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Novel genomic island modifies DNA with 7-deazaguanine derivatives

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The discovery of ∼20-kb gene clusters containing a family of paralogs of tRNA guanosine transglycosylase genes, called *tgtA5*, alongside 7-cyano-7-deazaguanine ($preQ_0$) synthesis and DNA metabolism genes, led to the hypothesis that 7-deazaguanine derivatives are inserted in DNA. This was established by detecting 2′-deoxy-$preQ_0$ and 2′-deoxy-7-amido-7-deazaguanosine in enzymatic hydrolysates of DNA extracted from the pathogenic, Gram-negative bacteria *Salmonella enterica* serovar Montevideo. These modifications were absent in the closely related *S. enterica* serovar Typhimurium LT2 and from a mutant of *S.* Montevideo, each lacking the gene cluster. This led us to rename the genes of the *S.* Montevideo cluster as *dpdA-K* for 7-deazapurine in DNA. Similar gene clusters were analyzed in ∼150 phylogenetically diverse bacteria, and the modifications were detected in DNA from other organisms containing these clusters, including *Kineococcus radiotolerans*, *Comamonas testosteroni*, and *Sphingopyxis alaskensis*. Comparative genomic analysis shows that, in Enterobacteriaceae, the cluster is a genomic island integrated at the *leuX* locus, and the phylogenetic analysis of the TgtA5 family is consistent with widespread horizontal gene transfer. Comparison of transformation efficiencies of modified or unmodified plasmids into isogenic *S.* Montevideo strains containing or lacking the cluster strongly suggests a restriction–modification role for the cluster in Enterobacteriaceae. Another $preQ_0$ derivative, 2′-deoxy-7-formamidino-7-deazaguanosine, was found in the *Escherichia coli* bacteriophage 9g, as predicted from the presence of homologs of genes involved in the synthesis of the archaeosine tRNA modification. These results illustrate a deep and unexpected evolutionary connection between DNA and tRNA metabolism.

DNA modification | restriction–modification | 7-deazaguanine | comparative genomics | queuosine

Hypermodifications of DNA requiring more than one synthetic enzyme are not as prevalent and chemically diverse as RNA hypermodifications, but around a dozen have been identified in DNA to date (1). The functions of most DNA hypermodifications are still not known, but some have roles in protection against restriction enzymes, whereas others affect thermal stability temperature, DNA packaging, or transcription regulation (2). For example, the hypermodified DNA base β-D-glucosyl-hydroxymethyluracil, or base J, is an epigenetic factor that regulates Pol II transcription initiation in kinetoplastids of trypanosomes (3). The recently discovered phosphorothioate (PT) modification of the DNA backbone in bacteria was found to perform different functions in different organisms (4–6). In *Salmonella* Cerro 87, PT occurs on each strand of a GAAC/GTTC motif as part of a restriction–modification (R–M) system, whereas in *Vibrio cyclitrophicus* FF75, which lacks PT restriction enzymes, PT occurs on one strand of $C_{ps}CA$ motifs, and the function remains unclear (6). In 2013, Iyer et al. described the computational prediction of 12 novel DNA hypermodification systems in phage and bacteria (7), demonstrating the potential diversity and complexity of modifications yet to be discovered.

7-Cyano-7-deazaguanine ($preQ_0$) is a common precursor of the widespread tRNA modifications queuosine (Q) and archaeosine (G^+) (8) and of pyrrolopyrimidines such as toyocamycin or tubercidin (9). In both Archaea and Bacteria, $preQ_0$ is synthesized from GTP in a pathway that has been fully characterized in the last 10 y (Fig. 1*A*). The first step catalyzed by GTP cyclohydrolase I (GCHI; FolE) is shared with the tetrahydrofolate synthesis pathway (10), and then three enzymes—6-carboxy-5,6,7,8-tetrahydropterin synthase (QueD), 7-carboxy-7-deazaguanine synthase (QueE), and 7-cyano-7-deazaguanine synthase (QueC)—lead to the formation of the $preQ_0$ moiety (11, 12) (Fig. 1*A*).

The synthesis of G^+ and Q diverge after the formation of $preQ_0$. In Bacteria, the tRNA guanosine (34) transglycosylase (bTGT; EC 2.4.2.29) enzyme that targets the G at position 34 of tRNAs with GUN anticodons (13) prefers the 7-aminomethyl-7-deazaguanine ($preQ_1$) base that is derived from $preQ_0$ in one step by NADPH-dependent enzyme $preQ_0$ oxidoreductase (QueF) (14), but it can

Significance

The discovery of a novel modification system that inserts 7-deazaguanine derivatives in DNA, modifications thought until now to occur only in RNA, is an excellent illustration of the power of biological evolution to alter the ultimate function not only of the distinct proteins but also of entire metabolic pathways. The extensive lateral transfer of the gene cluster responsible for this modification highlights its significance as a previously unrecognized foreign DNA defense system that bacteria and phages use to protect their genomes. The characterization of these DNA modification pathways also opens the door to novel tools to manipulate nucleic acids.

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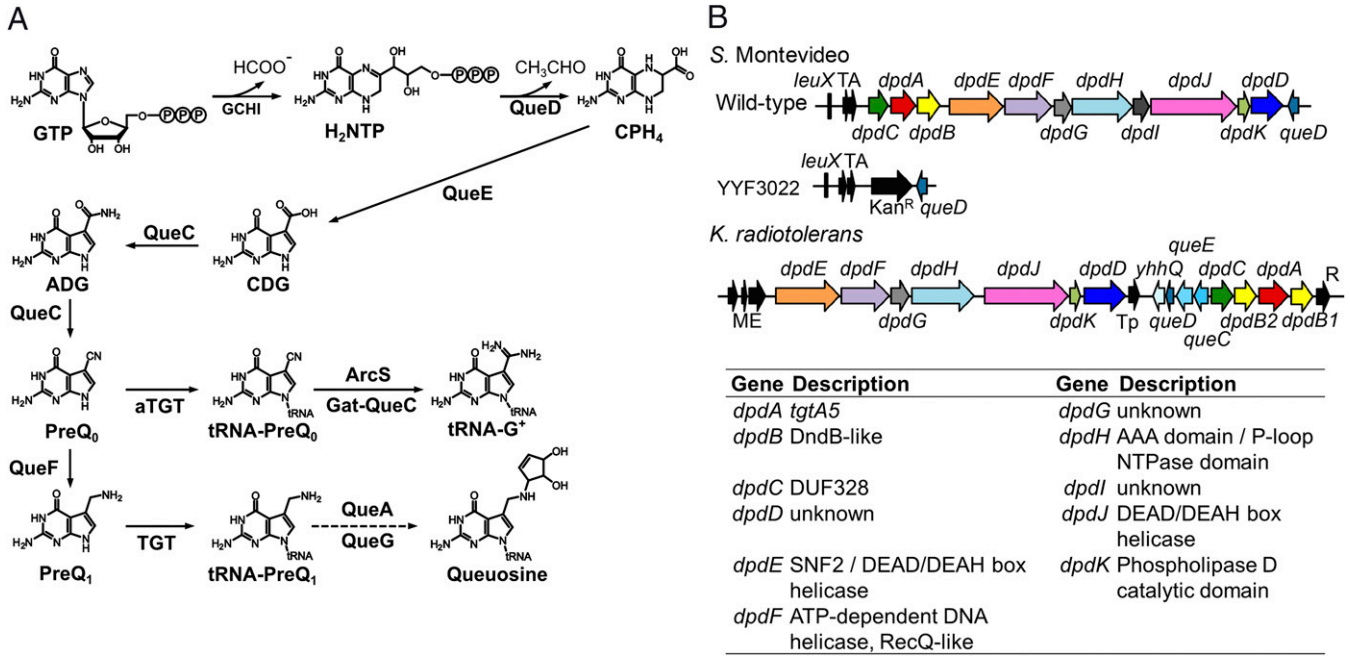

