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Discovery, properties, and biosynthesis of pseudouridimycin, an antibacterial nucleoside analog inhibitor of bacterial RNA polymerase

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Abstract

Pseudouridimycin (PUM) is a novel pseudouridine-containing peptidyl-nucleoside antibiotic that inhibits bacterial RNA polymerase (RNAP) through a binding site and mechanism different from those of clinically approved RNAP inhibitors of the rifamycin and lipiarmycin (fidaxomicin) classes. PUM was discovered by screening microbial fermentation extracts for RNAP inhibitors. In this review, we describe the discovery and characterization of PUM. We also describe the RNAP-inhibitory and antibacterial properties of PUM. Finally, we review available information on the gene cluster and pathway for PUM biosynthesis and on the potential for discovering additional novel pseudouridine-containing nucleoside antibiotics by searching bacterial genome and metagenome sequences for sequences similar to *pumJ*, the pseudouridine-synthase gene of the PUM biosynthesis gene cluster.

Introduction

New antibacterial drugs are urgently needed to address the threat of bacterial pathogens resistant to antibiotics in current clinical use. Unfortunately, the rate of discovery, development, and approval of new antibacterial drugs has dramatically declined, and most newly approved antibiotics represent incremental improvements of existing antibiotics and thus are likely to quickly succumb to existing resistance mechanisms [4, 5, 28].

A number of factors have contributed to the collapse of the pipeline of new antibacterial drugs, including economic barriers, regulatory barriers, and the perceived difficulty of identifying new classes of antibacterial compounds [12]. The situation has been exacerbated by the pharmaceutical industry's decision to de-emphasize natural-product screening in drug discovery [1, 7]. Natural products, particularly microbially produced natural products, were the sources of most current classes of antibacterial drugs [31, 36], but, during recent decades, the pharmaceutical industry has come to believe -- despite evidence to the contrary [14, 34, 44] -- that natural-product screening has been exhausted as a source of new drug leads.

Recently we reported the identification and characterization of pseudouridimycin (PUM), a novel microbially produced nucleoside-analog inhibitor of bacterial RNA polymerase

(RNAP) with broad-spectrum antibacterial activity and a low rate of resistance emergence [26]. We also have characterized the gene cluster and pathway for biosynthesis of PUM [37]. Here we describe the events that led to the discovery of PUM, summarize its properties and its biosynthesis, and add some personal observations on pitfalls and successes of natural-product screening programs.

Discovery

“I cipressi che a Bólgheri alti e schietti, Van da San Guido in duplice filar”. [“Tall and straight the cypress trees lead to Bolgheri, Gallant from San Guido in double file they go”. Giosuè Carducci, 1874–1886; translated by Laura Fullerton Gilbert, 1916]. The words of the poem, taught to every Italian schoolchild and remembered by almost every Italian adult, probably reverberated in the mind of the anonymous individual who, on 24 April 1991, collected a soil sample from beneath a cypress on the famed Viale dei Cipressi and who labeled the soil sample as “cipresso; Bolgheri/San Guido” [*i.e.*, collected by a cypress tree on the road from Bolgheri to San Guido, in central Tuscany, Italy]. The sample was collected on behalf of Gruppo Lepetit, the Milan-area pharmaceutical company that had discovered rifamycins in the 1950s and lipiarmycins in the 1970s. According to Lepetit records, *Streptomyces* sp. strain ID38640, the first microbial strain found to produce PUM, was isolated from that soil sample four months later, on 23 August 1991 (Figure 1, top). Coincidentally, on the same day, *Streptomyces* sp. ID38626, the second microbial strain found to produce PUM, was isolated from a soil sample collected under an olive tree from an unspecified location in France.

Four years later, in 1995, the Lepetit research center, which was soon to become an independent entity, first as Biosearch Italia and then as Vicuron Pharmaceuticals, started preparing a library of microbial-fermentation extracts, a library that eventually came to comprise 200,000 different microbial-fermentation extracts. The library was generated by fermenting 65,000 strains under different growth conditions, processing the resulting fermentation broths by solid-phase extraction, and storing aliquots of the dried extracts in 96-well microplates. The extracts then could be screened using any assay of choice.

