Flavin-dependent thymidylate synthase X limits chromosomal DNA replication

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We have investigated the hitherto unexplored possibility that differences in the catalytic efficiencies of thymidylate synthases ThyX and ThyA, enzymes that produce the essential DNA precursor dTMP, have influenced prokaryotic genome evolution. We demonstrate that DNA replication speed in bacteria and archaea that contain the low-activity ThyX enzyme is up to 10-fold decreased compared with species that contain the catalytically more efficient ThyA. Our statistical studies of >400 genomes indicated that ThyA proteins are preferred for the replication of large genomes, providing further evidence that the thymidylate metabolism is limiting expansion of prokaryotic genomes. Because both ThyX and ThyA participate in frequent reciprocal gene replacement events, our observations indicate that the bacterial metabolism continues to modulate the size and composition of prokaryotic genomes. We also propose that the increased kinetic efficiency of thymidylate synthesis has contributed to extending the prokaryotic evolutionary potential.

genome evolution | gene replacement | nucleotide metabolism | PBCV-1 | thymidylate synthesis

Contrarily to the deoxyribonucleotides dATP, dCTP, and dGTP, which can be produced directly by ribonucleotide reductase, *de novo* synthesis of dTTP requires the formation of thymidylate (dTMP). This essential DNA precursor is produced by the methylation of dUMP by thymidylate synthase. The mechanisms for *de novo* synthesis of DNA precursors were thought to be conserved in all free-living prokaryotes and eukaryotes. However, we identified a second family of thymidylate synthases [ThyX, also known as flavin-dependent thymidylate synthase (FDTS)] that is evolutionarily unrelated to the canonical thymidylate synthases ThyA. Approximately 30% of microbial species (based on completed genome sequences) depend on FDTS ThyX (EC 2.1.1.148) (1), whereas the canonical dTMP-forming enzyme ThyA (EC 2.1.1.45) is present in ≈70% of microorganisms. The key difference in ThyA and ThyX catalysis is related to the reductive mechanisms used for the reduction of the methylene group that serves as carbon source in the reaction. During ThyX catalysis, NAD(P)H is used as reductant, whereas ThyA uses methylenetetrahydrofolate (MTHF) not only as a one-carbon donor, but also as a source of reductive power (reviewed in ref. 2). Consequently, ThyX catalysis leads to the production of reduced tetrahydrofolate (THF), whereas ThyA produces oxidized dihydrofolate. These mechanistic differences of the two thymidylate synthases have clearly raised the possibility that the two enzymes might not be fully interchangeable *in vivo* (3).

Several arguments suggest that the evolutionary trajectories of ThyX and ThyA proteins differ. For instance, ThyA proteins form a highly conserved protein family, whereas ThyX sequences are much more divergent (1, 2). Notably, the high level of sequence conservation of ThyA proteins does not result from functional constraints because extensive mutagenesis studies performed on ThyA proteins identified only five critical residues for catalytic activity (4). Moreover, the sporadic and almost mutually exclusive phylogenetic distribution patterns of *thyX* and *thyA* are indicative of frequent lateral gene transfer and/or nonorthologous gene displacement events (1, 5). Environmental factors might also influence thymidylate synthase utilization because our sequence-similarity searches indicated that *thyX* genes are over-represented in the genomes of (hyper)thermophilic, microaerophilic, and anaerobic microorganisms (6). However, *thyX* can also be found in a number of mesophilic and aerobic organisms suggesting that these "environmental" factors are not sufficient to explain the complex distribution patterns of the two thymidylate synthases.

Using experimental and statistical analyses, we have addressed why these two analogous enzyme families appeared, and why both thymidylate synthase families have been maintained in current-day organisms.

Table 1. Catalytic parameters of PBCV-1 ThyX and *Lactobacillus casei* ThyA

Parameter*	PBCV-1 ThyX	*L. casei* ThyA
Reductant	NADPH/FAD	THF
k_{cat}, s^{-1}	0.35 (10%)	3.6 (100%)
K_m, mM		
dUMP	15	5
MTHF	20	10
NADPH	43	
k_{cat}/K_m^{dUMP}, $s^{-1}\cdot\mu M^{-1}$	0.023 (3.2%)	0.72 (100%)
k_{cat}/K_m^{MTHF}, $s^{-1}\cdot\mu M^{-1}$	0.0175 (4.8%)	0.36 (100%)

*Data for ThyX and ThyA enzymes are extracted from Graziani *et al.* (8) and Liu and Santi (31), respectively.

