *pykF* genes that were conditionally silenced due to the influence of TI between converging RNAPs on their expression, we integrated P_L -carrier DNA fragments convergently into the chromosome of an *E. coli* MG1655-based strain, downstream of *pykF* and face-to-face with its native promoter. In the presence of $\lambda cIts857$, more efficient *pykF* conditional (temperature-driven) silencing was achieved when the *rrnG* anti-termination sequence (termed *rrnG-AT* in Albrechtsen et al., [1991]) was placed downstream of P_L instead of the *nutL* sequence possessed by the native, (P_L -*nutL*) carrier, λ DNA fragment. The corresponding modification of the 5'-terminal part of P_L -originated nascent transcripts containing *rrnG-AT* could convert RNAP elongation complexes into forms that were resistant to Rho-dependent termination [Albrechtsen et al., 1990; Condon et al., 1995; Squires et al., 1993]. We anticipate that designs similar to the synthetic, anti-terminated, convergent transcription process described in this work could conditionally silence different target genes that are relevant to applied molecular microbiology and biotechnology.

Results

Structure of the *pykF* Gene in the Chromosomes Differed between Constructed Strains

E. coli mutants were constructed with alterations in the *pyk* genes and with a disruption in the *edd-eda* operon encoding the PYR kinases and enzymes of the Entner-Doudoroff pathway, correspondingly. λ Red-driven insertion of the set of DNA fragments carrying P_L as the convergent promoter was provided downstream of the putative Rho-independent terminator [Kingsford et al., 2007] of *pykF*, according to the procedure described by Datsenko and Wanner [2000] (fig. 2). For chromosomal rearrangements, the excisable marker $\lambda attL$ -Cm^R- $\lambda attR$ was used for selective DNA integration followed by curing the marker with λ Xis/Int recombinase, as described earlier [Katashkina et al., 2005]. Several of the strains possessed the *rrnG-AT* sequence instead of the native *nutL* sequence downstream of P_L (fig. 2), which may protect the P_L -originated *pykF* asRNA from Rho-dependent transcription termination. Strains with the convergently facing Rho-dependent terminator *rho_a* [Albrechtsen et al., 1990] downstream of *pykF* (fig. 2) were also constructed. In the latter strains, P_L -driven transcription could be terminated or anti-terminated at *rho_a*, depending on the presence of *rrnG-AT* in the nascent tran-

Fig. 1. Simplified scheme of PYR biosynthesis in *E. coli* using different carbon sources. Only genes that are mentioned in the text are indicated in the corresponding metabolic pathways. 6PG = Gluconate-6-phosphate; F6P = fructose-6-phosphate; G6P = glucose-6-phosphate; GAP = glyceraldehyde-3-phosphate; Glc = glucose; KD6PG = 2-keto-3-deoxy-gluconate-6-phosphate; MAL = malate; OAA = oxaloacetate; R5P = ribose-5-phosphate; Rib = ribose; TCA = tricarboxylic acid cycle; X5P = xylulose-5-phosphate.

script. The structures of the resulting insertions were confirmed by sequencing. The constructed strains are listed in table 1.

Effect of the Mutations on *pykF* Expression

The growth of the strains was tested on solid media supplemented with glucose or ribose as the sole carbon source. The strains that were modified only by 'simple' gene/operon deletions manifested growth features that accorded with their predicted ability [Ponce et al., 1995] to produce PYR in different branches of metabolism by PTS, PYR kinases, and/or in the Entner-Doudoroff pathway (fig. 1). A very poor, but detectable growth on ribose of the AK000 strain, with *pykAF* and *edd-eda* genes deleted, was considered as '−' in table 1, and could likely be explained by the possible formation of some amount of PYR due to

an activity of malic enzyme (product of *maeB* gene) [Siddiquee et al., 2004] in the PEP carboxylase (Ppc) – malate dehydrogenase (Mdh) – malic enzyme (Mae) shunt (fig. 1). AK002 and AK003, the Δ (*pykA*, *edd-eda*)-based strains carrying P_L as the convergent element to the *pykF* promoter, demonstrated the Pyk^- phenotype. Their growth on glucose was clearly poorer than the isogenic AK001

strain with the wild-type *pykF* gene (*pykF*-wt), and they were almost unable to grow on ribose, similar to the Δ *pykAF* strain, AK000. The AK002 and AK003 strains grown on glucose showed a residual PYR kinase activity that did not exceed 5% of the level in AK001 (table 1).