Gene	Description	Gene	Description
dpdA	*tgtA5*	*dpdG*	unknown
dpdB	DndB-like	*dpdH*	AAA domain / P-loop NTPase domain
dpdC	DUF328	*dpdI*	unknown
dpdD	unknown	*dpdJ*	DEAD/DEAH box helicase
dpdE	SNF2 / DEAD/DEAH box helicase	*dpdK*	Phospholipase D catalytic domain
dpdF	ATP-dependent DNA helicase, RecQ-like		

Fig. 1. Biosynthesis of 7-deazaguanine derivatives. (*A*) The biosynthetic pathways to queuosine and archaeosine in tRNA. ADG, 7-amido-7-deazaguanine; aTGT, archaeal TGT; CDG, 5-carboxydeazaguanine; CPH_4, 6-carboxytetrahydropterin; G^+, archaeosine; GCHI, GTP cyclohydrolase I (FolE); H_2NTP, Dihydroneopterin phosphate; TGT, tRNA-Guanine transglycosylase. (*B*) Gene clusters of *S.* Montevideo (GCA_000238535.2; TgtA5 UniProt ID E7V8J4) and *K. radiotolerans* (NC_009664; TgtA5 UniProt ID A6WGA1) *tgtA5*/*dpdA* and *S.* Montevideo mutant strain YYF3022 (Δ*dpdC-dpdD*::kan). Similar colors represent homologs. Red arrows indicate *dpdA*; black arrows represent genes predicted to be involved in HGT; gray arrows represent hypothetical proteins (hyp). int, integrase; ME, mobile element protein; R, resolvase; TA, toxin–antitoxin gene pair (*ccdA*, *ccdB*); Tp, transposase.

also use $preQ_0$ when $preQ_1$ is absent (14). Thus, $preQ_0$ is reduced to $preQ_1$ and inserted in tRNAs by bTGT (Fig. 1*A*). Two subsequent enzymatic steps, carried out by QueA and QueG, produce the final Q nucleoside (for recent review, see ref. 9). In Archaea, the tRNA guanosine (15) transglycosylase enzyme (aTGT; EC 2.4.2.48) is homologous to the bTGT enzyme and exchanges the G at position 15 with $preQ_0$ in nearly all tRNAs (15, 16). The $preQ_0$ is then modified to G^+ by different types of amidotransferases [archaeosine synthase (ArcS), QueF-like, and glutamine amidotransferase class-II (GAT)-QueC] (17, 18) (Fig. 1*A*). Although the bTGT and aTGT recognize a guanosine at different positions of the tRNA and use different substrates, key residues involved in base exchange and in zinc binding are conserved (19) (Fig. 2*A*). In addition, signature residues involved in the differences in 7-deazaguanosine substrate recognition between the bTGT and aTGT enzymes have been identified (19) (Fig. 2*B*). The role of 7-deazaguanosine derivatives as precursors of modified bases in tRNAs and of secondary metabolites is well established (9), and the $preQ_0$ molecule itself was recently found to have anticancer properties (20). These modified bases can also be detected in rRNA in vivo if labeled $preQ_1$ is fed to *Escherichia coli* (21) or inserted in DNA in vitro with the bTGT enzyme (22), but the biological relevance of these last two observations is not clear.

In the computational analysis of DNA modification systems by Iyer et al., the presence of divergent *tgt*-like genes and $preQ_0$ genes present in certain phage and bacteria clustering with ParB-like proteins and several families of helicases led the authors to hypothesize that these gene clusters encode a DNA modification system (7). Our own analysis of the distribution and physical clustering of *tgt* and $preQ_0$ synthesis genes, described here, led us to a similar hypothesis that a $preQ_0$ derivative would be found in DNA in specific bacteria and phages. This hypothesis was validated by the analysis of the DNA of organisms either possessing or lacking the gene cluster, leading to the discovery of complex modifications of DNA that had escaped identification to date and could be among the most chemically elaborate DNA modification systems found in nature thus far.

Results

A Bacterial TGT Variant, TgtA5, Must Be Involved in a $preQ_0$-Dependent Pathway Different from Q Synthesis. Analysis of the distribution of all Q synthesis genes in bacteria was performed using the "dpd cluster" subsystem in the SEED database (23). Analysis of ∼12,000 bacterial genomes showed that some organisms, such as pathogenic strains of *E. coli* (strain E22) or *Salmonella enterica* subsp. *enterica* serovar Montevideo, contained two homologs of the *tgt* gene, whereas most other sequenced *E. coli* or *Salmonella* species contained only one (*SI Appendix*, Table S1). Synteny analysis revealed that one of the two *tgt* genes clustered with the Q synthesis gene *queA* and encoded the experimentally characterized bTGT enzyme (24) (*SI Appendix*, Figs. S1 and S2*A*), whereas the other, that we named *tgtA5* (later changed to *dpdA*), was found in a different neighborhood context (Fig. 1*B*). Homologs of *tgtA5* were found in nearly 284 complete prokaryotic genomes (*SI Appendix*, Table S1 and Fig. S10 *A* and *B*), and the TgtA5 proteins possess divergent features from the bTGT that inserts $preQ_1$ at position 34 of tRNA (Fig. 2). Members of the TgtA5 family are larger proteins (average of ∼450 aa instead of ∼300 aa for bTGT), and only the core of TgtA5 shows significant similarity to the bTGT and aTGT enzymes (Fig. 2*B*). The key residues that catalyze the G exchange (Asp102 and Asp280 of *Zymomonas mobilis* bTGT and Asp95 and Asp249 of *Pyrococcus horikoshii* aTGT) (19), as well as the Zinc binding site ($CXCXXCX_{22}H$ motif), are conserved in TgtA5 (Fig. 2*B*). Analysis of the substrate binding pocket suggests that TgtA5 binds $preQ_0$, like the aTGTs. The critical residues for $preQ_0$ binding by aTGT are GVVPL[LM] at positions 196–201 of the *P. horikoshii* enzyme, differing from the bTGT $preQ_1$ binding pocket residues GLAVGE at position 230–235 of the *Z. mobilis* enzyme (19). Alignments of TgtA5 sequences with bTGT and aTGT showed the binding pocket residues resembled aTGT more than bTGT (G[ML]VPL[KR] in