Results and Discussion

Systematic activity measurements under aerobic and anaerobic conditions using both viral and bacterial ThyX proteins have suggested that flavin adenine dinucleotide (FAD) reduction by NAD(P)H or conformational changes associated with this reductive step limit the catalytic efficiency of ThyX catalysis *in vitro* compared with ThyA (7–11). This point is illustrated in Table 1 which shows that the catalytic efficiency of the viral ThyX protein is 3–5% of that which has been described for the *Lactobacillus casei* ThyA enzyme. This observation raised the unexplored possibility that intrinsic biochemical properties of ThyA and ThyX enzymes have influenced thymidylate synthase phylogeny and possibly the dynamics of prokaryotic genome

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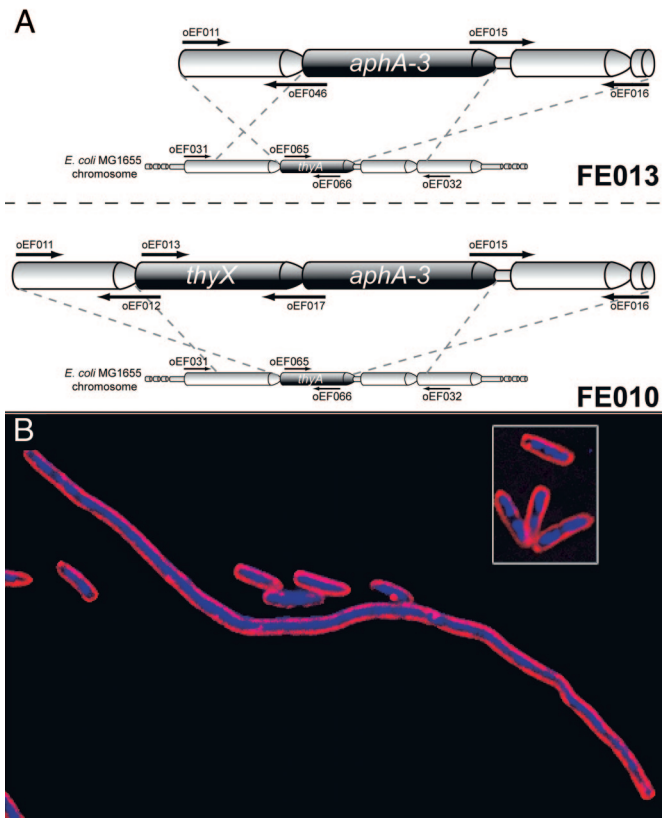

Fig. 1. Chromosomal replacement of *E. coli thyA* with *thyX* from PBCV-1. (*A*) Schematic representations of the chromosome of *E. coli strains* FE010 (Δ*thyA*::*thyX*) and FE013 (Δ*thyA*) (see *Materials and Methods* for details). DNA primers [supporting information (SI) Table S1] used for various PCRs are indicated with arrows. (*B*) Dual staining of *E. coli* cells grown under thymidine deprivation. The cell membrane is red (FM 4–64 staining) and DNA is blue (DAPI staining). The figure shows the representative field for the filamentous phenotype observed for FE010 (Δ*thyA*::*thyX*), whereas WT strain (*Inset*) did not filament under the same conditions.