Most screens at Lepetit, Biosearch Italia, and Vicuron in that period employed cell-free, target-based assays as primary screens, exploiting their high sensitivity and high specificity. Such screens identified two new classes of antibiotics with new, first-in-class chemical scaffolds: GE81112, a tetrapeptide inhibitor of bacterial translation initiation [27], and GE23077, a cyclic heptapeptide that potently inhibited bacterial RNAP [46]. However, it was soon realized that many compounds discovered by cell-free assays had little or no antibacterial activity, leading to internal discussions whether a screening program should favor chemical originality or antibacterial properties. In a compromise that had more to do with management issues than with scientific rigor, it was decided to screen against cell-free assays only those extracts that possessed an antibacterial activity. This led to the creation of suitable sub-banks, one of which consisted of extracts with growth inhibitory activity against *Staphylococcus aureus*.

During that period, a cell-free, target-based assay for RNAP-inhibitory activity had been implemented (the same assay used to discover GE23077 [46]). The assay used commercially available RNAP from *Escherichia coli* and a plasmid-based promoter-containing template. Selectivity of inhibition was ensured by performing a secondary assay using the structurally unrelated single-subunit RNAP from bacteriophage SP6 and only retaining hits that inhibited the bacterial RNAP but not bacteriophage SP6 RNAP. Product formation was measured by following the incorporation of radiolabeled UMP into newly synthesized RNA, and, to maximize radiochemical sensitivity, the assay conditions used near-saturating concentrations of ATP, CTP, and GTP (500 μ M each) and a limiting concentration of radiolabeled UTP (2 μ M). By pure coincidence, these assay conditions with subsaturating UTP were especially effective for identifying RNAP inhibitors that function by competition with UTP.

In the years 2003–2005, a set of ~3000 extracts with growth-inhibitory activity against *S. aureus* under standard CLSI conditions [30] was screened for RNAP-inhibitory activity. Two extracts, from *Streptomyces* sp. ID38626 and *Streptomyces* sp. ID38640, were found to selectively inhibit *E. coli* RNAP but not bacteriophage SP6 RNAP and to exhibit antibacterial activity against rifampicin-resistant *S. aureus* (Figure 1, bottom panel). For each of the two extracts, HPLC fractionation established that, surprisingly, the component responsible for the antibacterial activity against *S. aureus* was not the same as the component responsible for RNAP-inhibitory activity. The component responsible for the antibacterial activity against *S. aureus* was found to be identical to the previously known antibiotic lydicamycin [26, 37], and the component responsible for the RNAP-inhibitory activity was found to be the novel compound PUM (Figure 1, top), which has only weak, sub-threshold antibacterial activity against *S. aureus* under standard CLSI conditions ([26]; see below). Accordingly, the fact that the screen had employed a sub-library of extracts shown to have antibacterial activity against *S. aureus* under standard CLSI conditions appears to have been incidental to, and unnecessary for, the identification of PUM; a screen of equal size employing previously unscreened extracts presumably would have been equally likely to have identified PUM.

At the time that NAICONs acquired the microbial products assets from the former Vicuron in 2007 [25], preliminary information on the chemical structure of PUM had been obtained, as well as preliminary evidence for *in vitro* and *in vivo* antibacterial activity of PUM against streptococci. NAICONs entered into a collaboration with the Waksman Institute of Microbiology at Rutgers University in 2012 to undertake a full characterization of the properties and biosynthesis of PUM. The results are described in the following sections.

Properties

Mass spectrometry, multidimensional NMR spectroscopy, and confirmatory synthesis of the *N*-desoxy-derivative of PUM established the chemical structure of PUM as a formamidinylated, *N*-hydroxylated Gly-Gln dipeptide conjugated to 6'-amino-pseudouridine (Figure 1 [26]).

PUM selectively inhibits bacterial RNAP *in vitro*, with an IC₅₀ of ~0.1 μ M, and with selectivities for inhibition of bacterial RNAP vs. human RNAP I, II and III of ~600-fold, ~10-fold and ~90-fold, respectively [26].