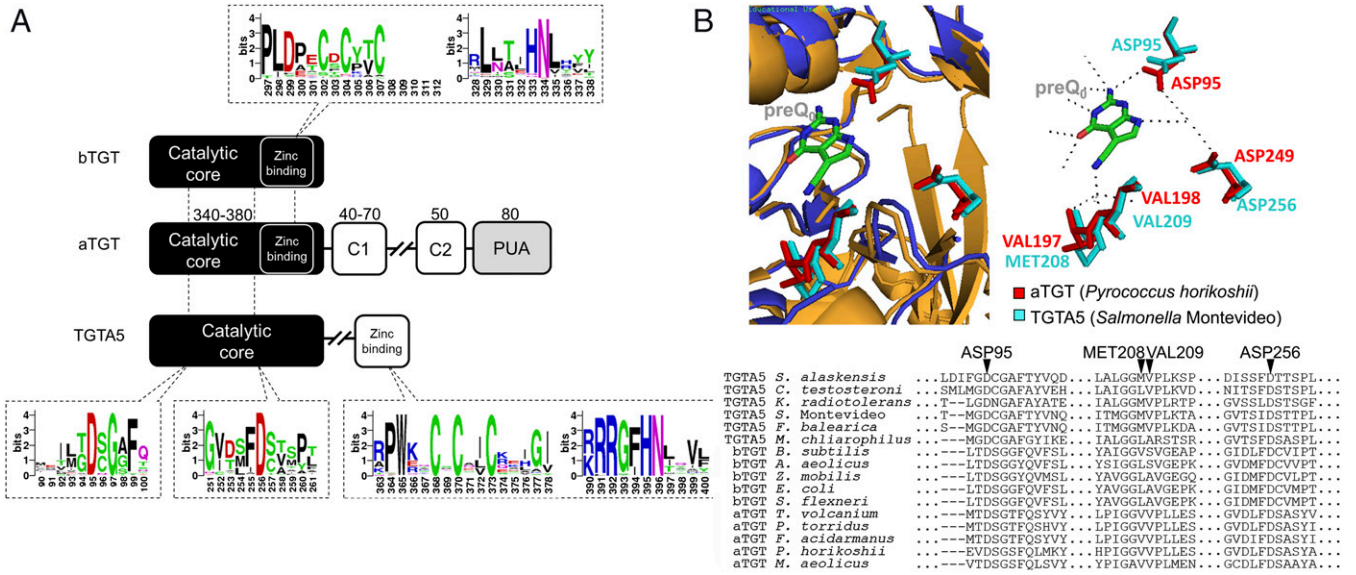

Fig. 2. Comparison of TGT and TgtA5 proteins. (*A*) Schematic representation of the domain architecture and arrangement of TgtA5 proteins and bacterial and archaeal TGT proteins (bTGT and aTGT, respectively). The numbering of the upper and lower logos refers to the *S*. Montevideo TGT and TgtA5 sequences, respectively. Sequence logos in dashed boxes show the two conserved Asp residues of TgtA5 and the zinc binding sites of bTGT (*Top*) and TgtA5 (*Bottom*). C1 and C2 represent C-terminal domains unique to aTGT (17, 19). (*B*) Model and alignments of proposed substrate-binding pocket of TgtA5. The aligned cartoon representation (*Top*) of the pockets of *S. Montevideo* TgtA5 and *P. horikoshii* aTGT (PDB ID code 1IT8) was produced by PyMol (version 1.3). The catalytic residues of aTGT, ASP95, VAL197, VAL198, and ASP249 (red) (18) and their TgtA5 counterparts ASP95, MET208, VAL209, and ASP256 (cyan) are indicated in stick models. Dashed lines among stick models indicate the catalytic residues interacting with $preQ_0$. Sequence alignment (*Bottom*) of select aTGT, bTGT, and TgtA5 proteins was performed using MUSCLE (53). Dots indicate regions intentionally deleted for this figure. Dashes indicate gaps in the sequence alignment. UniProt IDs for proteins included in multiple alignment are as follows: *S*. Montevideo TgtA5, E7V8J4; *F. balearica* TgtA5, E1SVY3; *S. alaskensis* TgtA5, Q1GPS0; *Comamonas testosteroni* TgtA5, H1RRG1; *K. radiotolerans* TgtA5, A6WGA1; *E. coli* bTGT, P0A847; *Z. mobilis* bTGT, Q8GM47; *Shigella flexneri* bTGT, Q54177; *Bacillus subtilis* bTGT, L8AMH3; *Aquifex aeolicus* bTGT, O67331; *P. horikoshii* aTGT, O58843; *Methanococcus aeolicus* aTGT, A6UVD8; *Thermoplasma volcanium* aTGT, Q977Z3; *Picrophilus torridus* aTGT, Q6L1W3; *Ferroplasma acidarmanus* aTGT, S0AQ23.

