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Research Article

Altering *Trichomonas Vaginalis* Codons Enhances Expression of The Trichomonad P270 Repeat Element in *Escherichia Coli*

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Abstract

The immunogenic protein of the *Trichomonas vaginalis* isolate NYH 286 called P270 (270-kDa) has 19 tandemly repeated elements (TRE), and each RE is 333-bp, which encodes for a protein of 11,770.90 daltons. Isolate NYH 286 trichomonads are infected with a dsRNA virus (TVV$^+$), and TVV$^+$ organisms undergo phenotypic variation between surface versus cytoplasmic placement of P270. These TVV$^+$ trichomonads are heterogeneous with fluorescent and non-fluorescent parasites by indirect immunofluorescence using the monoclonal antibody (MAb) C20A3 as a probe. The *p270* gene is single copy based on restriction enzyme analysis, and partial restriction using *Hind*III reveals a ladder pattern expected of TREs. Each TRE has the DREGRD epitope detected by MAb C20A3. As the cDNA encoding a protein of ~14-kDa within which is the ~11.8-kDa RE is poorly expressed by recombinant *E. coli* (r*E.coli*) the hypothesis was tested that the *T. vaginalis* codons for arginine (R) and glycine (G) translated by minor E. coli tRNA species decreased the synthesis of the RE. Therefore, the five codons of R^{109}, R^{112}, R^{116}, G^{119}, and G^{128} were altered with synonymous codons used by *E. coli*. The synthesis of the RE of the original plasmid (pORIG) expressed in r*E. coli* was then compared with r*E. coli* harboring the plasmid with less numbers of codons (called pLESS) used by the minor *E. coli* tRNAs. The control r*E. coli* with plasmid p21.b without insert, pORIG and pLESS all had similar growth kinetics, and bacterial lysates harvested at different times were evaluated by immunoblot for synthesis of the RE. The data show that the r*E. coli* with pORIG synthesized the RE only between 2-hours (h) and 8-h of growth. In contrast, r*E. coli* with pLESS permitted synthesis of the RE during the 30-h of bacterial growth. These data show for the first time the role of codon usage for expression of *T. vaginalis* proteins in r*E. coli*.

Keywords: Codon Usage; *Escherichia Coli* Codons; Gene Expression; P270; Silent Codon Changes; Tandemly-repeated Element; *Trichomonas vaginalis*; Transfer RNA.

Abbreviations: amp: ampicillin; bp: base pairs; *E. coli*: *Escherichia coli*; kb: kilobases; MAb: monoclonal antibody; Mr: electrophoretic mobility; MW: molecular weight; NC: nitrocellulose; P270: phenotypically varying high Mr protein; SDS-PAGE: sodium dodecylsulfate-polyacrylamide gel electrophoresis; TRE: tandemly-repeated element; tRNA: transfer RNA

Introduction

Trichomonas vaginalis remains the number one, non-viral sexually transmitted infection (STI) worldwide [1,2]. Indirect immunofluorescence (IF) using monoclonal antibody (MAb) C20A3 revealed naturally occurring isolates of *T. vaginalis* with trichomonads that were heterogeneous for the surface expression of a 270-kDa protein called P270 [3-5]. Flow cytometry experiments revealed that fluorescent organisms became non-fluorescent and vice-versa during in vitro batch culture [3], and this property of phenotypic variation was found to occur only for isolates of *T. vaginalis* harboring a dsRNA virus (TVV$^+$) [6,7]. This finding led to the discovery of two naturally occurring *T. vaginalis* isolate types defined as Type I without dsRNA virus (TVV$^-$) and Type II (TVV$^+$) [6]. Of particular interest is the finding that extended batch cultivation of TVV$^+$ parasites abort the virus thus creating TVV$^-$ like isogenic isolates which have lost the ability to undergo phenotypic variation [6-8], suggesting an important role for the virus in regulating surface placement of P270.

The large Mr P270 protein from representative TVV$^+$ phenotypically varying trichomonads was found to have an internal region comprised of tandemly repeated element (TRE) sequences

[9,10], and each TRE had the immunodominant DREGRD amino acid sequence detected by the MAb C20A3 [10-12]. Although the function of P270 remains undefined, recent work combining IF and immunoelectron microscopy has shown the existence of P270 on both the surface and inside the cytoplasm and peripheral vacuoles within the TVV^{+} parasites [8]. Importantly, P270 is stably conserved among fresh clinical isolates, which suggests an important function for P270 in the biology of *T. vaginalis*.

