Inhibitory Effect of Heterologous Ribosome Recycling Factor on Growth of Escherichia coli

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Received 27 January 2000/Accepted 11 July 2000

Ribosome recycling factor (RRF) of Thermotoga maritima was expressed in Escherichia coli from the cloned T. maritima RRF gene and purified. Expression of T. maritima RRF inhibited growth of the E. coli host in a dose-dependent manner, an effect counteracted by the overexpression of E. coli RRF. T. maritima RRF also inhibited the E. coli RRF reaction in vitro. Genes encoding RRFs from Streptococcus pneumoniae and Helicobacter pylori have been cloned, and they also impair growth of E. coli, although the inhibitory effect of these RRFs was less pronounced than that of T. maritima RRF. This suggests that these regions are important for the inhibitory effect of heterologous RRFs. We further suggest that bending and stretching of the RRF molecule at the hinge between two domains may be critical for RRF activity and therefore responsible for T. maritima RRF inhibition of the E. coli RRF reaction.

MATERIALS AND METHODS

Strains and plasmids. Table 1 shows all strains and plasmids used in this study. Vectors are pUC19 (Ap’), pET-11a (Ap’), and pET-24a(+) (Km’) (for carrying S. pneumoniae frr or H. pylori frr). Plasmid pHSG299 (Km’) (for carrying E. coli frr), pKA1 (pET-11a carrying T. maritima frr), pKA5 (pET-24a(+) carrying S. pneumoniae frr), pKA6 (pET-24a(+) carrying H. pylori frr), and pRR3 (pHSG299 carrying E. coli frr).

BL21(DE3)pLysS is a lysogen of lambda phage derivative DE3, which carries the gene for T7 RNA polymerase under the control of the inducible lacUV5 promoter in the chromosome. T7 RNA polymerase induced by the addition of IPTG (isopropyl-ß-D-thiogalactopyranoside) drove the expression of various frrs. BL21(DE3)pLysS contains plasmid pLysS expressing T7 lysozyme. This enzyme is a natural inhibitor of T7 RNA polymerase and reduces its ability to transcribe target genes in noninduced cells.

Oligonucleotide primers for PCR. All oligonucleotide primers for PCR were synthesized using Beckman Oligo 100M. The sense and antisense primers for cloning T. maritima frr were 5’-AGG GGA TAC ATT TGG GTA TTA ATC GTT CTA-3’ and 5’-GCA ACG TGC TGT GGG ATC ATG-3’. The sense and antisense primers for cloning S. pneumoniae frr were 5’-GGA TTA AGA AAG CAT ATG GCT AAC GCA-3’ and 5’-GAG TTT TTC TGT GGA TCC TTA GAC TCC-3’, respectively.

This paper describes the cloning, expression, and purification of RRF from Thermotoga maritima, which has been used for determination of the crystal structure (36). In addition, genes coding for RRF of Streptococcus pneumoniae and Helicobacter pylori were isolated and studied. In contrast to Pseudomonas aeruginosa frr, which functions in E. coli (29), we found that these other heterologous RRFs were toxic to E. coli. We suggest that freezing at the hinge region of the T. maritima RRF structure may be partly responsible for the toxic effects.
with the same antibiotics and shaken at 37°C to early mid-log phase (optical pLysS). The cultures were diluted 100-fold with fresh LB medium supplemented for pKA5, pKA6, pRR3, and pHSG299), and chloramphenicol (25
were ampicillin (50
overnight in Luria-Bertani (LB) medium containing the appropriate antibiotic to
pKA6, pRR3, pHSG299, or pRR2 was placed in BL21(DE3)pLysS (host), de-
dected by monitoring the sedimentation profile of the ribosomes in sucrose
ribosome isolated from the polysome can have any codon at this site. Since
real posttermination complex has a termination codon at the A site while the
be regarded as a model for the posttermination ribosomal complex. The only
on the P site, E site, and an empty A site. Each ribosome in this polysome can
puromycin. The resulting complex consists of ribosomes with deacylated tRNA
site. The polysomes have nascent growing polypeptides, which are removed by
the RRF reaction. The amount of preexisting monosomes was subtracted from the amount of monosomes present after the RRF reaction to calculate the conversion of polysomes to monosomes due to the RRF reaction. Details of the assay procedure have been described previously (9).