Thus, the P_L -carrier strains AK002 and AK003 did not differ from each other with respect to the *pykF*-silenced

Table 1. Description of the constructed strains, their growth on solid M9 minimal media with different carbon sources, and the detected level of *PykF* activity

Strain	Description	Comparative growth on carbon source ^a		<i>PykF</i> ^b mU/mg
		glucose	ribose	
AK111	MG1655 – wild type	+++++	+++++	NT
AK011	MG1655- Δ <i>pykA</i>	NT	NT	NT
AK101	MG1655- Δ (<i>edd-eda</i>)	NT	NT	NT
AK110	MG1655- Δ <i>pykF</i>	NT	NT	NT
AK010	MG1655- Δ (<i>pykA</i> , <i>pykF</i>)	NT	NT	NT
AK000	MG1655- Δ (<i>pykA</i> , <i>edd-eda</i> , <i>pykF</i>)	+++	–	5 ± 5
AK001	MG1655- Δ (<i>pykA</i> , <i>edd-eda</i>)	++++	++++	590 ± 30
AK002	AK001-(<i>pykF</i> ←[<i>nutL</i>]- P_L)	+++	–	10 ± 10
AK003	AK001-(<i>pykF</i> ←[<i>rrnG-AT</i>]- P_L)	+++	–	10 ± 10
AK004	AK001-(<i>pykF</i> ←[<i>rho_a</i>]-[<i>nutL</i>]- P_L)	++++	+++	480 ± 20
AK005	AK001-(<i>pykF</i> ←[<i>rho_a</i>]-[<i>rrnG-AT</i>]- P_L)	+++	–	10 ± 10
AK006	AK000:: λ cIts857			
AK007	AK001:: λ cIts857			
AK008	AK002:: λ cIts857			
AK009	AK003:: λ cIts857			

NT = Not tested.

^a Growth response was observed after 48 h of cultivation at 37°C on solid M9 minimal medium supplemented with either glucose (10 mM) or ribose (15 mM) as the sole carbon source.

^b *PykF* activity was measured for the cells grown at 37°C in liquid M9 minimal medium with glucose.

Fig. 2. a General scheme of stepwise Red-driven modifications of the strains based on AK001 with *pykF*-wt. GenBank accession numbers for the nucleotide sequences of chromosomal DNA fragments downstream of the *pykF* gene are indicated below the names of the corresponding strains. AK002 was obtained by inserting the (P_L -*nutL*) carrier λ DNA fragment linked with excisable Cm^R , followed by curing the marker with λ *attB* as a ‘scar’ sequence. The substitution of the *rrnG-AT* sequence for *nutL* was provided by the second Red modification, leading finally to the appearance of the second λ *attB* scar in the chromosome of AK003. AK004 and AK005 were obtained from AK002 and AK003, respectively, by inserting a DNA fragment consisting of the Rho-dependent transcription terminator *rho_a* [Albrechtsen et al., 1990]. **b** Structural details of the regulatory elements. The nucleotide sequence of λ *attB* is depicted as double-stranded DNA. Two core-type targets for λ Int-protein, B and B’, flanking the ‘overlap region’, O, comprise λ *attB*. Relative orientation of the λ Int bind-

ing sites is shown by arrows under the corresponding nucleotides in λ *attB* [Landy, 1989]. The probable, Rho-independent transcription terminator of *pykF*, T_{pykF} , is presented as the fragment of dsDNA, indicating the GC-rich inverted repeat and the (oligoT) tail in the sense strand. Features of the P_L structure [Giladi et al., 1992, 1996], including the P_L1 and P_L2 promoters, together with the sites for the IHF binding ($L1$, $L2$) and the O_L (O_L1 , O_L2 , and O_L3) operators (the targets for λ CI), are shown. The P_L -carrier DNA fragment that was initially inserted corresponded to the native λ DNA from nucleotide –240 up to +118 (numbers denoting the distance from P_L1 start site) and included the *nutL* site [Sanger et al., 1982]. The nucleotide sequences of *nutL* and *rrnG-AT* are depicted as single-stranded DNA. The ability of *boxB* to form a stem-and-loop structure is noted by the inverted pointers, and the sequences of *boxA* and *boxC* are indicated in rectangles [Li et al., 1984].