Early attempts to synthesize the RE as a recombinant protein by *E. coli* was met with limited success [9]. Expression of the RE was suboptimal, which did not permit for structure-function studies. In this paper, the hypothesis that the poor synthesis of the RE in r*E. coli* is due to the presence of *T. vaginalis* codons that are used by minor tRNAs of *E. coli* [13] was tested. Four arginine and two glycine codons were changed in the original cDNA encoding the RE (pORIG) with synonymous codons used by r*E. coli*. This resulted in a modified plasmid (called pLESS) with less numbers of codons that use minor E. coli tRNAs. Data presented here show the synthesis of the recombinant RE throughout the extended growth of r*E. coli* with pLESS compared with r*E coli* harboring the plasmid pORIG. These findings suggest that codon usage within trichomonad genes is an important variable that influences the kinetics and amount of recombinant protein produced r*E. coli*. The significance of our observations is discussed.

Methods

Screening of the transformants

Both blue-white and immunological screening of bacterial colonies using the MAb C20A3 [3,4] were performed by standard procedures [9]. White, immunoreactive clones were further evaluated through double restriction of purified plasmids (Qiagen Inc., Valencia, CA USA). Recombinant plasmids were verified by sequencing. Secondary structures of recombinant mRNAs were analyzed by PC/GENE (IntelliGenetics, Atlanta, GA USA).

pORIG and pLESS plasmids

Phagemid vector pcDNAII (Thermo Fisher Scientific, Waltham, MA USA) was employed for cDNA cloning. Subcloning of trichomonad cDNA required PCR amplification using the original cDNA clone [9] as template. Unidirectional ligation of the original cDNA into the plasmid (called pORIG) was assured by using a sense primer (ORIG) (5'-TTGAATTCCGGGATAACGTTAGA-3') designed to introduce an *Eco*RI restriction at the 5' site and an antisense primer (labeled orig) (5'-TTGATATCCCCTTGTTGTGCTGCGCG-3') to introduce an *Eco*RV restriction at the 3' site.

To decrease the number of codons in the original cDNA that use minor tRNAs for the pLESS plasmid for expression, the same orig sense primer was used, and a new antisense primer (labeled less)(5'-TTGATATCGCCTTGTTGTGCTGCGCGTAATGTGTC GCCTTTACTGCGAACGTTATCGCGACCTTCTCGAT-3') was made to replace five codons translated by minor *E. coli* tRNA species [13]. Three arginine codons (R^{109}, R^{112}, and R^{116})and two glycine codons (G^{119}, and G^{128}) of the original cDNA (Figure 1) [9] were changed with synonymous codons to replace the *T. vaginalis* codons. The synonymous codons were selected to avoid introducing secondary mRNA structural and translation initiation issues, which were analyzed as described above by PC/GENE. Hot start PCR amplification (Clontech Laboratories, Mountain View, CA USA) with three-step cycles was performed with the Ta individually set for each pair of primers. The amplification products were purified (Wizard PCR Preps, Promega, Madison, WI USA), digested with *Eco*RI and *Eco*RV restriction enzymes, and ligated into the *Eco*RI/ *Eco*RV-directed vector site. Transformation into the INVαF' host strain was performed as recommended by the manufacturer.

Recombinant E. coli and expression of the P270 repeat element (RE)