evolution. This could imply that the availability of dTMP in ThyA and ThyX organisms is dramatically different. To test this notion, we first constructed an *Escherichia coli* strain where the chromosomal copy of *thyA* was replaced with *thyX* encoding a highly active viral protein (*Materials and Methods*). This construct was marked with a nonpolar kanamycin selection marker (Fig. 1*A*). In the resulting strain FE010 (Δ*thyA*::*thyX*), the chromosomal copy of *thyX* is expressed from the native *thyA* promoter. In the presence of thymidine, FE010 and the *thyA*-deleted control strain FE013 grew similarly to the MG1655 WT strain. However, in thymidine-deprived solid and liquid growth media, FE010 grew poorly (0.36 doubling per hour) compared with the WT strain (0.63 doubling per hour), and strain FE013 (Δ*thyA*) failed to grow. Several experiments established that FE010 is impaired in DNA replication. For instance, we observed that under thymidine deprivation, FE010 cells form long filaments (Fig. 1*B*), similar to what is often found for *E. coli* DNA replication mutants. In addition, trichloroacetic acid precipitation measurements of the incorporation rate of radioactively marked thymidine into DNA showed that the relative DNA replication velocities of the WT and FE010 (Δ*thyA*::*thyX*) strains were 100% (corresponding to 0.17 fmol/min) and 12% (0.02 fmol/min), respectively. The strains overexpressing *thyX* from a low copy-number plasmid based on the T5 promoter transcription–translation system grew slowly (10) and had slowed-down DNA replication (0.05 fmol/min for FE023). Finally, the frequency of spontaneous rifampicin-resistant mutants of FE010 was increased by three orders of magnitude compared with the parental strain (data not shown). Altogether, these results indicate that chromosomally or plasmid encoded *thyX* replacing *thyA* limits *E. coli* DNA replication.

To investigate whether these results also hold true for organisms that naturally contain *thyX*, we measured the combined fork progression speed during *Helicobacter pylori* DNA replication. By using quantitative PCR, we determined a replication origin/terminus ratio of 2.7 ± 0.6 in midexponential phase cultures of *H. pylori*. This ratio corresponds to a replication period (C-period) of 230 min (see *Materials and Methods*), whereas the corresponding value is ≈40 min in *E. coli*. Accounting for the differences in genome size (*H. pylori* ≈1.7 Mbp; *E. coli* ≈4.6 Mbp), we estimate a mean DNA replication speed of ≈120 bp/s for *H. pylori*. This value is an order of magnitude lower than what has been measured for *E. coli* [1,870 bp/s (Table 2)]. Most notably, the low replication speed we have found for *H. pylori* has been observed in other bacteria and archaea that use ThyX for dTMP synthesis. Indeed, Table 2 shows that the average replication speed in a diverse group of ThyX organisms (135 ± 82 bp/sec) is 10-fold lower than in ThyA-containing species (1,301 ± 762 bp/s). This observation contradicts the current view

Table 2. Correlation between thymidylate synthase type and replication speed

Thymidylate synthase	Organism	Domain*	Genome size, Mbp†	Doubling time, min‡	C-period, min‡	Replication speed, bp/s‡	Ref.
ThyX	*Chlamydia trachomatis*	B	1.1	1,440	276	64	32
	Helicobacter pylori 26695	B	1.7	167	232	120	This work
	Prochlorococcus	B	1.7	1,020	≈240–360	95	33
	Synechococcus	B	2.4	360	≈240–360	135	33
	Pyrococcus abyssi	A	1.8	30	100	294	34
	Sulfolobus acidocaldarius	A	2.2	ND	ND	≈80–110	35
ThyA	*Bacillus subtilis*	B	4.2	26	55	1,277	36
	Brucella abortus	B	3.3	216	60	913	37
	Enteroccocus faecium	B	2.8	90	55	863	38
	Escherichia coli	B	4.6	20	41	1,870	39
	Staphyloccus aureus	B	2.8	120	120	392	40
	Vibrio cholerae	B	4.0	42	27	2,488	41

ND, not determined.
*Domain of the species is indicated (B for Bacteria and A for Archaea)
†Genome size data were collected from the IMG database
‡Doubling time, C-period, and replication speed were collected from studies cited.