Fig. 3. a Experimental strategy and RT-PCR analysis of RNAs extracted from AK001, AK002, and AK003 strains. The possible amplimers $L(i, j)$ obtained after exploiting $P(i)$ as a primer for reverse transcription and $P(j)$ for DNA amplification by RT-PCR are indicated. **b** Various amplimers corresponded to P_L -originated

asRNAs that were detected in the AK002 and AK003 strains. **c** Analyses of rather large fragments of sense and anti-sense *pykF* RNA. **d** RT-PCR-driven amplification of DNA fragments corresponded to mRNA that originated from the *pykF* start site and differed at the 3'-terminus.

phenotype achieved by constitutive P_L -driven convergent transcription. At the same time, the more efficient synthesis of *pykF* asRNA was detected by reverse transcription PCR (RT-PCR) in strain AK003, where it was originated from P_L -[*rrnG-AT*], than in the case of P_L -[*nutL*] in AK002 (fig. 3a, b). Indeed, rather short amplimers, L(1,4) and L(2,4) (up to 414 bp), corresponding to 5'-fragments of the *pykF* asRNA, were detected in extracts of both the AK002 and AK003 strains, but only AK003 retained asRNA extending from the 5'-terminus up to 1,199 nt in length as the template for RT-PCR³.

³ Later, the presence of rather large (1,120 nt in length) L(3,5) amplimer, corresponding to the central part of asRNA *pykF*, was demonstrated in extracts of AK002 (see next section), but again the extended transcription originating from P_L was more efficient in AK003 according to RT-PCR (data not shown).

In turn, the AK004 and AK005 strains, based on AK002 and AK003, correspondingly, and possessing *rho_a* as a Rho-dependent terminator for P_L -mediated convergent transcription, significantly differed in *pykF* gene expression. AK004 (*pykF*←[*rho_a*]-[*nutL*]- P_L) was able to grow on ribose as the carbon source and retained about 80–90% of the wild-type PykF activity (table 1), while AK005 (*pykF*←[*rho_a*]-[*rrnG-AT*]- P_L) had significantly impaired growth on ribose, and its PYR kinase activity did not exceed 5% of the control. We concluded that the *pykF* silencing in the latter case was caused by the convergent transcription by RNAPs that were resistant to Rho-dependent termination [Albrechtsen et al., 1990, 1991].

Decreased Level of Full-Sized mRNA Led to *pykF* Gene Silencing

RNA samples were prepared from the two strains, AK001 and AK002, with (*pykF*-wt) and with (*pykF*←

Fig. 4. a Structure of plasmid pPR53 [Mashko et al., 1990] carrying the cassette with the phage λ *clts857* gene and the gene for the hybrid Cro'-CAT protein under the transcriptional control of the λ P_R/O_R regulatory region (P_R). CAT is chloramphenicol acetyltransferase originally encoding by the *cat* gene from pBR325 [Bolivar, 1978]. The synthesis of the hybrid Cro'-CAT and the full-sized CAT could be seen by gel electrophoresis among the total cellular proteins that were extracted from the thermo-induced, plasmid-carrying *E. coli* cells. **b** Principal scheme of the cassette integration from pPR53 into the ϕ 80attB site of the *E. coli* MG1655 chromosome according to the dual in/out strategy [Minaeva et al., 2008].

[*nutL*]-P₁), correspondingly, grown on glucose, and *pykF* silencing was detected by RT-PCR according to the strategy presented in figure 3a. The presence of 1,120-nt fragments of mRNA and asRNA corresponding to the internal portion of the *pykF* gene was detected. The amplimers L(5,3) corresponded to mRNA fragments detected in the AK001 but not AK002 extracts. In contrast, L(3,5) for the asRNAs was only found in AK002 (fig. 3c).