**RESULTS**

**Purification of T. maritima RRF and the antigenic properties of purified T. maritima RRF.** E. coli BL21(DE3)pLysS cells harboring pKA1 carrying T. maritima frr were treated as described in Materials and Methods, and crude T. maritima RRF with a purity of 23.5% was obtained. For further purification, the crude T. maritima RRF was incubated at 75°C for 30 min to remove the bulk of the proteins from the host cells as shown in Fig. 1A. By this procedure, extracts (30.5 mg of protein of which 66.5% was RRF) were obtained. A similar treatment was equally effective for the purification of other T. maritima proteins expressed in E. coli (30, 39, 43). The heat-treated extract was then applied to a Sephadex G-100 column. The representative fractions were examined for purity as shown in Fig. 1B. The purified T. maritima RRF (5.5 mg/ml and 92.0% purity) was thus obtained with 25.0% recovery. Total recovery over the entire purification procedure was 15.8%.

The cross-reactivity of purified T. maritima RRF with an anti-E. coli RRF polyclonal antibody was examined as shown in Fig. 2. Although T. maritima RRF cross-reacted with the anti-E. coli RRF antibody, the reactivity was at least 300-fold less than that of E. coli RRF. T. maritima RRF is therefore only remotely related to E. coli RRF immunologically. It is noted that T. maritima RRF migrated more slowly than E. coli RRF (see also Fig. 6A), indicating that the band observed in lane 1

### TABLE 1. E. coli strains and plasmids

| Strains or plasmid* | Genotype or relevant characteristics | Reference or source |
|---------------------|-------------------------------------|---------------------|
| DH5α                | F<sup>+</sup> thi gal recA1 endA  Δ(lacZYA-argF)U169 deoR recA1 endA hsdR17<sup>(<i>K</i> <i>m</i> )<sup>1</sup> phoA supE44 Δ<i>h</i> i 1 gyrA96 relA1 | GIBCO |
| BL21(DE3)pLysS      | F<sup>−</sup> ompT <i>hsd</i><sup>R</sup><sub>Δ</sub><sup>1</sup> gal dcm (DE3)<sup>+</sup>/pLysS; Cp<sup>+</sup> | Novagen |
| **Plasmids**        |                                     |                     |
| pET-11a             | T7 promoter-driven high-efficiency protein expression and sequencing vector; encodes Ap<sup>+</sup> | Novagen |
| pET-24a (+)         | T7 promoter-driven high-efficiency protein expression and sequencing vector; encodes Km<sup>+</sup> | Novagen |
| pCA1                | Multicycopl vector; encodes Km<sup>+</sup> | Takara |
| pKA1                | pET-11a with T. maritima RRF gene insert; encodes Ap<sup>+</sup> | This study |
| pKA5                | pET-24a (+) with S. pneumoniae RRF gene insert; encodes Km<sup>+</sup> | This study |
| pKA6                | pET-24a (+) with H. pylori RRF gene insert; encodes Km<sup>+</sup> | This study |
| pRR2                | pUC19 derivative carrying E. coli RRF gene cistron with upstream and downstream flanking regions (including the promoter and the transcription terminator); encodes Ap<sup>+</sup> | This study |
| pRR3                | pHSG299 derivative carrying E. coli RRF gene cistron with upstream and downstream flanking regions (including promoter and transcription termination sequences); encodes Km<sup>+</sup> | This study |

* Boldface, empty vector, i.e., vector lacking an insert.
represents \textit{T. maritima} RRF and moved slower than that of \textit{E. coli} RRF.