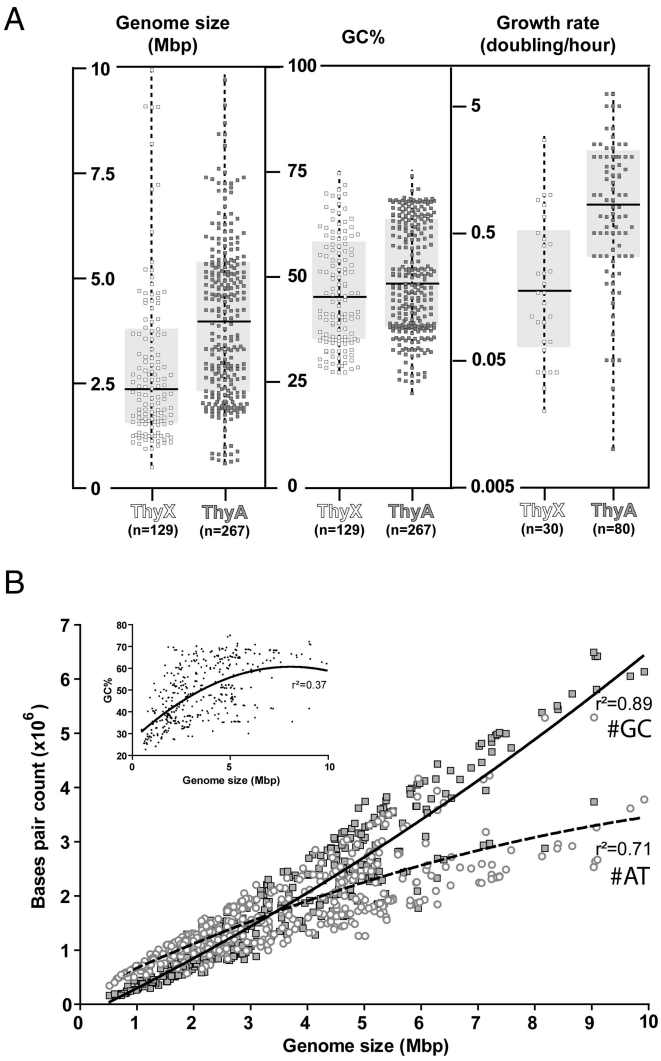

Fig. 2. Size distribution and nucleotide counts of *thyA*- and *thyX*-containing genomes. (*A*) Distributions of genome size, GC% and growth rate are shown. In each case, *thyX* and *thyA* containing organisms were analyzed individually. (*B*) A scatter plot demonstrating the relationship between the genome size and the nucleotide counts for 425 prokaryotic genomes is shown [GC count (filled squares); AT counts (open circles)]. The best polynomial nonlinear fits (second order) for GC (solid line) and AT (dotted line) counts are shown. (*Inset*) Plotting of the relative GC% as a function of genome size.

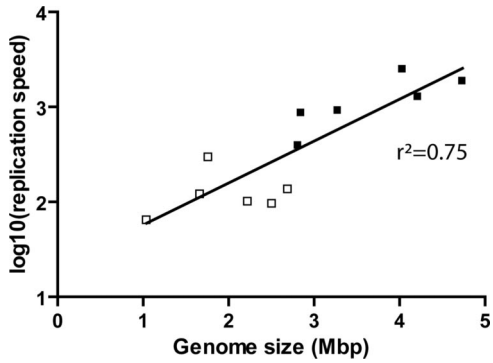

Fig. 3. Relation between mean replication speed (represented by a logarithmic scale) and prokaryotic genome size. ThyX (open squares) and ThyA (filled squares) containing organisms are indicated. A linear regression curve ($r^2 = 0.75$) is plotted. Data were extracted from Table 2.

that "high" and "low" replication speeds in prokarya reflect the differences in the molecular architectures of bacterial and archaeal replisomes, respectively. We propose the alternative explanation that the observed differences in replication speed result, at least in part, from dTMP availability influenced by the use of different thymidylate synthases. Earlier observations have indicated that in *E. coli*, a decreased replication rate results in a slower growth rate and decreased replicative fitness (12). The above suggestion and the significant growth difference of ThyX- and ThyA-containing species (Fig. 2*A*) raise the possibility that organisms with highly active ThyA could compete out catalytically less efficient ThyX species in natural populations.