The synthesis of *pykF* mRNA in AK001 and AK002 was evaluated by RT-PCR to detect the set of 3'-truncated *pykF* mRNA fragments. P6, P7, P8, or P5 primers were used as different probes for cDNA synthesis, followed by PCR using P9 as the second primer for DNA amplifica-

tion (fig. 3a). The set of *pykF* mRNA-mediated amplimers (up to 1,259 bp in length) were detected for both strains, but the longest fragment L(5,9) (1,326 bp) could only be seen clearly in AK001 (fig. 3d). Therefore, *pykF* silencing in AK002 (*pykF*←[*nutL*]-P₁) results from a significantly decreased level of the full-sized mRNA present in the growing cells.

Conditional *pykF* Silencing Is Dependent on the Activity of λ CI*ts857*

The (λ CI*ts857* – P_R>*cro'*-*cat*) cassette, taken from plasmid pPR53 [Mashko et al., 1990], provided the ts-dependent biosynthesis of the hybrid Cro'-CAT and the full-

Fig. 5. Effect of conditional *pykF* silencing depended on the cultivation temperature of the *cIts857* carrier cells. **a** Bacterial growth on ribose-containing agarose media. **b** Typical dependence of the specific CAT activity on the cultivation temperature is presented for AK007 for all four tested strains. **c** PYR kinase activities of the different strains show dependence on the cultivation temperature.

sized CAT protein (fig. 4a). Using a dual in/out strategy [Minaeva et al., 2008], this cassette was integrated at the $\phi 80attB$ site of the *E. coli* MG1655 chromosome (fig. 4b) followed by P1vir phage-mediated generalized transduction (P1-duction) of the cassette to strains AK000, AK001, AK002, and AK003, each differing in the structure of the *pykF* gene, and four new strains were obtained, numbered from AK006 through AK009 (table 1). The growth of the *cIts857* carrier strains was studied at different temperatures on agar plates with ribose. The levels of PYR kinase and CAT activities were determined in bacteria grown in liquid media with glucose. CAT was used as a distinctive reporter to evaluate ts-inducible P_L - or P_R -mediated gene expression.

At 30°C, when the repressor *CI*_{ts857} was active, AK008 (*pykF*←[*nutL*]- P_L) and AK009 (*pykF*←[*rrnG-AT*]- P_L) grew rather well on ribose, as did the (*pykF*-wt) strain, AK007 (fig. 5a). From the CAT activity (fig. 5b), we concluded that stepwise increases in the cultivation temperature up to 42°C led to the gradual derepression

of the P_L - and P_R -promoters. The growth of the tested strains on ribose was different. AK008 retained the residual, but visible, capacity to grow on ribose even at 42°C, although this growth was significantly worse than for the (*pykF*-wt)-carrying control. AK009 clearly manifested a conditionally-silenced *pykF* phenotype: it was practically indistinguishable from the AK007 at 30°C, and it already had poor growth on ribose at 42°C. The AK006 strain, carrying $\Delta pykF$, had a similarly poor growth at 42°C.

Increasing levels of CAT activity depended on increasing bacterial cultivation temperatures in all strains (fig. 5b). PykF activity responded differently: (a) its levels were almost identical at all temperatures in AK007 with *pykF*-wt, and (b) the obvious ts-dependent *pykF*-silencing could be seen in the two strains with P_L -mediated convergent transcription (fig. 5c). The value of specific PykF activity decreased with the cultivation time after thermoinduction for both AK008 (*pykF*←[*nutL*]- P_L) and AK009 (*pykF*←[*rrnG-AT*]- P_L) strains. (The data in fig. 5c

correspond to 3–3.5 h of cell growth at different experimental temperatures.) However, the detected level of PykF activity was lower for AK009 than for AK008 at early stages of cell cultivation at 37 and 42°C (data not shown). Thus, convergent transcription, protected from Rho-dependent termination, led to a more efficient conditional *pykF* gene silencing.