Inhibitory effect of \textit{T. maritima}, \textit{S. pneumoniae}, and \textit{H. pylori} RRF on the growth of \textit{E. coli}. We examined the effect of \textit{T. maritima} \textit{frr} expression on the growth of \textit{E. coli} (Fig. 3). The pET-11a vector carried \textit{T. maritima} \textit{frr} with the ampicillin resistance gene (Ap'). The expression of \textit{T. maritima} \textit{frr} was induced by the addition of IPTG. Induction of \textit{T. maritima} \textit{frr} inhibited the growth of \textit{E. coli} (Fig. 3A and D). The maximum killing effect was such that 90% of the viable counts were lost; the data represent typical results that were reproduced several times. The inhibitory effect depended on the concentration of the inducer, suggesting a dose-dependent relationship in the inhibition by \textit{T. maritima} \textit{frr}. The inhibitory effect appears to affect the viability of the host more than the increase in OD. Similarly, inactivation of temperature-sensitive (ts) RRF had a greater pronounced effect on viability than on the ODs of the cell cultures (17).

The presence of multicopy \textit{E. coli} \textit{frr} reversed the inhibitory effect of \textit{T. maritima} \textit{frr} (Fig. 3B and E). These cells were identical to those used in Fig. 3A but contained plasmid pRR3, which carried \textit{E. coli} \textit{frr} and kanamycin resistance. These figures show that a functional extrachromosomal \textit{E. coli} RRF overcame in large part the toxic effect of \textit{T. maritima} RRF. The empty vectors used in these experiments, as well as the presence of pRR3, had no deleterious effect on the host cells, as shown in Fig. 3C and F.

Figures 4 and 5 show similar but less-pronounced effects by \textit{S. pneumoniae} and \textit{H. pylori} \textit{frr}, respectively. In both cases, the addition of 1 mM IPTG exerted a deleterious effect, whereas, in the absence of IPTG, bacterial growth was identical to that for the controls (Fig. 4A and C and 5A and C). The effect of \textit{H. pylori} \textit{frr}, however, was so mild that it did not reduce the viable count but only retarded the increase (Fig. 5A and C). These toxic effects were significantly reduced when plasmids carrying \textit{E. coli} \textit{frr} were present simultaneously (Fig. 4B and D and 5B and D). Since the expression of \textit{E. coli} \textit{frr} carried by pRR2 did not depend on adding inducer (Fig. 6A, lane 3) (13, 38), the constant presence of excess RRF is not toxic.

Evidence for the expression of heterologous RRF in \textit{E. coli}. The preceding results can be interpreted by assuming that each of the plasmids carrying non-\textit{E. coli} \textit{frr} expresses the corresponding RRF. Indeed, plasmids pKA1 (pET-11a carrying \textit{T. maritima} \textit{frr}) and pRR3 (pHSG299 carrying \textit{E. coli} \textit{frr}) expressed the respective RRFs (Fig. 6A, lane 2). The relative amounts of these RRFs were in the ratio of 10 (\textit{E. coli})/8 (\textit{T. maritima}) (average of the densities of the bands on three different gels). Because \textit{E. coli} RRF migrated slightly faster than that of \textit{T. maritima}, one can detect the presence of both. That the slower-moving band corresponds to \textit{T. maritima} RRF is clear from lanes 3 to 5. We conclude from this experiment that expression of the \textit{E. coli} \textit{frr} gene neutralizes the deleterious effect of \textit{T. maritima} RRF.