PUM exhibits *in vitro* antibacterial activity against drug-sensitive, drug-resistant, and multi-drug-resistant *Streptococcus* spp. *in vitro*, with MICs of ~4 to ~6 μ g/mL. PUM also exhibits *in vitro* antibacterial activity against drug-sensitive, drug-resistant, and multi-drug-resistant *Staphylococcus* and *Enterococcus* spp., with MICs from ~4 to ~16 μ g/mL, but does so only in assays using aged Müller-Hinton II broth, and not in assays using freshly prepared Müller-Hinton II broth [26]. (The observed medium-dependence of the antibacterial activity against *Staphylococcus* and *Enterococcus* spp. implies that a component present at different levels in fresh and aged media affects activity against these microorganisms. We do not know the identity of this component, but we speculate that higher levels of pyrimidine nucleosides or pyrimidine nucleotides in fresh medium could competitively interfere with PUM uptake.) PUM also exhibits *in vitro* antibacterial activity against some Gram-negative bacteria, including *Moraxella catarrhalis*, with MICs of ~2 μ g/mL. PUM undergoes decomposition in most growth media, with a half-life of ~12 h [26]. Accordingly, in all of the above cases, broth-microdilution endpoint assays, which have a run time of 16–24 h [30], corresponding to ~1.3 to ~2 PUM half-lives, underestimate the absolute antibacterial activities of PUM, and broth-microdilution growth-curve assays [17], which have shorter run times, need to be performed to obtain accurate estimates of absolute antibacterial activities of PUM [26].

The *in vitro* antibacterial activity of PUM is unaffected by addition of 30% human serum, indicating that PUM does not stably interact with serum proteins.

PUM clears infection *in vivo* in a mouse *Streptococcus pyogenes* peritonitis model, with ED₅₀s of ~9 mg/kg upon intravenous administration and ~30 mg/kg upon subcutaneous administration, demonstrating *in vivo* efficacy and tolerability in a mammal [26].

PUM exhibits no cross-resistance with the rifamycin RNAP inhibitor rifampin (Rif), exhibits additive antibacterial activity when combined with Rif, and exhibits resistance rates that are only ~1/10 those of Rif and lipiarmycin, suggesting that PUM inhibits RNAP through a binding site and mechanism different from those of Rif and lipiarmycin [26; D. Degen and R.H.E., unpublished data].

Isolation and sequencing of PUM-resistant mutants indicates that all mutants conferring 4-fold PUM-resistant mutants contain mutations in the *rpoB* gene (encoding RNAP β subunit) or the *rpoC* gene (encoding RNAP β' subunit), confirming that RNAP is the functional cellular target of PUM [26]. Substitutions conferring PUM resistance are obtained at just four sites: β residues 565, 681, and 684 and β' residue 786 (residues numbered as in *E. coli* RNAP). The number of sites of substitutions conferring PUM resistance is only ~1/10 the number of sites of substitutions conferring Rif-resistance, consistent with the observation that resistance rates for PUM are only ~1/10 those for Rif.

The sites of substitutions conferring PUM-resistance form a single discrete cluster in the three-dimensional structure of bacterial RNAP (“PUM target”; Figure 2). The PUM target

overlaps the RNAP active-center NTP addition site (also referred to as “A site” or “i+1 site”), suggesting that PUM inhibits RNAP by interfering with function of the NTP addition site (Figure 2). The PUM target is different from, and does not overlap, the targets of the RNAP inhibitors Rif [6, 13, 19], lipiarmycin [23, 39], myxopyronin [3, 29, 39], streptolydigin [41, 42], CBR703 [2, 11], salinamide [8], and $N\alpha$ -aroyl- N -aryl-phenylalaninamides [22], and, correspondingly, PUM does not exhibit cross-resistance with any of these RNAP inhibitors (Figure 2). The PUM target partly overlaps the target for GE23077 [46], and, correspondingly, PUM does exhibit partial cross-resistance with GE23077 (Figure 2 [26]).

Five lines of biochemical evidence indicate that PUM functions as a nucleoside-analog inhibitor that competes with UTP for occupancy of the RNAP NTP addition site [26] (1) PUM inhibits nucleotide addition; (2) high concentrations of UTP, but not GTP, ATP, or CTP, overcome transcription inhibition by PUM; (3) PUM inhibits transcription only with templates that direct incorporation of U; (4) in single-nucleotide-addition assays, PUM inhibits incorporation of U--but not G, A, or C; and (5) in multiple-nucleotide-addition assays, PUM inhibits incorporation of U--but not G, A, or C. The single and multiple nucleotide-addition assays further indicate that transcription inhibition by PUM requires a template position that directs incorporation of U preceded by a position that directs incorporation of G, A, or U.