Discussion

Integration of the Clts857-governed, P_L downstream *pykF*, face-to-face with its native promoter, led to conditional gene silencing. With constitutive P_L -mediated convergent transcription, the basal level of PykF activity did not exceed 5% of that seen in the wild type. This effect could result from the following: (a) *cis*-encoded asRNA hybridization with mRNA; (b) impaired mRNA transcription caused by TI between converging RNAPs, or (c) both of these mechanisms. These mechanisms are frequently considered in relation to the results of convergent transcription [Elledge and Davis, 1989; O'Connor and Timmis, 1987].

The majority of natural *cis*-encoded asRNAs are small (~50–300 nt), highly structured molecules that bind their target RNAs, thereby regulating target gene expression [Brantl, 2007]. For proper inhibition, the asRNA molar concentration must be in significant excess over that of the target [Brantl and Wagner, 1996; Brenner and Tomizawa, 1991; Nakashima and Tamura, 2009]. The hybridization between asRNA and mRNA occurs via a complex pathway that usually has several steps and is initiated by specifically arranged complementary structures [reviewed in Brantl, 2002, 2007].

Thus, we doubted that the strong *pykF* silencing resulted from the inhibitory effect of *cis*-encoded asRNA, which would need to hybridize with mRNA without any specific targeting. On the other hand, the repression potential of TI caused by converging RNAPs may be very large (over a 100-fold range) [Elledge and Davis, 1989]. Moreover, there was a strikingly strong dependence upon convergent promoters strength for the TI-driven repression; changes in promoter initiation rates of two- or threefold often had profound effects. Indeed, a rather high residual activity of PykF was detected in the present study at moderate temperatures around 37°C (fig. 5). This partial derepression of the P_L -mediated transcription was equivalent to the decreased quasi-strength of the convergent promoter.

The detection of 5'-proximal fragments of *pykF* mRNA in the silenced conditions can be explained by two molecular mechanisms. Regarding specific effects of *cis*-encoded asRNA, the RNase-mediated fast degradation of the duplex may have occurred between asRNA and the 3'-end of the mRNA, whereas if TI played the main role in *pykF* silencing, the head-on EC collision mechanism between the converging RNAPs may have led to formation of the truncated mRNA of the target gene. Certainly, the two other known TI mechanisms, 'promoter occlusion' and 'sitting duck' interference, where mRNA synthesis cannot be initiated, may additionally increase the efficiency of silencing. Indeed, the existing theoretical models of TI mention the great impact of EC collisions on the interference efficiency not only when the 'aggressive' convergent promoter is very strong but also when the interfering promoters are far apart [Sneppen et al., 2005].

At present, it is impossible to investigate the fate of the RNAPs once they have undergone collision. In vitro, both RNAPs, which are in converging complexes that have collided, usually stall against each other and remain bound to the DNA [Crampton et al., 2006]. At the same time, the simplest explanation of the in vivo reversibility of TI [Ward and Murray, 1979] is that both of the RNAPs that have collided are released from the DNA template. However, the available data to date do not exclude the possibility that in each EC collision only one RNAP dissociates based on the complex stability. The complexes are not equivalent even in vitro because regulation of transcription elongation appears to be governed by the ability of RNAP to adopt multiple conformational states that are induced, in particular, by template DNA and binding NTP [Foster et al., 2001]. The asymmetry of the TI could be significantly enhanced in vivo in the presence of different transcription elongation factors [Borukhov et al., 2005], due to the possible cooperative 'push' from following RNAPs that depends on the density of elongating complexes [Epshtein et al., 2003; Epshtein and Nudler, 2003]. It could also be enhanced when asRNA-synthesizing RNAPs are much more sensitive to possible Rho-dependent termination [Banerjee et al., 2006; Nudler and Gottesman, 2002] than RNAPs participating in mRNA synthesis, usually coupled with translation in the bacterial cell.