In a similar manner, under the conditions where the plasmid carrying \textit{E. coli} \textit{frr} reduced the deleterious effect of \textit{S. pneumoniae} \textit{frr}, the expression of both RRFs indeed took place.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Purification of \textit{T. maritima} RRF. (A) A bacterial crude extract was heated at 75°C for 30 min, and the crude extracts obtained before (pre) and after (post) the heat treatment were analyzed by SDS–15% PAGE followed by staining with Coomassie brilliant blue R. (B) The heated extracts were loaded on a Sephadex G-100 superfine (Pharmacia Biotech) column, and representative fractions were examined by SDS-PAGE as described for panel A. MW, molecular weight markers (GIBCO).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Antigenic cross-reactivity of \textit{T. maritima} RRF with anti-\textit{E. coli} RRF antibody. Purified RRFs were analyzed by SDS–15% PAGE, and the Western blotting was performed with anti-\textit{E. coli} RRF antibody. Lane 1, \textit{T. maritima} RRF, 3,000 ng; lane 2, \textit{E. coli} RRF, 30 ng; lane 3, \textit{E. coli} RRF, 10 ng; lane 4, prestained protein marker, broad range (BioLabs).}
\end{figure}
The relative amounts of these RRFs in this cell were 7 (S. pneumoniae) to 10 (E. coli) (data not shown). Although H. pylori RRF shows the weakest inhibition of E. coli growth, this is not due to a weak expression of H. pylori RRF, as shown in lane 3 in Fig. 6B. We conclude that the relative amount of H. pylori RRF expressed was similar to that of T. maritima RRF (compare Fig. 6B and A).

Inhibition of the E. coli RRF reaction by T. maritima RRF. We examined the effect of T. maritima RRF on the in vitro RRF assay using naturally occurring polysomes (9). In this system, polyribosomes isolated from growing E. coli cells were treated with puromycin to remove the nascent peptide. We regard the resulting complex of ribosome, tRNA, and mRNA as a model substrate for the posttermination ribosomal complex. Disassembly of this model posttermination complex converts the polysomes into monosomes (8).

Table 2 shows that the strongest inhibition (65%) of E. coli RRF by T. maritima RRF was observed when the largest amount of T. maritima RRF (10-fold larger than the amount of E. coli RRF) was added. Equal amounts of T. maritima RRF and E. coli RRF in the reaction mixture yielded only a slight (7%) inhibition. This result suggests that the affinity of T. maritima RRF for the E. coli polysomes must be less than 10% that of E. coli RRF. T. maritima RRF did not disassemble the posttermination complex of E. coli (Table 2).

DISCUSSION

It is known that P. aeruginosa RRF (29) and the l-lactate dehydrogenase gene from T. maritima (30) function in E. coli. We therefore expected that heterologous RRFs would function in E. coli because their sequences are very similar to that of E. coli RRF (Fig. 7). Contrary to this expectation, expression of the genes coding for these RRFs was deleterious to E. coli. We suggest that this toxic effect is due to an inhibitory action of a heterologous RRF on the reaction catalyzed by E. coli RRF for the following reasons.

First, the toxic effects of the heterologous fr depend on the extent of induction of these heterologous RRFs by IPTG. Second, analysis of the E. coli extract harboring the heterologous fr showed the presence of the corresponding heterologous RRF. Third, pure heterologous RRF represented by T. maritima RRF inhibited the in vitro E. coli RRF reaction. Fourth, the heterologous RRF, represented by T. maritima RRF, was close enough to E. coli RRF immunologically to be cross-reactive. Although cross-reactivity does not necessarily indicate a similar structure, it suggests that the heterologous RRF can compete with E. coli RRF because of the structural similarities of these two proteins. Fifth, and most importantly, the simultaneous expression of an excess amount of E. coli RRF overcame the toxic effect of the heterologous RRF. In addition, T. maritima frr did not complement LJ15 (17), an E. coli mutant carrying ts RRF at 47°C (the nonpermissive
We previously reported that spinach RRF exerted a deleterious effect on a mutant E. coli carrying a ts RRF (32). The finding reported here represents the first observed inhibitory effect of a heterologous RRF on wild-type E. coli.

Figure 7 shows the amino acid sequences of RRF from E. coli (13), P. aeruginosa (29), T. maritima (36; see Addendum in Proof), S. pneumoniae, and H. pylori (42). In the five sequence segments (a to e) there are many amino acids of E. coli RRF identical to those of P. aeruginosa RRF but the RRFs of the other species showed only a few identical amino acids. This suggests that these segments may be responsible for the inhibitory effect of the heterologous RRFs.