Recombinant *E. coli* (r*E. coli*) INVαF' (Thermo Fisher Scientific) with the plasmids pORIG and pLESS (Figure 1) as well as control *E. coli* with p21.b were grown in Luria-Bertani (LB) medium and/or on LB agar plates supplemented with 60μg/ml of ampicillin (amp) as described recently [14-16]. Twenty milliliters of LB medium were then inoculated with equal amounts of bacteria and incubated at 37°C for up to 32-h on a shaker at 250rpm. At 2-h intervals, an aliquot of each culture with equal OD_{600} was centrifuged, and pellets were frozen at -20°C until used for electrophoresis and immunoblotting as described below. Also, the plasmids from an equal volume of each r*E. coli* with identical OD_{600} were purified [9,14,15]. Both purified plasmids and cDNAs were quantitated after electrophoresis in agarose gels to ensure the r*E. coli* possessed equivalent amounts of pORIG and pLESS plasmid DNA. This was done to be able to accurately compare the P270 REs synthesized by r*E. coli*.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on lysates derived from frozen pellets of each r*E. coli* with the p21.b control plasmid and the pORIG and pLESS plasmids obtained above. Pellets were thawed with electrophoresis dissolving buffer, as before [9,12,14]. Stained gels for r*E. coli* was obtained for time points of 2-h up to 30-h to insure equal amounts of protein were added to lanes of all time points for SDS-PAGE and subsequent immunoblotting onto nitrocellulose. Duplicate gels after SDS-PAGE were transferred onto nitrocellulose for probing with the MAb C20A3 that detects the TRE DREGRD (Figure 2) conserved within the RE as described before and using established protocols [9,10,12,14-16].

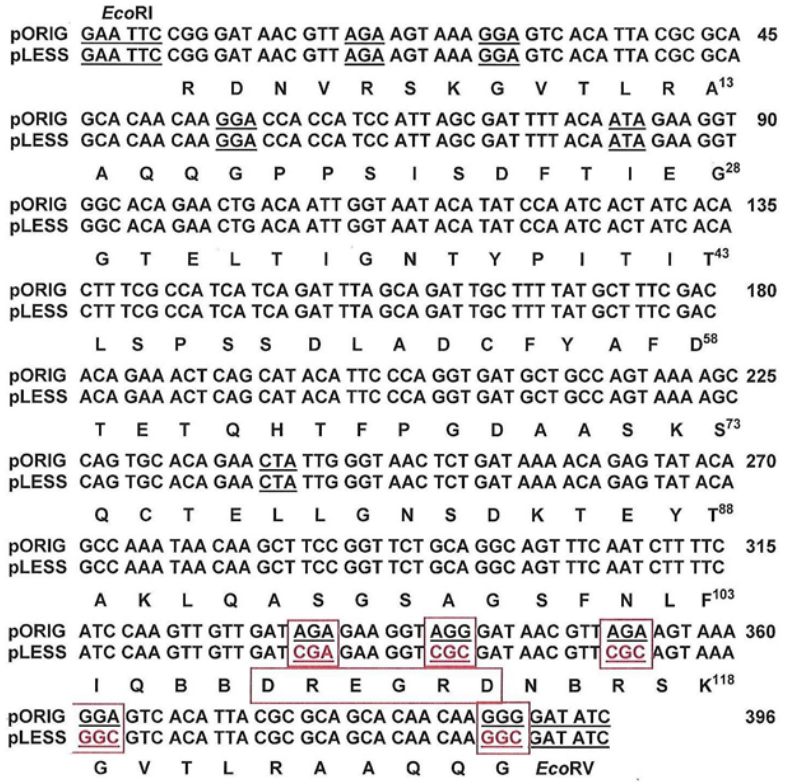

Figure 1: Nucleotide and amino acid sequences of the cDNAs of plasmid pORIG as in Figure 2 and of plasmid pLESS with altered codons. The designation pLESS denotes a decrease in codons that utilize minor tRNAs in *E. coli*. The altered codons for R109, R112, R116, G119, and G128 are in red, underlined and boxed in red to reflect the translation by alternative tRNA species by *E. coli*. The replaced synonymous codons retain the same amino acids. The DREGRD epitope as above for Figure 2 is also highlighted and boxed in red.