In this study, efficient *pykF* silencing was initially achieved by constitutive P_L -mediated convergent transcription. The frequency of transcription initiation from P_L is in the range of 5–15 transcripts/min [Liang et al., 1999], the mRNA chain elongation rate is in the range

40–55 nt/s [Bremer and Dennis, 1996], and the length of the *pykF* mRNA is approximately 1,700 nt. From these facts, we expect that about 3–10 RNAPs participate in the convergent transcription at any given moment; thus, each could collide with RNAPs originating from *pykF* mRNA synthesis if the EC collision frequency depends only on the strength of convergent promoters. It could be further proposed that the ECs were equivalent and at least one, or possibly both, of the RNAPs that collided then dissociated from the template with equal probability. However, the following facts then cannot be explained: (a) the detection of rather large 3'-truncated, *pykF* mRNA fragments (with $L > 1,200$ nt) in AK002 (*pykF*←[*nutL*]-P_L), and (b) the slight decrease in the *pykF* expression level that was detected in *cIts857* carrier strains AK008 (*pykF*←[*nutL*]-P_L) relative to AK009 (*pykF*←[*rrnG-AT*]-P_L) under partial P_L derepression conditions.

TI could be completely abolished if a fraction of the converging RNAPs terminated transcription in an untimely manner and did not participate in EC collisions. Such transcription attenuation would be formally equivalent to a significant decrease in the convergent promoter strength.

P_L was inserted downstream of the putative Rho-independent *pykF* terminator (fig. 2). According to the known computer algorithms [Kingsford et al., 2007; Lesnik et al., 2001] we did not find clear Rho-independent terminators for the convergent transcription of *pykF*⁴, but the transcription might still be terminated by the Rho-dependent mechanism.

P_L-mediated transcription in AK002 (*pykF*←[*nutL*]-P_L) was not protected by the λN anti-terminating system [Chattopadhyay et al., 1995; Mogridge et al., 1995, 1998], as was in a previous study [Ward and Murray, 1979]. At the same time, the λ *boxA* from the *nutL* site in the (P_L-*nutL*)-carrier constructions (fig. 2) seemed to closely resemble the *rrn boxA* which, in turn, was shown to be sufficient to prevent Rho-dependent termination [Condon et al., 1995; Squires et al., 1993]. Nevertheless, inserting *rho_a* interrupted the effect of *pykF* silencing in AK004, where P_L-*nutL* was responsible for convergent transcription. However, this terminator only slightly decreased the

level of *pykF* silencing in AK005, where the 5'-terminus of the P_L-originating nascent RNA carried the *rrnG-AT* sequence. These data correlated with the known findings that just a slight difference in *boxA* sequences finally determined the assembly pathways leading to the controlled formation of different RNA protein anti-termination complexes involved in vivo in the transcription of the *rrn* genes or in N-dependent phage λ development [Greive et al., 2005; Luo et al., 2008].

It seems that transcription initiated in the bacterial chromosome and protected by λN-mediated or other systems, which could override all types of terminators [King et al., 1996; Marr et al., 2001; Nickels et al., 2002; Oberto et al., 1993], might be toxic for the cell. Nevertheless, the conversion of converging RNAP to a form that is only resistant to Rho-dependent termination was considered to be a reasonable hypothesis for the improvement of the gene silencing effect caused by artificial convergent transcription. Comparing the effects of *pykF* silencing for strains AK004 (*pykF*←[*rho_a*]-[*nutL*]-P_L) and AK005 (*pykF*←[*rho_a*]-[*rrnG-AT*]-P_L), we conclude that our results confirm this proposal. Untimely termination of convergent transcription, which often occurs, may explain why such an obvious, TI-based approach has not yet received broad application for target-gene silencing in molecular microbiology.

TI caused by EC collisions between converging RNAPs is the most probable reason for the *pykF* silencing that was detected. Nevertheless, our results could not conclusively rule out a role for strictly *cis*-asRNA effects. Even in the latter case, efficient, anti-terminated, engineered asRNA synthesis seems essential. Therefore, we recommend this novel design using convergent transcription that is protected from Rho-dependent termination to silence any gene target. It may be especially useful when stationary, growth-dependent [Miksch et al., 2005] and/or metabolically regulated promoters of moderate strength [Farmer and Liao, 2000], not as 'aggressive' as P_L, are used as the convergent silencers.