We have recently resolved the crystal structure of T. maritima RRF prepared as described in this paper; it consists of two domains, domain I being a long three-helix bundle and domain II being a three-layer β-α-β sandwich (36). Recent work on E. coli RRF (26) confirmed this structure, which essentially agrees with the computer-predicted secondary structure (3) of RRF (16). These two domains are connected through the hinge region. Out of the five segments marked in Fig. 7, segments a and b are in domain II while the remaining three are in domain I.

A heterologous RRF may act on E. coli RRF directly and inactivate it by forming an inactive complex. Our preliminary nuclear magnetic resonance studies on RRF (24), however, indicate that RRF tends to stay as a monomer. In addition, it takes at least a 10-fold-higher molar concentration of heterologous RRF for a 50% inhibition of E. coli RRF (Table 2).

Since RRF is a nearly perfect mimic of tRNA, we postulated that it behaves like tRNA on the ribosome (36). Our mutation data (17) support this hypothesis. It is therefore more likely that the heterologous RRF competes with E. coli RRF for the ribosomal A site (the site at which aminoacyl tRNA binds to the ribosome).

**Table 2. Inhibition of the E. coli RRF reaction by T. maritima RRF**

| Amt (μg) of RRF of: | % Conversion of polysome to monosome | % Inhibition |
|---------------------|-------------------------------------|-------------|
| E. coli T. maritima |                                     |             |
| 5                   | 0                                   | 19.8 0.0    |
| 5                   | 2.5                                 | 19.3 2.6    |
| 5                   | 5                                   | 18.3 7.2    |
| 5                   | 50                                  | 6.9 65.3    |
| 0                   | 50                                  | ~8.8 NA^b   |

^a The E. coli RRF reaction was performed as described in Materials and Methods. The values were adjusted for the amount of monosome (38.3%) previously existing in the substrate.
^b NA, not applicable.

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^a The E. coli RRF reaction was performed as described in Materials and Methods. The values were adjusted for the amount of monosome (38.3%) previously existing in the substrate.
^b NA, not applicable.
It should be noted that less pronounced inhibitory effects by 37°C. For inhibition by these RRFs and a-e regions may also play important roles.

Third, the hinge region represents the high-mobility agent is required for crystallization of S. pneumoniae (DDBJ accession no. AB010087); Spne, H. pylori (GenBank accession no. J05113); Paer, T. maritima (GenBank accession no. AB010087); Tmar, S. pneumoniae (TIGR microbial database; http://www.tigr.org/db/dmdb/dmdb.html); Hpyl, H. pylori (GenBank accession no. P66398).

Why then does the A site-bound T. maritima RRF not work for E. coli? We may speculate that RRF must bend at the hinge region during its action. It is possible that T. maritima RRF bound at the A site to function at 37°C. The following observations lead to this speculation. First, crystalization of E. coli RRF depends on denaturing β-D-maltopyranoside, which fits into the pocket of the hinge region of RRF (26), probably fixing the molecule in one form. Second, no such agent is required for crystallization of T. maritima RRF at room temperature (37), probably because this RRF keeps itself in one form due to its nonflexibility at room temperature. This is conceivable because T. maritima RRF is probably designed to be bendable at the hinge region at 80°C but not at 37°C. Third, the hinge region represents the high-mobility region of this molecule (estimated from the crystal structure) despite the fact that it is not near the N or C terminal. It should be noted that less pronounced inhibitory effects by S. pneumoniae and H. pylori RRFs probably do not involve this mechanism because their RRFs are designed to function at 37°C. For inhibition by these RRFs and T. maritima RRF, the a-e regions may also play important roles.

ACKNOWLEDGMENTS

We thank Karl O. Stetter and Robert Huber of the University of Regensburg, Regensburg, Germany, for providing DNA of T. maritima, James Koesis of Jefferson Medical College for critically reading the manuscript and for linguistic help, and Yun-Wen Shaw for clerical assistance.

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