YbeA is the $m^3\Psi$ methyltransferase RlmH that targets nucleotide 1915 in 23S rRNA

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ABSTRACT

Pseudouridines in the stable RNAs of Bacteria are seldom subjected to further modification. There are 11 pseudouridine (Ψ) sites in *Escherichia coli* rRNA, and further modification is found only at Ψ 1915 in 23S rRNA, where the N-3 position of the base becomes methylated. Here, we report the identity of the *E. coli* methyltransferase that specifically catalyzes methyl group addition to form m³ Ψ 1915. Analyses of *E. coli* rRNAs using MALDI mass spectrometry showed that inactivation of the *ybeA* gene leads to loss of methylation at nucleotide Ψ 1915. Methylation is restored by complementing the knockout strain with a plasmid-encoded copy of *ybeA*. Homologs of the *ybeA* gene, and thus presumably the ensuing methylation at nucleotide m³ Ψ 1915, are present in most bacterial lineages but are essentially absent in the Archaea and Eukaryota. Loss of *ybeA* function in *E. coli* causes a slight slowing of the growth rate. Phylogenetically, *ybeA* and its homologs are grouped with other putative S-adenosylmethionine-dependent, SPOUT methyltransferase genes in the Cluster of Orthologous Genes COG1576; *ybeA* is the first member to be functionally characterized. The YbeA methyltransferase is active as a homodimer and docks comfortably into the ribosomal A site without encroaching into the P site. YbeA makes extensive interface contacts with both the 30S and 50S subunits to align its active site cofactor adjacent to nucleotide Ψ 1915. Methylation by YbeA (redesignated RlmH for *r*RNA *l*arge subunit *m*ethyltransferase *H*) possibly functions as a stamp of approval signifying that the 50S subunit has engaged in translational initiation.

Keywords: RNA mass spectrometry; helix 69; pseudouridine methylation; RluD; ribosomal subunit interface

INTRODUCTION

The pivotal roles carried out by rRNAs during protein synthesis on the ribosome (Noller 2005) are supported by a range of nucleotide modifications that are added posttranscriptionally (Grosjean 2005). In *Escherichia coli* there are 11 modified nucleotides in 16S rRNA and 25 in 23S rRNA, and these modifications consist almost exclusively of pseudouridines and methylations on bases and ribose (Rozenski et al. 1999; Ofengand and Del Campo 2004; Andersen and Douthwaite 2006). In principle, methylation and pseudouridylation have opposing effects on the structure of the rRNA and its interactions with other ribosomal components. Base methylation creates a hydrophobic patch that improves the potential for base stacking, but removes an electrostatic interaction site and might sterically hinder the close approach of other components or ligands; ribose methylation can have similar effects while at the same time stabilizing the phosphodiester backbone. Pseudouridines, on the other hand, gain an extra site for electrostatic interaction after the N1-nitrogen, which was originally an integral part of the uridine glycosidic bond, swaps its role with the C5-carbon and becomes free to make a hydrogen bond. Notably, the N3-nitrogen remains in the same position in both uridine and pseudouridine (Fig. 1). Methylation and pseudouridylation rarely happen together at the same nucleotide, and in Escherichia coli RNAs there

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Abbreviations: AdoMet, S-adenosylmethionine; COG, Cluster of Orthologous Genes; MALDI, matrix assisted laser desorption/ionization; MS, mass spectrometry; m³U, 3-methyluridine; m³ Ψ , 3-methylpseudouridine; *m*/*z*, mass (in daltons) to charge ratio; RFM, Rossmann-fold methyltransferase; *rlmH*, rRNA large subunit methyltransferase gene *H* (synonym, *ybeA*); SPOUT, SpoU and TrmD methyltransferases.

Article published online ahead of print. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.1198108.



FIGURE 1. Chemical structures of 3-methylpseudoridine $(m^3\Psi)$, 3-methyluridine (m^3U) , and 1-methylguanosine (m^1G) .

is but a single example: of the 11 pseudouridines in *E. coli* rRNA, only position 1915 is subjected to further modification, and this occurs via addition of a methyl group to the base N-3 position to form $m^3\Psi$ 1915 (Kowalak et al. 1996).