Experimental Procedure

Recombinant DNA Techniques, Bacterial Strains and Growth Condition

Construction and restriction analysis of the recombinant DNAs, agarose gel electrophoresis, Ca²⁺-dependent transformation, and electroporation of *E. coli* cells were all carried out using routine experimental protocols [Sambrook and Russell, 2001]. Restriction endonucleases, T4 DNA ligase (Fermentas, Lithuania), and AccuTaq DNA polymerase (Sigma, USA) were used according to the manufacturer's instructions. Sigma products were

⁴ The native Rho-independent terminator of the *pykF* gene was calculated to possess a rather high 'termination score' even for convergent transcription, according to the algorithm developed by Kingsford et al. [2007].

used to isolate plasmid DNA and extract DNA fragments from agarose gels.

All of the strains tested were derived from *E. coli* MG1655 as follows: (a) Red modifications of the chromosome were prepared according to Datsenko and Wanner [2000], exploiting the phage λ site-specific Xis/Int system instead of FLP recombinase, as described earlier [Doroshenko et al., 2007]; (b) the λ Cts857 carrier cassette from pPR53 [Mashko et al., 1990] (fig. 4a) was inserted into the native ϕ 80attB site of the *E. coli* MG1655 genome according to Minaeva et al. [2008] (fig. 4b), followed by a combining of the chromosomal modifications using (c) P1-duction [Miller, 1972]. The plasmid pKD46 [Datsenko and Wanner, 2000], carrying arabinose-inducible λ Red genes, was used to provide the Red recombination. The pMW118- λ attL-Cm^R- λ attR [Katashkina et al., 2005], λ Cts857 DNA (Fermentas), and chromosomal DNA from *E. coli* MG1655 were used as templates to construct amplimers with excisable Cm^R, P_L-promoters and with *rrnG*-AT sequence, respectively. Plasmid pMW- λ int-xis [Doroshenko et al., 2007] encoding the λ Xis/Int recombinase was used to eliminate all DNA fragments that were flanked by λ attL/R sites at the final steps of the chromosomal modifications. For ϕ 80-driven integration, plasmids pAH123 ([Haldimann and Wanner, 2001] GenBank accession number AY048726) and pAH162- λ attL-Tc^R- λ attR [Minaeva et al., 2008] were used as the 'helper' plasmid and 'integrative vector' [Minaeva et al., 2008], respectively. The latter plasmid possessed a Pir⁺-dependent replication function [Bowers et al., 2007]; therefore, the *E. coli* CC118 λ pir⁺ strain [Herrero et al., 1990] was used as a recipient and as a host strain for propagation of the conditionally replicated recombinant plasmids constructed using this vector.

Several steps were required to insert P_L downstream of *pykF*, followed by the introduction of *rrnG*-AT and *rho_a* sequences. DNA fragments (λ attR-Cm^R- λ attL)-P_L and (λ attR-Cm^R- λ attL)-(*rrnG*-AT) were obtained by PCR using DNA fragments containing one of the regulatory elements and the excisable Cm^R as the selective marker. The linkage of (λ attR-Cm^R- λ attL)-P_L was provided by ligation of the *Kpn*I-produced ends of the amplimers, and *Bgl*II was used for the joining of (λ attR-Cm^R- λ attL)-(*rrnG*-AT) that followed. These restriction sites were predesigned into the corresponding PCR primers. The DNA fragments obtained were introduced by the Red system, downstream of the *pykF* gene in the *E. coli* MG1655 chromosome (fig. 2). The marker was excised from the chromosome to complete the desired rearrangements. The residual λ attB site was the 'scar' sequence at each step of such marker curing. Later, the amplimer with *rho_a* was also inserted. The structures of all stepwise Red-inserted DNA fragments were confirmed using PCR-mediated sequencing.