The crystal structure of a PUM-inhibited transcription complex confirms that PUM is a nucleoside-analog inhibitor that competes with UTP for occupancy of the RNAP NTP addition site (Figure 3 [26]). PUM binds to the RNAP NTP addition site. The pseudo-U base moiety of PUM makes Watson-Crick base pair with a DNA template-strand A in a manner equivalent to the base moiety of an NTP; the ribose sugar moiety of PUM makes interactions with the RNAP NTP addition site in a manner nearly equivalent to the sugar moiety of an NTP; the glutamine moiety of PUM makes interactions that mimic interactions made by an triphosphate moiety of an NTP; and the N -hydroxy and guanidinyll moieties of PUM interact with the RNA nucleotide base-paired to the preceding DNA template position (RNA 3' nucleotide), with the N -hydroxy making an H-bond to the 3' OH of the RNA 3' nucleotide, and with the guanidinyll moiety making H-bonds to the 5' phosphate of the RNA 3' nucleotide and to the base of the RNA 3' nucleotide (Figure 3).

The crystal structure accounts for the observed specificity of inhibition for template positions that direct incorporation of U preceded by template positions that direct incorporation of G, A, or U [26]. The Watson-Crick base pair by the pseudo-U base moiety of PUM confers specificity for a position directing incorporation of U, and the H-bond donated by the guanidinyll moiety of PUM with the base moiety of the RNA 3' nucleotide confers specificity for a preceding position directing incorporation of G, A, or U (each of which contains an H-bond acceptor at the appropriate position).

The crystal structure also accounts for the selectivity of transcription inhibition by PUM [26]. All RNAP residues contacted by PUM are conserved across Gram-positive and Gram-negative bacterial RNAP, but four of these RNAP residues are not conserved in human RNAP I, II, and III.

The crystal structure also explains the small size of the PUM resistance spectrum (four RNAP residues). In addition to making direct contacts with the four RNAP residues at substitutions conferring PUM-resistance are obtained, PUM makes direct contacts with ten RNAP residues that comprise functionally critical residues of the RNAP active center that cannot readily be substituted without compromising RNAP activity [20, 35, 38, 40, 45, 46]. In short, PUM interacts with a “privileged target” for which most residues have functional constraints that preclude substitution to yield viable resistant mutants.

Finally, the crystal structure potentially enables rational design of PUM analogs having increased potency and increased selectivity [26]. Initial hit-to-lead and lead-optimization chemistry efforts corroborate the importance of the *N*-hydroxy, glutamine, and guanidinyll moieties of PUM and indicate that the glutamine C(O)NH₂ moiety of PUM can be replaced by C(O)NHR.

Biosynthesis

PUM is a peptidyl nucleoside, a member of a family of microbial natural products that interfere with different biological processes, including bacterial peptidoglycan biosynthesis, bacterial teichoic-acid biosynthesis, bacterial and fungal protein synthesis, and fungal chitin synthesis [32, 43]. However, in contrast to most known members of this family of microbial natural products, PUM is a “*C*-nucleoside,” in which a carbon atom of the base moiety is connected with the sugar moiety, rather than an “*N*-nucleoside,” in which a nitrogen atom of the base moiety is bonded to the sugar moiety (Figure 1).

From a draft genome sequence of *Streptomyces* sp. ID38640, we identified the *pum* gene cluster responsible for PUM biosynthesis as a 20-kb segment comprising fifteen open reading frames (ORFs), including an ORF encoding a putative pseudouridine synthase and an ORF encoding a putative glycine amidinotransferase (Figure 4 [37]). After developing methods for genetic manipulation of *Streptomyces* sp. ID38640, we constructed knockout mutants of seven *pum* ORFs in *Streptomyces* sp. ID38640, demonstrated loss of PUM production in the knockout mutants, identified the PUM precursors and intermediates guanidineacetic acid (GAA), 5'-amino pseudouridine (APU), Gln-APU and *N*-desoxy-PUM that accumulate in knockout mutants, and assessed rescue by feeding putative PUM precursors and intermediates to knockout mutants [37]. Based on the results, we deduced the likely pathway of PUM biosynthesis (Figure 4 [37]).

This work provides the first biosynthetic pathway for a *C*-nucleoside antibiotic and reveals three unexpected features: (1) production of free pseudouridine by a pseudouridine synthase (PumJ), (2) nucleoside activation by the oxidoreductase PumG and the aminotransferases PumI, which are unrelated to equivalent enzymes involved in the biosynthesis of *N*-nucleoside antibiotics; and (3) peptide-bond formation by the amide ligases PumK and PumM, proteins that show similarity to ATP-dependent carboxylate-amine ligases containing an ATP-grasp domain (Figure 4).