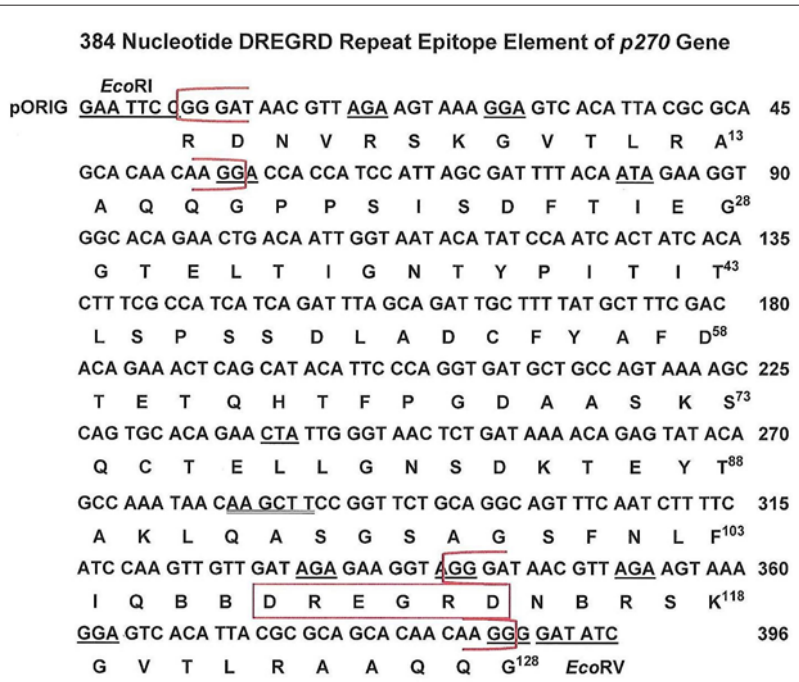

Figure 2: Open reading frame and amino acid sequence of the cDNA called pORIG of the P270 TRE, as before [9]. The two brackets shown in red highlight the 49 nucleotides (17 amino acids) that are repeated within the cDNA. The amino acid DREGRD boxed in red is the tandemly repeated epitope within the *p270* gene that is detected by the monoclonal antibody (MAb) C20A3 [8,9,11]. The restriction sites of *Eco*RI and *Eco*RV for cloning purposes are shown at the 5' and 3' ends of the cDNA, respectively.

Results

The p270 gene and tandemly repeated elements (TREs)

As in earlier reports for a *T. vaginalis* Type II (TVV+) isolate [10,11] the organization of the *p270* gene and TREs for the long-term grown NYH 286 isolate were characterized. Figure 3 (part a) illustrates the *p270* gene with the internal large TRE of 333-bp defined by the restriction enzyme *Hind*III [9,10] that encodes for a protein of ~11.8-kDa, consistent with the *p270* gene of a fresh clinical isolate [10,11]. Parts b and c are two different representative experiments of *T. vaginalis* DNA digested with *Eco*RI, which shows the *p270* gene is single copy consistent with an earlier report [10]. Digestion to completion with *Hind*III shows a single band of the expected size for part b (lane 1) and part c (lanes 1a through 1c). Partial digestion with HindIII shows the ladder pattern as expected for a repeat element within the p270 gene [12].