TgtA5; Fig. 2*B*). Modeling of the *S*. Montevideo TgtA5 protein with the aTGT structure with $preQ_0$ in the binding pocket demonstrated the similar placement of these binding pocket residues compared with the aTGT (Fig. 2*B*), supporting our hypothesis that TgtA5 binds $preQ_0$. Finally, *tgtA5* clusters with the $preQ_0$ synthesis genes in 94% of analyzed genomes, but it does not cluster with *queF*, suggesting that the substrate is $preQ_0$ or a derivative, and not $preQ_1$. Sequence and genome context analyses predict that TgtA5 recognizes $preQ_0$ as a substrate like the aTGT enzymes, but because TgtA5 proteins lack the tRNA binding PUA domain found in aTGTs (25), analyses strongly suggest that TgtA5 proteins do not target tRNAs (Fig. 2*A*). Also, *tgtA5* genes are found in organisms that lack the canonical Q synthesis gene *tgt*, such as *Kineococcus radiotolerans* (*SI Appendix*, Table S1), and we confirmed that *K. radiotolerans* lacked Q and $preQ_0$ in tRNA (*SI Appendix*, Fig. S1*A*). Finally, the *tgt* gene was deleted in the *S*. Montevideo strain that contained both a *tgt* and a *tgtA5* gene, and tRNA extracted from the Δ*tgt* strain (YYF3020) lacked Q (*SI Appendix*, Figs. S1*B* and S2), confirming that TgtA5 is not involved in incorporation of Q in tRNA.

Strong physical clustering was observed between *tgtA5* with homologs of a DndB-like protein that is involved with the PT modification of DNA in bacteria (5) (Fig. 1*B* and *SI Appendix*, Table S1). These *dndB*-like genes were present in 123 of 134 (92%) *tgtA5* gene clusters analyzed, and in 21 clusters (15%), there were two distinct copies of this gene flanking the *tgtA5* gene (Fig. 1*B* and *SI Appendix*, Table S1 and Fig. S4). According to HHPred analysis and previous work by Iyer et al. (7), DndB proteins contain a domain belonging to the superfamily of ParB nucleases involved in chromosome and plasmid partitioning (26, 27), suggesting a role in DNA recognition and binding. Studies have shown that DndB negatively regulates the PT modification (28–30) by binding to the promoter region and regulating expression of the DndBCDE operon (30). The *S*. Montevideo DndB-like protein (renamed DpdB) has 23% amino acid identity to the DndB protein of *S*. Cerro. Despite the low similarity, several residues are conserved among the PT-related DndB and TgtA5-clustered DndB-like proteins, including a QR doublet near the N terminus and a FXXXN motif near the middle of the sequence. Most striking, however, is the strictly conserved DGQQR motif in nearly all of the TgtA5-clustered DndB-like proteins, which differs by one residue from the DGQHR motif conserved among the DndB proteins involved in PT modification (*SI Appendix*, Fig. S3*A*). Although the PT-related DndB and the TgtA5-clustered DndB-like (DpdB) proteins share conserved motifs, they separate on a phylogenetic tree (*SI Appendix*, Fig. S3*B*), indicating that they comprise two subfamilies of the DndB-like family.

Strong physical clustering was also observed with *tgtA5* and several other genes (Fig. 1 and *SI Appendix*, Figs. S4 and S5). Two genes of unknown function, which we call *dpdC* and *dpdD*, were present in 88% and 90% of the clusters analyzed, respectively. DpdC was predicted to encode a DUF328 domain-containing protein, and DpdD has little similarity to any known protein, although a small portion of the C terminus matched a DUF2325 domain (*SI Appendix*, Fig. S5 and Table S2). In 98% of the bacteria analyzed, the *tgtA5* genes also clustered with several other putative DNA-binding enzymes, including a member of the DEAD/DEAH box helicase family, a SNF2-type helicase, and a RecQ-like Superfamily II DNA helicase (Fig. 1 and *SI Appendix*, Figs. S4 and S5 and Tables S1 and S2). This grouping allowed us to speculate that TgtA5 is involved in introducing $preQ_0$-like modifications in DNA, a hypothesis previously proposed by Aravind and colleagues (7).

Nine of the analyzed genomes contained much larger TgtA5 proteins (~700 aa). The TgtA5 domain of these proteins is

similar to the rest of the TgtA5 family. The N-terminal half of the protein contains a DUF328 domain, like the one present in the DpdC of the *S.* Montevideo cluster. The genomic context of the longer *tgtA5* genes includes $preQ_0$ synthesis genes, similar to the other *tgtA5* genes; however, one noticeable difference is the absence of some or all of the three genes conserved in the other clusters, the *dndB*-like *dpdB*, and *dpdC* and *dpdD* (see the *Meiothermus chliarophilus* DSM 9957 cluster in *SI Appendix*, Fig. S4 and others in *SI Appendix*, Fig. S6). Some of the long *tgtA5* clusters encode a similar SNF2-type helicase, RecQ-like helicase, phospholipase-like domain-containing protein, and a DpdD, whereas others have other putative DNA-binding proteins and helicases, suggesting that the long TgtA5 protein is also involved in introducing a modification into DNA in these organisms.

The *tgtA5* Cluster Is Responsible for the Insertion of $PreQ_0$ and of 7-Amido-7-Deazaguanine in DNA. To test the hypothesis that 7-deazaguanine derivatives were inserted in the DNA of organisms that encode the *tgtA5* cluster, a mass spectrometry-based approach was used to analyze DNA from two closely related Gram-negative bacteria possessing and lacking this gene cluster (*S.* Montevideo and *S.* Typhimurium LT2, respectively) and from the Gram-positive bacteria *K. radiotolerans* that also encodes the cluster (Fig. 1*B*). The strategy for discovering the 2'-deoxynucleosides was based on an initial presumption of the presence of 2'-deoxynucleosides containing any of the six 7-deazaguanine nucleobase structures formed in the tRNA queuosine biosynthetic pathway shown in Fig. 1*A*: 2'-deoxyCPH_4, 2'-deoxyCDG, 2'-deoxyQ, 2'-deoxy$preQ_0$, 2'-deoxy$preQ_1$, and 2'-deoxyG^+. A search for each candidate 2'-deoxynucleoside was conducted by neutral loss analysis mass spectrometry, in which product ions resulting from loss of a 2-deoxyribose during collision-induced dissociation could be traced back to the original 2'-deoxynucleoside eluting from the HPLC column at a specific retention time. In both *S.* Montevideo and *K. radiotolerans*, small amounts of putative 2'-deoxy$preQ_0$ (d$preQ_0$) and a stronger signal for putative 2'-deoxyCDG (dCDG) were detected (*SI Appendix*, Fig. S7). Subsequent structural analysis revealed that the prediction of dCDG was incorrect and that the signal at *m/z* 311 was actually the M+1 isotopomer for a 2'-deoxy-7-amido-7 deazaguanosine (dADG; Fig. 3*B*). The identities of d$preQ_0$ and dADG were established by fragmentation analysis using high mass-accuracy quadrupole time-of-flight (QTOF) mass spectrometry (*SI Appendix*, Fig. S8) and by comparison with synthetic standards. Standards were also used to rule out detectable levels of d$preQ_1$ and 2'-deoxyArch (dG^+) (*SI Appendix*, Fig. S8*A*). Using these standards, the optimal mass transitions (Fig. 3*B* and *SI Appendix*, Fig. S8 *B–D*) and retention times (*SI Appendix*, Fig. S9) of the modified 2'-deoxynucleosides were determined. Quantitative analysis by external calibration revealed ~1,600 dADG modifications per 10^6 nt in *S.* Montevideo and ~1,300 per 10^6 nt in *K. radiotolerans* (Fig. 3*C*). d$preQ_0$ levels were found to be significantly lower at 10 and 30 d$preQ_0$ per 10^6 nt, respectively (Fig. 3*C*). These results suggest that dADG is the main product of *tgtA5* cluster, with d$preQ_0$ appearing as a side product.