To address why ThyX enzymes are nevertheless used in a large number of species, we determined the genome size and guanine and cytosine (GC) composition of 425 prokaryotic organisms as a function of their thymidylate synthase use. When these genomic features were separately plotted by using a box-and-whiskers graph (Fig. 2*A*), we observed only a small difference in GC percentage between ThyX and ThyA-containing organisms. The striking observation is that although the genome sizes of *thyA* organisms were widely distributed, with a median size of 3.9 Mb, the size distribution of *thyX*-containing genomes was significantly narrower, with a median size of 2.3 Mb [$P < 0.0001$ (a nonparametric two-tailed Mann–Whitney test)]. We also plotted the number of different nucleotides as a function of the genome size (Fig. 2*B*). The nonlinear polynomial fit of the combined guanine and cytosine count, as a function of genome size, had a high goodness-of-fit value of $R^2 = 0.89$ and revealed an obvious gradual increase in all analyzed genomes. Although the counts of adenine and thymidine nucleotides ($R^2 = 0.70$) also showed an initial increase, starting from a genome size of ≈3 Mb, this increase was less important. This observation is unexpected because synthesis of dATP and dTTP precursors requires less energy than the production of dGTP and dCTP precursors (13). Notably, our observations suggest that prokaryotic genomes have enlarged mainly by increasing their GC content (GC%) to overcome the "AT-barrier" and are likely to be at the origin of the much weaker positive correlation that has been observed for the relative GC% and the average genome size (Fig. 2*B*) (14, 15). Further support for this notion is shown in Fig. 3, which depicts a positive correlation between the replication speed and genome size in prokarya. Altogether these observations predict that organisms with large genomes that thrive in complex and variable environments should show an increased GC content.

Although reciprocal gene replacements implicating *thyX* and *thyA* are occurring frequently and randomly, in the light of functional constraint imposed by the nature of the thymidylate synthase, the likelihood for observing replacement of *thyA* by *thyX* in natural populations is small. This constraint, however, does not operate in species with a relatively small genome, explaining why the catalytically less efficient ThyX proteins are still present in 30% of prokaryotic organisms (based on completed genome sequences). Our work also provides the hypothesis that thymidylate synthase use globally affects prokaryotic genome dynamics. Our data predict that massive gene duplications or lateral gene transfer into *thyX*-containing species should be counterselected. Moreover, the replacement of *thyA* by *thyX* coincides with the well documented case of reductive genome evolution observed in *Rickettsia* species (16). Even though this correlation does not prove causation, future experimental work will be needed to address the possibility that an analogous increase in mutation rate, as observed for the strain FE010 (Δ*thyA*::*thyX*), could accelerate genome reduction as has been proposed for *Pelagibacter* and *Prochlorococcus* species that both contain *thyX* (17). The observation that some cyanobacteria have

strikingly large, AT-rich genomes suggests that alternative strategies to enlarge prokaryotic genomes exist.

In conclusion, our combined experimental results and statistical analyses have indicated that metabolic enzymes can shape prokaryotic genomes. Because the work reported here indicates that ThyX enzymes limit DNA replication kinetics *in vivo*, our observations suggest that the increased catalytic efficiency of ThyA enzymes was necessary to increase the prokaryotic evolutionary potential.

Materials and Methods

Bacterial Strains and Growth Conditions. Bacterial strains used in this study are listed in Table S2. *E. coli* cells were grown at 37°C in either thymidine-containing LB medium or thymidine-free M9 minimal medium supplemented with 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 0.2% casamino acids (Difco) or thymidine-free L+ medium (18). When necessary, thymidine (50 μg/ml) and isopropyl D-thiogalactopyranoside (1 mM) were added to the growth medium. *H. pylori* 26695 was grown on either blood agar base two (Oxoid) plates supplemented with 10% defibrinated horse blood (Oxoid) or in liquid culture in brain-heart infusion broth (Oxoid) supplemented with 10% FBS (Gibco BRL). An antibiotics–fungicide mix consisting of vancomycin (final 10 μg/ml), polymyxin B (2.5 units per liter), trimethoprim (5 μg/ml), and fungizone (2.5 μg/ml) was added. Plates were incubated at 37°C in a microaerobic atmosphere in jars by using CampyGen (Oxoid). Liquid cultures were shaken at 175 rpm.