The mechanisms by which rRNAs are post-transcriptionally modified in Bacteria are fundamentally different from those operating in Archaea and Eukaryota. Pseudouridylations and 2'-O-methylations make up the bulk of eukaryal rRNA modifications, and most are added by modification enzymes that are not specific by themselves but need to be guided to target nucleotides by complementary sequences in snoRNAs (Lafontaine and Tollervey 1998; Kiss 2001). Comparable mechanisms have more recently been found in the Archaea (Tran et al. 2004). In contrast, Bacteria lack such guide RNAs and, consequently, each bacterial rRNA methylation requires its own specific enzyme (Andersen and Douthwaite 2006; Sergiev et al. 2007). The requirements for synthesis of pseudouridines in Bacteria are (almost) as stringent. For instance, E. coli pseudouridine synthases are usually specific for one uridine or a limited set of uridines that occupy a similar structural context (Ofengand and Del Campo 2004); and this latter type is exemplified by RluD, which converts 23S rRNA nucleotides U1911, U1915 and U1917 to pseudouridines.

Modifications in rRNA generally cluster within regions of the ribosome that perform key functions (Brimacombe et al. 1993; Decatur and Fournier 2002; Ofengand and Del Campo 2004) and ¥1911, ¥1915, and ¥1917 are no exception. These nucleotides are located at the end of hairpin 69, where they are exposed to the solvent at the 50S subunit interface (Ban et al. 2000; Harms et al. 2001); hairpin 69 interacts with helix 44 in 16S rRNA to form interbridge B2a during association with the 30S subunit (Yusupov et al. 2001; Schuwirth et al. 2005; Korostelev et al. 2006; Selmer et al. 2006). Various studies have demonstrated the functional importance of hairpin 69 (Ali et al. 2006) and of individual nucleotides within this hairpin (O'Connor and Dahlberg 1995; Liiv et al. 2005; Hirabayashi et al. 2006; O'Connor 2007) and the modifications at these nucleotides (Gutgsell et al. 2001; Johansen et al. 2006; Ejby et al. 2007). However, the exact role of $m^3\Psi 1915$ remains to be established, as does the identity of the methyltransferase that modifies this nucleotide.

It was speculated recently that the putative methyltransferase YbeA might be responsible for the $m^3\Psi$ 1915 methylation (Tkaczuk et al. 2007). Bioinformatics analyses showed that YbeA belongs to the Cluster of Orthologous Genes COG1576, within the SPOUT superfamily of AdoMetdependent RNA methyltransferases (Tkaczuk et al. 2007). No member of COG1576 had yet been functionally characterized, although their closest relatives in the SPOUT superfamily are the TrmD-type m¹G methyltransferases (COG0336) and, slightly more distant, the Trm10p-type m¹G methyltransferases (COG2419). The SPOUT superfamily also includes RlmE-type m³U methyltransferases (COG1385). The $m^{3}U$ and $m^{1}G$ methyltransferases modify equivalent nitrogen atoms in uracil and the pyrimidine ring of guanine (Fig. 1). As none of the m¹G RNA methyltransferases remain undiscovered in E. coli, YbeA was viewed as a reasonable candidate for the $m^3\Psi 1915$ methylation.

In this study, we used MALDI mass spectrometry to compare the rRNA methylation pattern in a *ybeA*-knockout strain to that in wild-type *E. coli* cells. A recombinant version of the enzyme was expressed in the knockout strain to determine whether the original wild-type rRNA methylation pattern could be restored. These experiments show unequivocally that YbeA catalyzes the $m^3\Psi1915$ methylation. Docking simulations indicate that YbeA binds as a homodimer to the ribosomal A site at the interface between the 30S and 50S subunits and recognizes its substrate in a manner that has not previously been seen among the rRNA methyltransferases.

RESULTS AND DISCUSSION

In silico identification of YbeA as an $m^3\Psi$ RNA methyltransferase candidate

Methylation at the N3 of pseudouridine has been observed only at nucleotide $m^3\Psi$ 1915 in 23S rRNA, and at present this modification appears to be unique. Thus, with no enzyme precedent for $m^3\Psi$ methylation, we looked at enzyme groups that are phylogenetically close to the known m³U RNA methyltransferases. These latter enzymes are exemplified by RlmE, which is responsible for m³U1498 modification in 16S rRNA (Basturea et al. 2006) and belongs to the Cluster of Orthologous Genes family COG1385 within the SPOUT superfamily of AdoMetdependent methyltransferases (Tkaczuk et al. 2007). Sequence analyses of SPOUT proteins identified two major lineages: one groups several families of known 2'-Omethyltransferases together with the RlmE-type m³U methyltransferases; the second lineage contains TrmD-type and Trm10p-type m¹G methyltransferases and the bacterial cluster COG1576, in which YbeA is the sole E. coli member (Tkaczuk et al. 2007).