The crucial role in the PUM biosynthetic pathway is played by PumJ (Figure 4). PumJ appears to be a pseudouridine synthase that generates free pseudouridine [37], in contrast to

all previously identified pseudouridine synthases, which generate tRNA-linked pseudouridine [15]. Knocking out *pumJ* abolishes PUM production and results in accumulation of GAA. Feeding free pseudouridine to the *pumJ* knockout mutant rescues PUM production, indicating that free pseudouridine is efficiently taken up and converted into PUM [37].

We hypothesize that PumJ generates free pseudouridine by catalyzing *N*- to *C*-isomerization of an activated uridine derivative. Specifically, we hypothesize that the adenylate kinase PumH first generates a phosphorylated uridine derivative, that the pseudouridine synthase PumJ then catalyzes *N*- to *C*-isomerization to yield a phosphorylated pseudouridine derivative, and that the hydrolase PumD finally releases pseudouridine.

In view of its crucial role in the pathway, PumJ could be a diagnostic for biosynthetic gene clusters for other pseudouridine-containing *C*-nucleoside antibiotics [37]. PumJ is distantly related to the TruD family of pseudouridine synthases, which carry out the *C*-isomerization of uracil-13 in tRNAs [10]. From a phylogenetic analysis of ~7,000 TruD homologues across bacterial genomes, PumJ and its homologs clade in a separate branch, clearly divergent from the main TruD branch (Figure 5). Interestingly, the PumJ homologs all are encoded by genes embedded in likely biosynthetic gene clusters. These likely biosynthetic gene clusters can be subdivided into three main groups: (1) a group, found mainly in *Streptomyces* spp., that comprises near-identical copies of the *pum* biosynthetic gene cluster, with occasional additional genes or gene variations; (2) a group, found in taxa spanning the *Actinobacteria* and the γ -*Proteobacteria*, that usually shares a core set of enzymes in addition to PumJ, and frequently also contains homologs of the PumK carboxylate-amine ligase, the PumM peptide-bond synthetase, and the PumG aminotransferase; and (3) a group, found mainly in myxobacteria, that encodes a PumJ homolog and includes genes encoding homologs of the α and β subunits of sarcosine oxidase and a homolog of adenylate cyclase.

Concluding remarks

Serendipity--and perhaps poetic inspiration along the Viale dei Cipressi--played roles in the discovery of PUM.

There were two key elements of serendipity. First, the RNAP assay conditions in our screen-- with near-saturating ATP, CTP, and GTP concentrations and limiting UTP concentrations--turned out to have been especially sensitive for detection of an RNAP inhibitor, such as PUM, that functions as a competitive inhibitor of UTP [26]. Second, the PUM producer strains identified in our screen, *Streptomyces* spp. ID38626 and ID38640, turned out to co-produce lydicamycin, a natural product having high antibacterial activity against *S. aureus*, and therefore were included in our primary screen of extracts having antibacterial activity against *S. aureus*, and passed our secondary screen for antibacterial activity against rifampicin-resistant *S. aureus*, despite the fact that PUM itself does not have high antibacterial activity against *S. aureus* under standard CLSI conditions [26]. It should be noted, however, that all screening programs have one or more elements of serendipity, and it is important to appreciate early on the structural originality of a newly discovered compound. When sufficient amounts of purified compound are available, appropriate tests

can be performed, allowing detailed characterization of the desired bioactivity, as with PUM, or leading to unexpected other bioactivities [e.g., 18]. Having the compounds in hand can thus allow additional testing as new targets, concepts, and ideas develop.