To confirm the role of the *tgtA5* cluster in the insertion of d$preQ_0$ and dADG in DNA, a *S.* Montevideo derivative (YYF3022) with a 21-kb deletion eliminating nearly the entire cluster was constructed (Fig. 1*B* and *SI Appendix*, Fig. S2). Both the d$preQ_0$ and dADG modifications were absent in genomic DNA extracted from YYF3022 (Fig. 3*C*).

This discovery led us to rename the genes of the *S.* Montevideo cluster as *dpdA-K* (Fig. 1*B*), with dpd standing for 7-deazapurine in DNA.

The *dpd* Cluster Is Horizontally Transferred and Found in Genomic Islands. Analysis of the taxonomic distribution of the *dpdA* (*tgtA5*) gene (Fig. 4) showed that this gene is evenly distributed along the bacterial tree, suggesting either an ancestral origin in Bacteria accompanied by massive independent losses along the diversification of Bacteria or a more recent origin with propagation through horizontal gene transfers (HGTs). The phylogenetic analysis of the DpdA/TgtA5 homologs and the discrepancies observed between the topology of the resulting Bayesian and maximum likelihood DpdA/TgtA5 trees with the currently recognized systematics (compare Fig. 4 with *SI Appendix*, Fig. S10 *A* and *B*), such as the nonmonophyly of Gammaproteobacteria or Archaea or the robust grouping of *Herbaspirillum massiliense* (Betaproteobacteria) with *Spirosoma spitsbergense* (Bacteroidetes) and a Verrucomicrobia bacterium but not with other Betaproteobacteria (bootstrap value, 100%; posterior probability, 1.00; *SI Appendix*, Fig. S10 *A* and *B*, respectively), strongly favor the HGT hypothesis.

To confirm that the presence of *dpdA* is diagnostic of the presence of $preQ_0$ and ADG in DNA, we analyzed the genomic DNA from a diverse set of organisms harboring the cluster (*SI Appendix*, Fig. S4 and Fig. 4). As shown in Fig. 3*C*, *M. chliarophilus*, *Comamonas testosteroni*, *Sphingopyxis alaskensis*, and *Ferrimonas balearica* all harbor dADG in DNA but in different quantities. Unlike the other strains analyzed, the DNA from *M. chliarophilus* contained only ADG and no detectable $preQ_0$ (Fig. 3*C*). *M. chliarophilus* was the only strain tested with a long version of the *dpdA* and no *dpdBCD* genes (*SI Appendix*, Fig. S4).

In the *S.* Montevideo and *E. coli* strains that harbor the *dpd* cluster, it is inserted adjacent to the LeuX locus (*SI Appendix*, Fig. S11), a region that had been previously identified as highly variable (31). A pair of 19-bp direct repeats flanking the *dpd* cluster was identified (*SI Appendix*, Fig. S11), again indicative of a genomic island (32, 33). More recently, the sequencing of a large number of *Salmonella* strains confirmed this region as a novel variable island (SGI2) that contained different types of restriction systems, toxin/antitoxin modules, and mobile elements (34). Of the sequenced *S.* Montevideo strains analyzed, 92% contain the *dpdA* cluster at the SGI2 position.

Unlike the *dpd* islands of *S.* Montevideo and *E. coli*, neither tRNA genes nor direct repeats were identified in the region surrounding the *K. radiotolerans dpd* cluster. However, the region is flanked by mobile element proteins (e.g., a transposase-like protein and resolvase protein), and the IslandViewer software (35) identified this cluster as a genomic island. Additionally, the GC content of the ORFs is lower in this region compared with the rest of the genome (66% GC vs. 74% GC).

The *dpd* Cluster of *S.* Montevideo Encodes an R–M System. The discovery of a horizontally transferred DNA modification cluster logically suggested a potential role as a novel R–M system. To test this hypothesis, we compared the transformation efficiencies of the isogenic strains *S.* Montevideo WT and YYF3022 lacking the *dpd* cluster. pUC19 was propagated in each strain, and following extraction, 10 ng of the plasmid DNA was used to transform the WT and YYF3022 strains by electroporation. As shown in Fig. 5, pUC19 DNA extracted from YYF3022 transformed the WT strain with 100-fold less efficiency than pUC19 DNA extracted from the WT strain, indicating the unmodified plasmid is restricted in the WT host. The pUC19 extracted from YYF3022 transformed the YYF3022 strain with about 1,000-fold higher efficiency than the WT strain, suggesting that one or more of the genes in the *dpd* cluster was responsible for this restriction. No difference was seen with the plasmid extracted from the WT strain. Liquid chromatography (LC)–MS/MS analysis confirmed that d$preQ_0$ and dADG were present in the pUC19 extracted from *S.* Montevideo WT and not the mutant strain (Fig. 3*C*).

$PreQ_0$ Derivatives in Phage. R–M systems and genomic islands are often transferred through phage transduction. Several examples of phage-encoded *tgt*-like genes and $preQ_0$ genes have already been reported in the literature, including Mycobacteriophage Rosebush (36), *Streptococcus* phage Dp-1 (37), and the *E. coli*