Primers that were used to delete genes were designed to replace almost all of the coding sequences (usually 12 codons from both ends were retained) of each gene/operon with the excisable Cm^R marker. The deletions marked by Cm^R were transferred using P1-duction followed by λ Xis/Int-dependent excising of the marker to combine the mutations in one strain. The resulting mutants were checked by PCR to confirm their genetic structure. Primers were designed to provide the deletions 'in frame' after excising the marker and preserving the λ attB site as the scar sequence. The sequences of these primers are available from the corresponding author.

Bacteria were grown on M9 minimal media agar [Sambrook and Russell, 2001], supplemented with glucose (10 mM) or ribose

(15 mM) as the sole carbon source. Bacteria were alternatively cultivated in LB broth or M9 minimal glucose-containing liquid media. Ampicillin (100 mg/l) was added to maintain the Ap^R plasmid carrier strains. Chloramphenicol (25 mg/l) or tetracycline (12.5 mg/l) was added to media as required to select for the corresponding markers in the bacterial chromosome.

Reverse Transcription PCR

RT-PCR was performed as previously described [Doroshenko et al., 2007]. Briefly, total RNA was isolated from *E. coli* cells, its concentration was determined using the absorbance at 260 nm, the RNA integrity was monitored electrophoretically, reverse transcription of *pykF* RNA was carried out, and the resulting cDNA was subsequently amplified by PCR using different primers designed for testing *pykF* cognate RNAs, as presented in figure 3a. The following primers were used:

- P(1): 5' - atgccgtggtgccgttgaaa → 3';
- P(2): 5' - cgtaccgaccgcgtgatgaa → 3';
- P(3): 5' - tccgcaccatgaaactggaaggcgg → 3';
- P(4): 5' - ttacaggacgtgaacagatgcg → 3';
- P(5): 5' - ttgtgtgccagaccgctctgc → 3';
- P(6): 5' - ggaagcagcaacaagtctacg → 3';
- P(7): 5' - tgggtgcatccgggaagtattt → 3';
- P(8): 5' - tctctttaacaagctgcggcaca → 3';
- P(9): 5' - gaccaaattgtttgaccatcgg → 3'.

Equal quantities (about 100 ng) of the total RNA were used to prepare cDNA for comparative evaluations, and the final PCR-amplified DNA probes that were loaded on the gels were additionally normalized using the *rrsA* gene (encoding 16S rRNA) as an internal control in each sample. The reproducibility of this procedure was determined by performing two separate cDNA synthesis experiments using the RNA extracted for each strain. Similar results were obtained for the qualitative transcription levels of each of these duplicate experiments.

Control reactions to monitor DNA contamination were conducted with prepared RNAs in mock RT reactions without the presence of AMV reverse transcriptase.

Enzyme Assays

Overnight cultures of the strains grown on M9 minimal liquid medium with glucose at 30°C were diluted to a starting OD₅₉₅ of 0.1, and the cells were grown at different experimental temperatures up to OD₅₉₅ ~ (0.5–0.6), harvested, washed, and sonicated. Spectrophotometric measurements were made using a Synergy 2 multi-detection microplate reader (BioTek Instruments, Inc., USA). Total protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, USA) in accordance with the manufacturer's instructions.

PykF activity was measured from the production of PYR in a coupled system with lactate dehydrogenase (LDH) and NADH [Malcovati and Valentini, 1982]. The reaction buffer contained 100 mM Tris-HCl pH 7.5, 10 mM KCl, 15 mM MgCl₂, 5 mM ADP, 0.15 mM NADH, 10 U/ml LDH, 1 mM fructose-1,6-biphosphate, and 1 mM PEP. All reagents were purchased from Sigma. PYR kinase activity was measured spectrophotometrically by the reduction in NADH in the probes.

CAT activity was assayed according to the standard spectrophotometric method [Shaw, 1975] based on detection of produced

thionitrobenzoic acid at 412 nm, as described in detail by Minaeva et al. [2008].

One unit of specific enzyme activity was defined as the amount of enzyme required to convert 1 μ mol substrate into the specific product per minute per milligram of protein. Each measurement was performed in triplicate.

SDS-PAGE of the Cellular Proteins

Protein electrophoresis was performed in 0.1% SDS-12% PAGE [Laemmli, 1970] followed by staining with Coomassie R-250.

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