Despite the fact that PUM was discovered and reported only very recently, three lines of evidence suggest that PUM may be a relatively widely distributed microbial secondary metabolite. First, as summarized in the section on Discovery, two different microbial strains producing PUM were identified from a screening just 3000 extracts (hit rate = 0.7%; Figure 1). Second, as summarized in the section on Biosynthesis, near-identical copies of the *pum* biosynthetic gene cluster are found in at least seven sequenced *Streptomyces* spp. [37]. Third, PUM appears to have been independently isolated--but assigned an incorrect structure--by another laboratory. The *Streptomyces albus* DSM 40763 natural product “strepturidin,” which was reported by Süssmuth and co-workers in 2014 and assigned a structure different from that of PUM [33], has a molecular formula, mass-spectrometric fragmentation profiles, and ¹H- and 2D-NMR spectra identical to those of PUM [26], and therefore it appears highly likely that “strepturidin” in fact is PUM [S.M., unpublished data]. According to the DSMZ webpage for *Streptomyces albus* DSM 40763, the strain was isolated from “mouldy hay” at an unspecified location at an unspecified date before 1990--presumably in the 1950s or 1960s--by Philip H. Gregory and deposited in the culture collection of the Waksman Institute of Microbiology at Rutgers University [“IMRU”; <https://www.dsmz.de/catalogues/details/culture/DSM-40763.html>]. Ironically, thus, a strain that produces PUM appears to have been first deposited in the culture collection of the same unit of the same university that, decades later, housed part of the research team that discovered and characterized PUM from the strain collected on the Viale dei Cipressi.

The discovery and characterization of PUM underscores the fact that RNAP is a favored target for antibacterial microbial natural products. In addition to rifamycins, lipiarmycins, and PUM, at least a dozen other classes of antibacterial microbial natural products function as RNAP inhibitors, interacting with at least seven different, largely non-overlapping, binding sites on RNAP [Figure 2; 16, 23, 24, 39 and references therein]. We believe it will be possible to discover yet more novel classes of antibacterial RNAP inhibitors from microbial sources, for example, by employing assays expressly designed to find additional nucleoside-analog RNAP inhibitors [9].

The characterization of the *pum* cluster with the encoded pseudouridine synthase PumJ has revealed an additional key enzyme in specialized metabolism and hinted at the possibility of discovering novel *C*-nucleoside antibiotics structurally and possibly functionally unrelated to PUM. This offers the exciting possibility of targeted discovery of novel *C*-nucleoside antibiotics through targeted genome mining. A recent example highlights the fact that there are *many* microbial natural products to be discovered, even in highly exploited taxa such as *Bacillus* [21].

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Fig. 1. PUM producer strain *Streptomyces* sp. ID38640 (top left), structure of PUM (top right) and the screening program that led to PUM discovery (bottom). Y and N denote selection and non-selection criteria, respectively

Fig. 2.

Location of PUM target (blue) in three-dimensional structure of bacterial RNAP [29] (gray; β' non-conserved region and σ omitted for clarity). Black circle denotes active-center region; violet sphere active-center Mg^{2+} (I). The location of the Rif (red), lipiarmycins (cyan), streptolydigin (yellow), and myxopyronins targets (magenta) are shown for comparison. Figure adapted from reference [26]

Fig. 3.

Structural basis of transcription inhibition by PUM [26]. Structures of *T. thermophilus* transcription initiation complex containing PUM (RPO-GpA-PUM). Left, stereodiagram of interactions. Green, PUM carbon atoms; pink, RNA carbon atoms; gray, RNAP carbon atoms; red, blue, yellow, and orange, oxygen, nitrogen, sulfur, and phosphorous atoms; dashed lines, H-bonds. Right, summary of interactions. Red dashed lines, H-bonds; blue arcs, van der Waals interactions. Residues numbered as in *T. thermophilus* RNAP and, in parentheses, *E. coli* RNAP. Figure from reference [26]

Fig. 4.

Gene cluster and proposed pathway for PUM biosynthesis [37]. Enzymes in brackets have not yet been targeted by knockouts of the corresponding genes. X and Y can either be H, phosphate, or diphosphate. The pathway assigns biosynthetic roles for nine proteins: PumH and PumD (orange), and PumJ (red), involved in formation of pseudouridine (PU); PumI and PumG (light green), involved in formation of 5'-amino pseudouridine (APU); PumK and PumM (light brown), involved in the formation of the two amide bonds; PumN (dark green), involved in the formation of guanidineacetic acid (GAA); and PumE (blue), involved in N-hydroxylation. The other genes from the *pum* cluster are predicted to be involved in transport (*pumB* and *pumL*, purple), regulation (*pumC*, dark brown) or are of unassigned function (*pumA*, *pumF* and *pumO*, grey). Figure adapted from reference [37]

Fig. 5.
Phylogenomic analysis of TruD homologs, highlighting the clade comprising the three groups of sequences most related to PumJ, colored red, green and blue. See text and reference [37] for details. The end products of TruD and PumJ are also illustrated