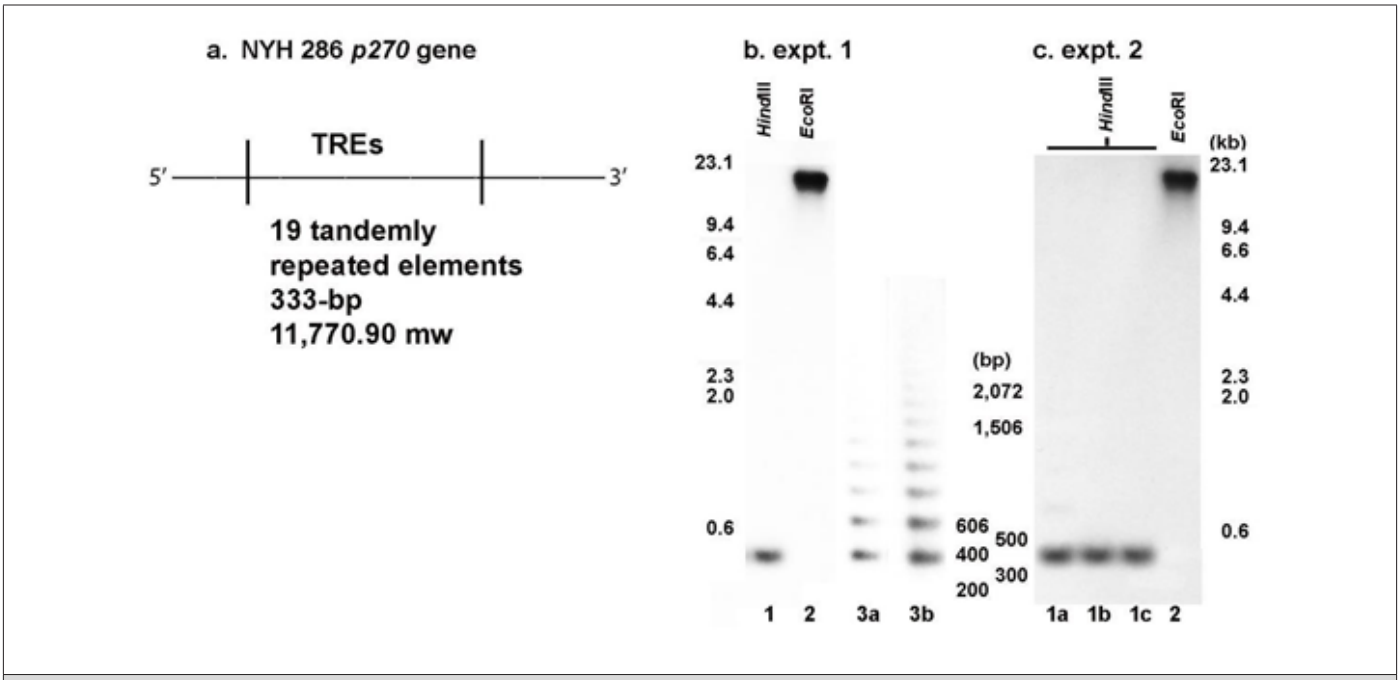

Figure 3: The *T. vaginalis* NYH 286 isolate p270 gene (part a) and restriction enzyme digestion of the purified trichomonad DNA (parts b and c). Part a presents the organization of the p270 gene within which are the 19 tandemly repeated elements (TREs) of 333-bp defined by *Hind*III and that encode for a RE of ~11.8-kDa in size. This gene organization is consistent with an earlier published report [11]. Parts b and c present the complete restriction enzyme digestion of DNA with *Eco*RI (expt. 1 and 2, lanes 2). The complete digestion with *Hind*III is shown in lane 1 for expt. 1 and lanes 1a through 1c for expt. 2. Complete digestion for the two representative experiments was performed identically. For both experiments, the expected large size of the p270 gene of ~24-kb in size for *Eco*RI-digested DNA and the 333-bp size for *Hind*III-digested DNA were obtained, as evidenced by the size markers and consistent with earlier reports [9-11]. Lanes 3a and 3b of expt. 1 is of partial *Hind*III restriction to illustrate the 333-bp TRE ladder compared to the full digestion single band end product as shown in lane 1 of expt. 1 and lanes 1a-1c of expt. 2.

Plasmids pORIG and pLESS encoding the TRE and codon usage

Figure 2 presents the 384 nucleotide RE of the *p270* gene that is withing the 396 nucleotide cDNA described before [9]. Flanking the DNA is an *Eco*RI and *Eco*RV restriction sites at the 5' and 3' ends, respectively, used for cloning into pORIG. The brackets in red shows the repeated 49 nucleotide (17 amino acids) that flank the cDNA, and the immunodominant epitope DREGRD detected by MAb C20A3 is boxed in red. This original 384bp cDNA (pORIG) encodes for a protein of ~14.2-kDa as described before [9].

Comparison of pORIG with the pLESS cDNA having five less *T. vaginalis* codons that use minor tRNAs of *E. coli* is shown in Figure 1. The modified codons R^{109}, R^{112}, R^{116}, G^{119}, and G^{128} are in red, underlined and boxed in red. As for Figure 2, the DREGRD epitope is boxed in red. In a separate experiment, the codons R^{5}, G^{8}, and G^{17} in pLESS were also modified, but these changes did not increase the amount of RE that is synthesized and are not included here.

Synthesis of the P270 TRE

The synthesis of the *p270* RE first required showing that the growth kinetics of each r*E. coli* with the respective plasmids (p21.b, pORIG and pLESS) were similar, and the growth curves of rE. coli is shown in Figure 4. Each r*E. coli* reached similar OD_{600} densities. This was preparatory to performing SDS-PAGE and immunoblotting using as probe the MAb C20A3. The r*E. coli* was harvested at 2-h or 4-h intervals until 30-h of growth.