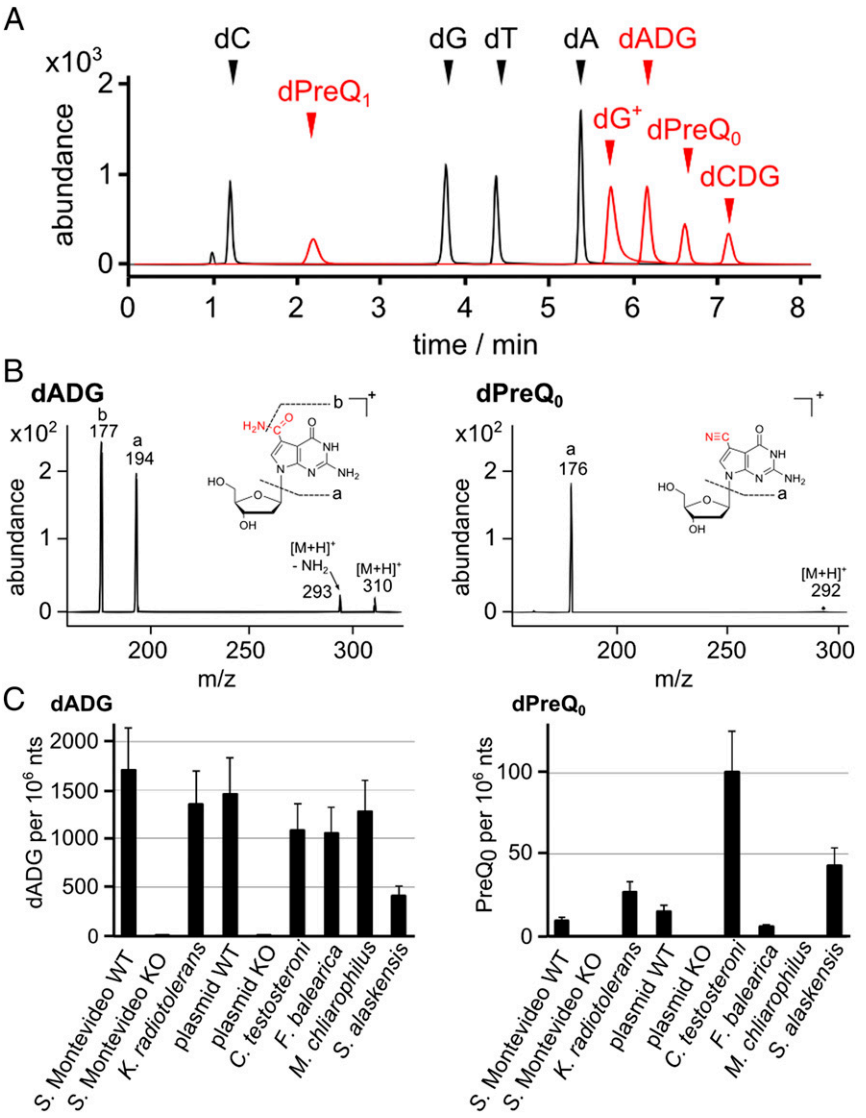

Fig. 3. Detection and quantification of 2′-deoxy-7-deazaguanosine derivatives by LC–MS/MS. (*A*) The LC–MS/MS analytical method is illustrated with an extracted ion chromatogram showing the HPLC retention of the various 7-deazaG–modified (red) and canonical (black) 2′-deoxynucleosides. Abundance denotes arbitrary units of signal intensity. (*B*) MS/MS fragmentation patterns for synthetic dADG and dPreQ$_0$. Abundance denotes arbitrary units of signal intensity. (*C*) Detected quantities in DNA samples of various bacterial species displayed as modification per 10^6 nucleotides.

phage 9g (38) (Fig. 6*A* and *SI Appendix*, Fig. S12). In the characterization of phage 9g, Kulikov et al. (38) speculated that the restriction endonuclease-resistant nature of the phage DNA suggested it was heavily modified, and they proposed that *tgt* and preQ$_0$ synthesis genes were involved in inserting Q into the DNA. To evaluate the prevalence of *tgt* paralogs and PreQ$_0$ synthesis genes in phages, a similarity-based search was performed on all available phage genomes in the National Center for Biotechnology Information database. This revealed 36 bacteriophages and two archaeal viruses that encode a Tgt-like protein (*SI Appendix*, Table S5). Multiple sequence alignments of the phage Tgt-like proteins allowed the identification of catalytic residues (two conserved Asp residues) and of the preQ$_0$-binding pocket (*SI Appendix*, Fig. S13) (19). The zinc-binding residues that are conserved in the aTGT, bTGT, and TgtA5 families were not found in the phage homologs; however, a His residue (H196 of phage 9g Tgt-like protein) is conserved specifically in the phage enzymes.

The preQ$_0$ biosynthesis pathway genes (*folE*, *queD*, *queE*, and *queC*) were identified in 16 of Tgt-containing phages (*SI Appendix*, Table S5), three of which contained a homolog of Gat-QueC that is involved in the synthesis of archaeosine in some archaea (18). Two of the phages harbored a QueF homolog involved in preQ$_1$ synthesis. Finally, one phage, phi13:1, encoded the three first genes of the preQ$_0$ synthesis pathway (*folE*, *queD*, and *queE*) but no *tgt* homolog (*SI Appendix*, Table S5).

The genomic contexts of the phage preQ$_0$/*tgt* clusters are different from those found in bacteria, however many of them encode DNA processing enzymes and could therefore insert 7-deazaguanosine derivatives in DNA. Some of these phages encode a homolog of ParB, an enzyme important for DNA binding and segregation (26), as previously pointed out by Aravind's group (7) (e.g., Mycobacteriophage Rosebush). Other phages encode potential helicases and nucleases near the preQ$_0$ cluster [e.g., Gp11 of 9g and Gp39 of JenKI contain SnfII-like domains (39), Gp11 of Dp-1 is a RecU-like protein (40), Gp40 of JenK1 encodes a putative exonuclease (41), and Gp10 of Dp-1 is a Cas4-like protein (42); *SI Appendix*, Fig. S12]. The presence of these nucleases is indicative of possible defense systems. The nature of the exact modification might differ with the specific phage, as the preQ$_0$