Considering that no m¹G RNA methyltransferases remained to be discovered in *E. coli* and that the uridine/pseudouridine

N3-atom can be regarded as being in a structural context that is chemically similar to the guanosine N1-atom (Fig. 1), we deemed YbeA and its COG1576 homologs to be worthy candidates for the task of $m^3\Psi$ methylation. The *ybeA*-knockout was obtained from the Keio collection (Baba et al. 2006). In strain K-12 BW25113 $\Delta ybeA$, the putative methyltransferase gene has been replaced with a kanamycin resistance cassette. We confirmed by PCR analysis of the relevant chromosome region that the *ybeA* gene sequence had been displaced.

Detection of a minor growth defect in *ybeA*-knockout cells

The growth rate of the *ybeA*-knockout was measured in rich medium at 37°C to determine whether it differed from that of wild-type or *xylA*-knockout cells. Triplicate measurements in independent cultures under the same conditions showed that the doubling time of the wild-type cells is 34 ± 2 min, *xylA*-knockout cells double in 35 ± 1 min, and *ybeA*-knockout cells take 37 ± 2 min. From these simple growth experiments, we could not with any certainty say whether the doubling times were significantly different.

Growing cells in competition with each other for 50–100 generations in the same culture normally reveals small differences in their doubling times. The wild-type cells were clearly fitter and began to outgrow the *ybeA*-knockout already after the first growth cycle of 10 generations (Fig. 2). To test whether the reduced fitness of *ybeA* cells might merely be due to the cost of expressing the kanamycin resistance gene, growth competition was repeated against *xylA*-knockout cells. The *xylA* cells have the same resistance cassette as the *ybeA*-knockout, and the lack of ability to metab-



FIGURE 2. Growth of the *ybeA*-knockout ($\Delta ybeA$) in competition against wild-type cells or against *xylA*-knockout cells ($\Delta xylA$). Equal numbers of viable cells were present at the start of the first growth cycle; each growth cycle represents approximately 10 cell divisions. The values for the relative proportions of cells are averages of duplicate measurements.

Making a straightforward extrapolation from the curves in Figure 2 (i.e., taking no account of potential differences in lag phase times or viability), the growth rate of the *ybeA*knockout is 5.4% slower than wild-type cells and 2.5% slower than *xylA* cells. If the doubling time of the wild type is set to 34.0 min, the doubling times of the *xylA*- and *ybeA*knockouts would be 34.7 and 35.5 min, respectively. Thus, the presence of the kanamycin resistance gene does indeed have a biological cost, while loss of *ybeA* function makes its own individual contribution toward slowing growth.

Identification of the YbeA methylation site

The N3-position of uridine and pseudouridine (Fig. 1) is a hydrogen bond donor in canonical Watson–Crick basepairing interactions, and thus substitution of the N3proton with a methyl group eliminates its availability for base pairing. The loss of base-pairing ability can be exploited for rapid detection of $m^3\Psi$ (and m^3U) using primer extension analysis, where the progress of reverse transcriptase is impeded by these modifications. We also considered the proviso that the sequence in question at the apex of helix 69 contains three Ψ A dinucleotides, and these are linked by notoriously labile phosphodiester bonds. As reverse transcriptase is stopped equally well by backbone cleavage as by $m^3\Psi$ methylation, we initially opted for an analytical method that could unequivocally establish the presence or absence of a methyl group.