Molecular Biology Techniques. The replacement of the complete *thyA* gene of *E. coli* strain MG1655 by the *Paramecium bursaria Chlorella* virus 1 (PBCV-1) *thyX* gene, encoding the most catalytically active ThyX protein isolated to date, was performed as follows. First, *thyX* and 500-bp DNA fragments flanking *thyA* were amplified by PCR (primers used are indicated in Table S1). The kanamycin cassette *aphA-3* was obtained by SmaI digestion of pUC18K2. The deletion construct (Fig. 1*A*) was assembled in two sequential PCRs. The obtained PCR product was introduced into the chromosome of *E. coli* by the use of the lambda-red recombination system (19, 20). This system uses the thermosensitive pKOBEG plasmid that carries the λ phage red$\gamma\beta\alpha$ operon under the control of a pBAD promotor. Transformants were selected with kanamycin in the presence of thymidine (replacement efficiency $\approx 7.10^3$ CFU/μg of linear DNA). The *thyA*-deleted strain, FE013, was constructed as above without insertion of *thyX*. Both constructs were confirmed by PCR and DNA sequencing (data not shown).

Fluorescence Microscopy. *E. coli* strains MG1655 and FE010 were diluted in thymidine-deprived L+ medium to an $OD_{600} \approx 0.2–0.3$ and incubated at 37°C with agitation. One-milliliter culture aliquots were removed and stained as described (21). Briefly, cell membranes were stained with *N*-(3-triethylammonium-propyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide (FM 4-64) at 0.01 $\mu g.\mu l^{-1}$, and the nucleoid was stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) at 2 $\mu g \cdot ml^{-1}$. FM 4-64 stains lipid membranes and emits a red fluorescence (excitation/emission ≈515/640 nm); DAPI is a nucleoid stain with blue fluorescence (excitation/emission ≈350/470 nm). The stained cells were observed by using a Leica DM RXA microscope. Images were captured with a CCD camera 5 MHz Micromax 1300Y (Roper Instruments). The final reconstructed images were obtained by deconvoluting Z-series with Metamorph software (Universal Imaging).

Runout Replication Assay. *E. coli* strains were grown in LB medium for 12–14 h and diluted 10-fold by using M9 minimal medium. At the beginning of the exponential phase, rifampicin (50 μg/ml final; Sigma), uridine (50 μg/ml final; Sigma), and 3 μCi of [*methyl*-^{3}H]-thymidine (84 Ci/mmol; Amersham) were added. Incorporation of radioactive thymidine was measured by scintillation counting after trichloroacetic acid precipitation of whole cells.

Determination of Ori/Ter Ratio and Replication Period (C-Period). For the experimental quantification of the relative ratios of *H. pylori* origin and terminus regions [previously identified by using bioinformatic analyses (22)], DNA was extracted from midexponential cultures ($OD_{600} \approx 0.8$) by using QIAamp DNA Mini kit (Qiagen). The primers used (oEF102, oEF103, oEF106, and OEF107) for the quantification reactions are indicated in Table S1. Quantitative PCR was performed in a MiniOpticon real-time PCR detection system (Bio-Rad) by using SYBR green PCR MasterMix Q-PCR (Bio-Rad). Observed cycle thresholds and PCR efficiencies allowed determination of a calibrator-normalized relative quantification of the replication origin over terminus for each sample by using Opticon Monitor Software version 3.1.32 (Bio-Rad). The replication time (C in the following equation) was calculated from the origin/terminus ratio by using the formula $O/T = 2^{\tau/C}$, where τ is the doubling time (23, 24).

Statistical Analysis. We analyzed several genomic characteristics of 425 complete prokaryotic genomes (Table S3) that were retrieved in March 2006 from the Integrated Microbial Genomes (25) and the Cluster of Orthologous Groups databases (26, 27). Automated analyses were completed and validated by manual investigation. In particular, the presence and/or absence of both thymidylate synthases was confirmed by using BLAST and PSI-BLAST tools (28, 29). The maximum growth rates of prokaryotes used in this study were taken from ref. 30. Statistical analyses were performed by using GraphPad Prism version 4.00 for Windows.

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