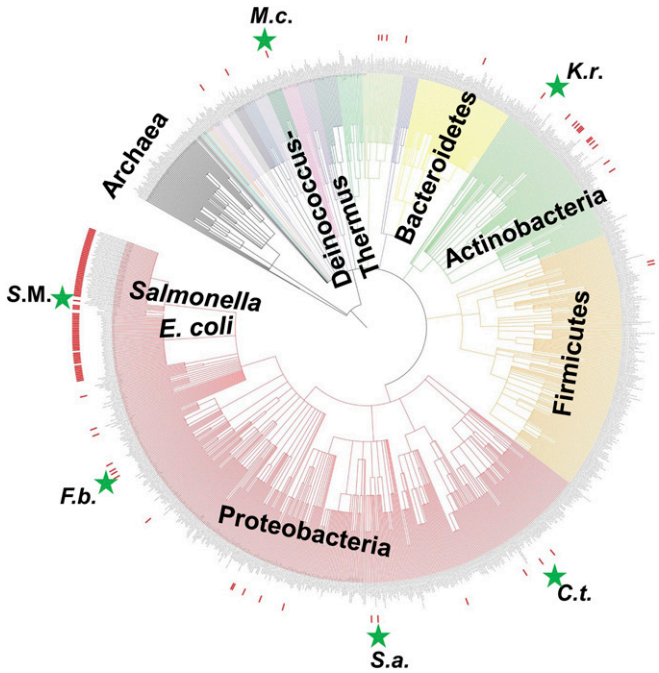

Fig. 4. Taxonomic distribution of TgtA5. Taxonomic tree of ~1,000 representative prokaryotes generated using iToL. Red bars indicate the presence of *tgtA5* in species. Stars indicate organisms for which $preQ_0$ and/or ADG were detected in DNA. *C.t.*, *C. testosteroni*; *F.b.*, *F. balearica*; *K.r.*, *K. radiotolerans*; *M.c.*, *M. chliarophilus*; *S.a.*, *S. alaskensis*; *S.*M., *S.* Montevideo.

pathway is found in Rosebush or BCD7, the $preQ_1$ pathway in Dp-1, and the archaeosine pathway in 9g (*SI Appendix*, Table S5).

To test the hypothesis that some of these phages contained $preQ_0$ derivatives, DNA from phage 9g, a phage predicted to insert archaeosine because of the presence of the *gat-queC* gene, was isolated and subjected to LC–MS/MS analysis as described above. As expected, 2'-deoxy-archaeosine (dG^+) was found in the phage DNA (Fig. 6*B*). dG^+ quantities were extremely high, allowing quantification by both MS/MS and UV analysis that revealed a conversion of dG to dG^+ by 25% and 27%, respectively.

Discussion

The discovery of 7-deazaG derivatives in DNA of diverse bacteria and phages is a compelling example of the power of coupling in silico predictive approaches with bioanalytical validation (43, 44). These modifications would not have been identified if we had not purposely looked for them in very specific organisms. It is also another unexpected example of the crosstalk between RNA and DNA metabolism and of the strong evolutionary links found between RNA and DNA modifying enzymes (1). This crosstalk is advantageous in allowing plasticity in evolution of nucleic acid modification enzymes but also poses specificity problems that could lead to erroneous modification of a nontarget nucleic acid. It is poorly understood how bTGT enzymes, which, unlike aTGT proteins, lack any RNA-binding domains, recognize their RNA targets and also modify DNA in artificial situations. It will be interesting to understand if the TgtA5/DpdA family has evolved specific DNA-binding recognition or if they require the help of accessory proteins in the cluster.

We have clearly demonstrated that the DpdA-containing genomic islands are involved in inserting 7-deazapurine derivatives in DNA; however, the roles of the specific genes in the cluster and mechanism for modification are still to be elucidated. The well-characterized $preQ_0$ synthesis genes, either encoded in the cluster or elsewhere on the chromosome, produce the ADG and $preQ_0$. It was recently shown that ADG is an intermediate in the QueC-mediated reaction from CDG to $preQ_0$ (45) (Fig. 1). ADG is the result of amidation of CDG, a reaction that occurs more rapidly compared with the subsequent dehydration to $preQ_0$. DpdA is most likely involved in exchanging guanine in DNA for ADG or $preQ_0$ in a base exchange reaction similar to bTGT and aTGT, but biochemical studies are required to test this hypothesis and determine if it can do it alone or if it requires other proteins encoded within the cluster. Because the majority of the detected modifications were dADG, it is likely that ADG is the preferred substrate of DpdA, and $dPreQ_0$ may be present as the result of nonspecific insertion by DpdA. The presence of only dADG, and not $dPreQ_0$, in the DNA of *M. chliarophilus* suggests that its DpdA has stricter substrate specificity. The DpdA of *M. chliarophilus* is a larger protein, with an additional 300 amino acids at the N terminus, and the substrate binding pocket of the longer DpdA proteins has a slightly different sequence motif (GGLAR vs. GGMVP of other DpdAs). The LA residues resemble the bTGT $preQ_1$ binding pocket rather than the aTGT $preQ_0$ pocket and may confer specificity for ADG.

The *dpdB* gene encodes a member of the DndB-like family. The DndB proteins appear to have a role in regulation of the PT modification of DNA. Deletion of *dndB* homologs in *Streptomyces lividans* and *Salmonella* Cerro led to increased PT modification (28,

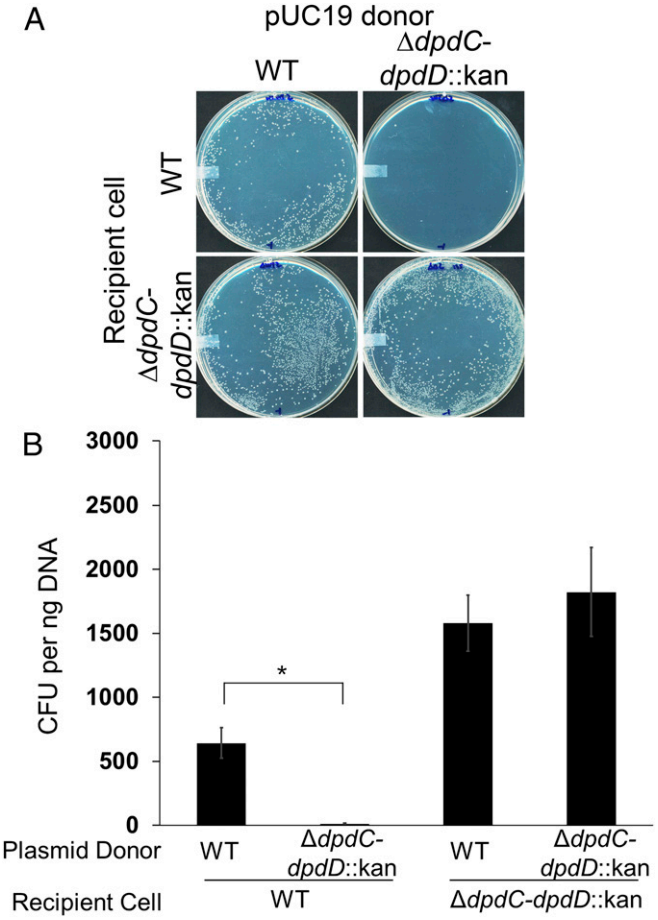

Fig. 5. Transformation efficiency of modified and unmodified pUC19 DNA. (*A*) *S.* Montevideo WT and YYF3022 (Δ*dpdC-dpdD*::kan) transformed with 10 ng pUC19 extracted from either WT (modified) or Δ*dpdC-dpdD*::kan (unmodified) on LB agar plates containing ampicillin. (*B*) Transformation efficiencies of modified versus unmodified pUC19 in WT and Δ*dpdC-dpdD*::kan. Transformation efficiency per 1 ng DNA was calculated per 10^6 viable cfu. The average of three experiments is shown, with error bars representing SE (*$P < 0.05$, two-tailed Student's *t* test).