MALDI mass spectrometry was applied to determine any difference between methylation patterns in the rRNAs from the wild type and the ybeA-knockout. The MALDI-MS approach used here functions optimally on RNA fragments with lengths in the tri- to decanucleotide range, where masses can be accurately measured to within 0.2 Da (Andersen et al. 2004). Thus, molecules as large as the 16S and 23S rRNAs need to be fragmented to yield specific oligonucleotides of suitable size for analysis. In the present study, a 58-nucleotide (nt) sequence from G1891 to G1948 in 23S rRNA was isolated by hybridization to a complementary oligodeoxynucleotide (Fig. 3), and was then fragmented further by RNase digestion. After RNase A digestion, Ψ 1915 ends up in an dinucleotide fragment $(A\Psi)$ whereas, following RNase T1 digestion, Ψ 1915 is in an 11-nt fragment Ψ 1911–G1921 that has a unique m/z of 3519.5 (unmethylated) or m/z of 3533.5 (methylated). In principle, these large oligonucleotides can be identified directly following RNase T1 digestion of total rRNA without prior purification (Madsen et al. 2003). However, isolation of a discrete rRNA region (with the 58-mer) markedly improves the signal-to-noise ratio and gives unambiguous spectra.



FIGURE 3. MALDI-MS analyses of E. coli 23S rRNA around helix 69. The 23S rRNA fragment from nucleotides 1891 to 1948 was isolated from E. coli YbeA⁺ and YbeA⁻ strains and was then digested with RNase T1. (A) MS spectrum of RNA from the YbeA⁺ strain. The empirically measured masses for RNase T1 digestion products of trinucleotides and larger are shown above the peaks and match well with the theoretical values (the theoretical monoisotopic masses are given in the box). In this spectrum, RNase T1 digestion gave rise mainly to products with cyclic phosphates (>p) and a minor amount of linear phosphate products; the linear products, with 18 Da greater masses, can seen immediately to the right of the main peaks. Adducts are occasionally evident: e.g., the m/z 1004.1 peak is UAG>p with a sodium ion (+23 Da). (B) Hairpin 69 region showing the sequence from C1892 to G1948 isolated for MS analysis. The location of the hairpin 69 region is boxed in the outline of the 23S rRNA domain IV secondary structure (Noller 2005). (C) Enlargement of the spectral region above m/z 3500 from the YbeA⁺ rRNA. The 11-nt fragment containing m³ Ψ 1915 runs at m/z 3533.5, with a minor linear phosphate component at m/z 3551.5. These peaks are preceded by a small, but reproducible, top at m/z 3519.5, which is the unmethylated fragment (the linear phosphate component is at m/z 3537.5 and hidden in the ¹³C isotope tail of the more abundant m/z 3533.5 peak). The m/z 3519.5 peak indicates that the in vivo methylation by YbeA was incomplete. (D) The same spectral region from the YbeA⁻ strain shows that the RNA is entirely in the unmethylated form at m/z3519.5 (with the minor linear phosphate component visible at m/z3537.5). None of the spectral data have been smoothed.

RNase T1 digestion of the isolated 23S rRNA fragment from the wild-type strain produced the m/z 3533.5 peak, which has a cyclic phosphate, and a small amount of the linear fragment at m/z 3551.5 (Fig. 3C). Both these peaks are consistent with the Ψ 1911 to G1921 sequence containing one methyl group. However, a minor quantity of this 11-mer oligonucleotide flew at m/z 3519.5, indicating that methylation in wild-type cells was less than stoichiometric. In the *ybeA*-knockout strain, the 11-mer oligonucleotide flew exclusively at m/z 3519.5 (with a small amount of the linear fragment at m/z 3537.5), showing that in the absence of YbeA there was no methylation in this RNA sequence (Fig. 3D). The m/z 3533.5 peak was subjected to further analysis by tandem MS (data not shown), and the site of YbeA methylation was localized to nucleotide 1915.

Stoichiometry of methylation at Ψ 1915 by YbeA

The MALDI-MS analyses on the wild-type and ybeAknockout rRNAs were confirmed by reverse transcriptase primer extension followed by gel analysis, which gives a clear visual illustration of the methylation site (Fig. 4). There is a small amount of reverse transcriptase readthrough past $m^{3}\Psi$ 1915 (which is stopped at the next modification site, m²G1835), and this is consistent with the minor proportion of unmethylated rRNA seen with MS for the wild-type rRNA (Fig. 3C). The loss of methylation in the ybeA-knockout strain was shown to be rescued by addition of a functional, recombinant version of ybeA on a plasmid (Fig. 4). It could be rationalized that Ψ and $m^{3}\Psi$ could rotate around the glycosidic bond (Fig. 1) and present a syn conformation that might facilitate reverse transcriptase read-through. Even so, there are two reasons why we do not believe this happens to any appreciable degree: First, the proportion of unmethylated Ψ 1915 in YbeA⁺ cells estimated by MS and primer extension matches fairly well; second, semi-empirical ab initio calculations using AM1, PM3, and MNDO Hamiltonians suggest that $m^{3}\Psi$ is unlikely to attain a conformation capable of productive base pairing. In the latter case, no account could be taken for possible water-backbone bridges in the RNA (Sumita et al. 2005) or for interactions within the reverse transcriptase active site. However, for lack of contradictory evidence, we presently equate the amount of reverse transcriptase read-through with the proportion of unmethylated Ψ 1915.