Figure 5 presents the Coomassie brilliant blue-stained gels (labeled a) and immunoblots (labeled b) after SDS-PAGE of total proteins of *E. coli* harboring the plasmids p21.b control (part A), pORIG (part B) and pLESS (part C). For each r*E. coli* throughout the

growth period, stained gels of total *E. coli* proteins were identical, showing that lanes for each time period were loaded with the same amount of r*E. coli* protein. Not unexpectedly, the immunoblot for control r*E. coli* with p21.b was unreactive with the MAb C20A3 as evidenced by the absence of any protein band (part A). Ponceau-staining of the nitrocellulose showed equal amounts of total r*E. coli* proteins as the stained gels. For the r*E. coli* with pORIG (part B), the MAb C20A3 reacted with the RE protein of the expected ~14-kDa in size but only for the 2-h to 8-h time period. On the other hand, the nitrocellulose with proteins of r*E. coli* with pLESS (part C) showed the RE protein throughout the growth curve up to 30-h. These data suggests strongly that the changes in the codons to those utilized by *E. coli* resulted in more effective synthesis of the RE. For r*E. coli* with pORIG or pLESS, there was no evidence of overexpression of P270 in stained gels.

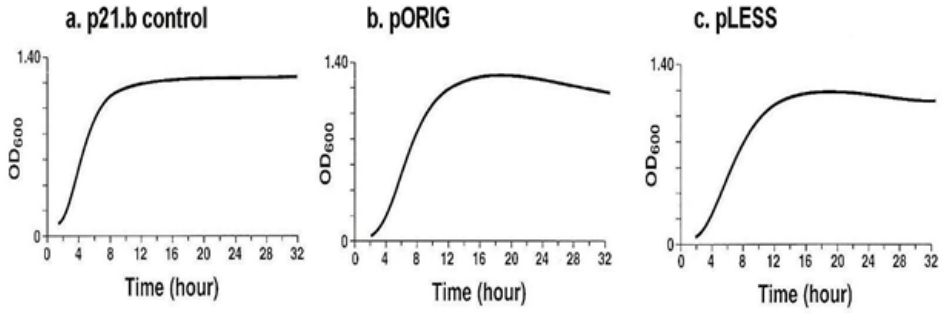

Figure 4: The growth curves of respective *E. coli* control with plasmid without insert (a, p21.b), original plasmid (b, pORIG) and plasmid with modified codons (c, pLESS). Each recombinant *E. coli* reached similar OD600 densities, and recordings were carried out every 2-h until 12-h after which bacterial growth densities were recorded every 4-h up until 32-h. This was necessary because immunoblotting after SDS-PAGE of total *E. coli* proteins was carried out up to 30-h for expression of the P270 cDNA encoding the RE.