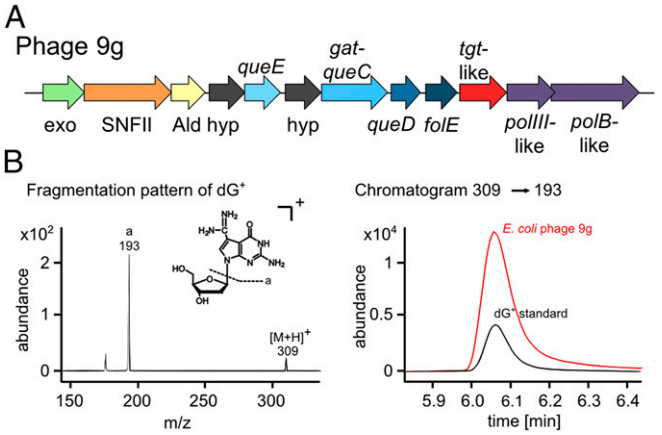

Fig. 6. Phage 9g DNA is modified with dG+. (*A*) Gene cluster of phage 9g (NC_024146) *tgt*-like. Ald, aldolase; exo, Cas4-like exonuclease; hyp, hypothetical; *polB*-like, DNA polymerase B; *polIII*-like, DNA polymerase β-subunit; SNFII, superfamily II-like helicase. (*B*) MS/MS fragmentation pattern of synthetic dG^+ (*Left*) used for subsequent detection of dG^+ in *E. coli* phage 9g DNA (*Right*).

29, 46). A recent study revealed that DptB (DndB homolog) of *S*. Cerro binds to the promoter region and negatively regulates the expression of the DptBCDE operon (30). It is possible that the DndB-like DpdB protein has a similar role in regulating the expression of the *dpd* modification genes.

The roles of DpdC and DpdD remain a mystery, and experimental studies are now underway. Very few hints as to a function have been derived from bioinformatics analyses. Although HHpred analysis identified a DUF328 domain present in DpdC, this domain has no known function. In a computational analysis of potential DNA modification clusters, Iyer et al. (7) predicted the DpdC protein to have an activity similar to ArcS, the amidotransferase that modifies $preQ_0$ to archaeosine, based on long-range similarities. In our analysis of bacterial DNA, we did not detect archaeosine. Based on gene clustering, we predict that DpdC may be important in insertion of the modification, as it nearly always encoded adjacent to *dpdA* and/or *dpdB*. As for DpdD, only 90 residues of DpdD match a DUF2325 domain of unknown function, and we believe this is a completely novel protein family. The *dpdD* gene is present alongside the helicase-like genes and putative DNA-binding genes, leaving the possibility open that DpdD could be involved in the restriction system.

If these genomic islands do indeed encode R–M systems, as suggested by the reduced transformation efficiency of plasmids lacking the modification, the nucleases catalyzing cleavage of the unmodified DNA remain a mystery. Bioinformatic analyses of the genes in the clusters provided several candidates, although none stand out as a true endonuclease. We are currently working to elucidate the protein(s) involved in the restriction.

Finally, the discovery of archaeosine in DNA of phage 9g is another demonstration of the spectrum of DNA modifications that could occur in nature. It is unclear if the archaeosine modification of 9g is part of a restriction system. No restriction enzyme has been identified, although the SnfII-like protein encoded near the modification genes (Fig. 2) could be a potential candidate. Another role for this modification could be to provide resistance to restriction systems, essentially acting as an antirestriction system (47). The phage 9g DNA is resistant to most restriction enzymes tested (38), suggesting the presence of modified bases inhibits recognition or cleavage by these enzymes.

Further studies are needed to elucidate the roles of the 7-deazaguanine derivatives in bacterial and phage DNA, with potential functions varying among R–M systems, antirestriction systems, epigenetic marks, and unforeseen protective roles, as these modifications were found in organisms like *K. radiotolerans* that can resist radiation stress (48). We foresee that the molecular characterization of the enzymes involved in the synthesis, recognition, and cleavage of 7-deazaguanine derivatives in DNA could open the door to both biotechnological and antibacterial applications.

Materials and Methods

Bioinformatic Analyses. Taxonomic distribution and physical clustering analysis of *tgtA5* and $preQ_0$ synthesis genes was performed on the public SEED server (pubseed.theseed.org/SubsysEditor.cgi) (23, 49). Results of the analysis are available in the dpd cluster subsystem and summarized in *SI Appendix*, Table S1. The taxonomic distribution of *tgtA5* was then visualized using the Interactive Tree of Life (iToL, itol.embl.de) (50). Further details on all bioinformatic analyses can be found in *SI Appendix, SI Materials and Methods*.

Strains, Media, and Growth Conditions. Strains used in this study are listed in *SI Appendix*, Table S3. *S.* Montevideo strains were routinely grown in LB (Tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L) at 37 °C. All other strains were grown in media and conditions as detailed in *SI Appendix, SI Materials and Methods*. *S*. Montevideo deletion constructs were made using the linear recombination method described by Datsenko and Wanner (51). Oligonucleotides used for deletion and confirmation of mutants are listed in *SI Appendix*, Table S4. Further details can be found in *SI Appendix, SI Materials and Methods*.

Plasmid Restriction Test. Restriction of the plasmid pUC19 was tested as described in ref. 52 and is detailed in *SI Appendix, SI Materials and Methods*.

DNA Preparation. Total DNA was extracted from bacteria and phage with phenol-chloroform followed by alcohol precipitation, as detailed in *SI Appendix, SI Materials and Methods*.

DNA Analysis. DNA was enzymatically hydrolyzed to 2′-deoxynucleosides as described in *SI Appendix, SI Materials and Methods*. Modified 2′-deoxynucleosides were initially detected by LC–MS/MS, with subsequent structural corroboration of $dPreQ_0$ and dADG by LC–QTOF. Quantification of $dPreQ_1$, $dPreQ_0$, dADG, dCDG, and dG^+ was achieved by LC–MS/MS using external calibration curves. Details can be found in *SI Appendix, SI Materials and Methods*.

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