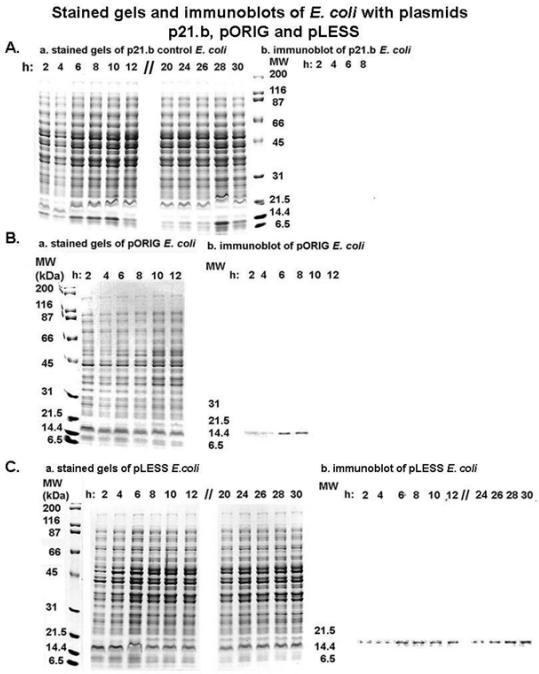

Figure 5: Coomassie brilliant blue-stained gels (labeled a) and immunoblots (labeled b) after SDS-PAGE of total proteins of *E. coli* harboring the plasmids p21.b control (part A), pORIG (part B) and pLESS (part C). For experiments with the three plasmids, the stained gels show that equal amounts of protein were added to wells for each time point for each r*E. coli* (Part A, Part B and Part C, respectively). Part A. Recombinant *E. coli* with control p21.b was grown for up to 32-h. *E. coli* were harvested at the indicated times and total proteins were electrophoresed and stained. Duplicate gels were immunoblotted onto nitrocellulose membranes and probed with the P270 TRE MAb C20A3 that detects the epitope DREGRD (Figures 2 and 3). The blot for only 2-h through 8-h is shown, although no proteins were detected at all time points. Part B. Same as part A except using r*E. coli* with pORIG. Immunoblots with MAb C20A3 showed a protein band with the expected MW of ~14-kDa for the cDNA encoding the TRE, as before [9,13]. Only up to 12-h for the stained gel is presented as no protein was evident after 8-h in the immunoblot. Part C. Same as part A except with r*E. coli* with pLESS. The immunoblot probed with MAb C20A3 shows the presence of recombinant RE throughout the 30-h growth of rE. coli beginning at 2-h through 30-h. For *E. coli* with pORIG or pLESS, there was no evidence of overexpression of P270 in stained gels. The experiments were performed no less than on three different occasions. Importantly, for each of the nitrocellulose blots, Ponceau staining showed total proteins as seen for the stained gels presented in part a.

Discussion

In this report we wanted to test the hypothesis that the poor synthesis of the *p270* tandemly repeated element was the result of *T. vaginalis* using codons for arginine and glycine translated by rare *E. coli* tRNAs [13,17-21]. This is the first time that r*E. coli* expressing a RE of *p270* from pLESS with three altered arginine and two altered glycine codons is shown to be synthesized throughout the bacterial growth kinetics compared with pORIG-expressing r*E. coli* (Figure 5). Indeed, our observations reinforced the idea that the presence of rare codons in the cloned trichomonad cDNA directly affects amounts of recombinant protein synthesized in r*E. coli*. The examination of 29,845 codons from 80 partial and complete sequences of protein-coding *T. vaginalis* genes in GenBank further revealed that 2% of leucine, 4% of isoleucine, 11% of glycine, and 25% of arginine residues were encoded by codons rarely used in *E. coli* [13,17-21]. Thus, it is conceivable that RE gene expression in pORIG is influenced by different rates of translation resulting from the amounts of minor tRNAs.

The level of gene expression depends on amount and translational frequency of full-length mRNA [21]. Both pORIG and pLESS cDNAs were ligated into identical sites of lacZ gene, and consequently expression of both proteins was under the control by the same promoter. Importantly, the vector does not express the lac repressor, further affirming that both cDNAs were under the control of equal elements within the plasmid and cDNA. Two classes of protective elements have been shown to stabilize mRNAs in *E. coli*. One class of elements are sequences in the 5'-UTRs, and the second group includes stem-loop structures from the 3'-UTRs [19]. It is equally noteworthy that as no sequence changes were done within the 5'-UTRs of the cDNAs, evaluation of the 3'-UTR regions of the mRNAs from pORIG and pLESS did not detect any differences in the secondary structure. Furthermore, although silent codon changes influence mRNA stability via variations of ribosome traffic [21,22], it has been established that abundance of different tRNA species varies with the growth rates of *E. coli* [23]. Therefore, the data on RE synthesis in pLESS versus pORIG may be explained by numerous variables as mentioned here. This notwithstanding, our work suggests that the use by *T. vaginalis* of tRNAs that are minor in *E. coli* is likely to negatively affect successful stable expression of recombinant trichomonad proteins. Our results may have bearing on the efficient synthesis of this RE and other trichomonad proteins in r*E. coli*, and synthesis of the *T. vaginalis* proteins is a prerequisite first step in structure-function characterization of virulence factors and, therefore, of proteins important to the host-parasite interrelationship.

Conclusion

The ability to express proteins of *T. vaginalis* in r*E. coli* is preparatory for structure-function studies that permit understanding of the role of such proteins in virulence and mechanisms of disease pathogenesis for this STI caused by this ancient protist. This work examined for the first time the role of codons used by trichomonads in the poor expression of proteins in r*E. coli*. Future successful synthesis of heretofore poorly-expressed *T. vaginalis* virulence factors in r*E. coli* via alteration of codons will contribute to our overall understanding of the biology of this STI agent and of the ability of this organism to infect human hosts.

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