Significant read-through past Ψ 1915 was observed in the complemented strain (Fig. 4) even though it contained much larger quantities of YbeA enzyme than wild-type cells. We have previously observed for other 23S rRNA methyltransferases, which also fail to completely methylate all the rRNA molecules under normal growth conditions, that stoichiometric rRNA methylation can be achieved if the methyltransferase is expressed in larger quantities. In the case of YbeA, the proportion of 23S rRNA without



FIGURE 4. Gel autoradiograms of primer extension showing the hairpin 69 region of the rRNAs. (Lane 1) rRNA was isolated from wild-type *E. coli* (YbeA⁺, as in Fig. 3C); (lane 2) rRNA from the *ybeA*-knockout (YbeA⁻, as in Fig. 3D); (lane 3) rRNA from the *ybeA*-knockout strain complemented with the plasmid-encoded *ybeA* gene. The transcription stop caused by the N3-methylation at Ψ 1915 and the read-through bands are arrowed. Our reasoning (see text) is that reverse transcriptase cannot transcribe past m³ Ψ , and thus the read-through band is indicative of unmethylated RNA. The rRNA template for the dideoxy-sequencing reactions (lanes *C*,*U*,*A*,*G*) was from the *ybeA*-knockout.

 $m^{3}\Psi1915$ methylation is seen under logarithmic growth and remains constant even when the intracellular concentration of YbeA is increased (Fig. 4). The explanation that fits best with this observation and the enzyme-target docking (described below) is that the unmethylated faction reflects recently synthesized 23S rRNA molecules that have not yet reached a later stage of assembly where they are recognized by YbeA as suitable substrates.

Sequence analysis of YbeA orthologs

Numerous common motifs are evident in the SPOUT superfamily; some of these motifs are involved in the binding of AdoMet, while others are unique to COG1576 and are probably related to the specific function of YbeA (Fig. 5). YbeA orthologs are present in most major bacterial lineages, and the phylogenetic tree derived from the alignment matches well with the phylogeny of the host organisms (Tkaczuk et al. 2007). In addition to Bacteria, orthologs of *ybeA* are evident in the chloroplasts of numerous plant species; however, these organelles are regarded as being of bacterial origin, and there is no evidence of $m^3\Psi$ methylation within the cytoplasmic protein synthesis ma-

chinery of Eukaryota. This modification is also essentially absent in the Archaea; however, solitary *ybeA* orthologs have been found within the *Euryarchaeota*, and the significance of this observation remains unclear. On balance, we effectively regard YbeA as being a bacterial enzyme.

None of the organisms mentioned above possesses more than a single COG1576 member, and there is no evidence of species-specific duplication or horizontal gene transfer. Taken together, this would suggest that all YbeA orthologs have the same function. The current extent of the databases shows that every genome that contains a *ybeA* ortholog also contains an ortholog of *rluD*. However, the converse is not true, and *rluD* is found in the genomes of Actinobacteria, Cyanobacteria, and Spirochaetes despite their lack of ybeA. This indicates that Ψ formation at nucleotide 1915 is independent of YbeA methylation, whereas the action YbeA might require the prior formation of a Ψ substrate. As the preferred substrate for RluD is the assembled 50S subunit (Leppik et al. 2007; Vaidyanathan et al. 2007), YbeA would methylate Ψ 1915 at the same or a later stage of ribosome maturation. Consistent with this, the accompanying article (Ero et al. 2008) shows that YbeA activity requires prior conversion of nucleotide 1915 to pseudouridine and for Ψ 1915 to be presented within the context of the 70S ribosome.

A mechanistic model for YbeA activity

To date, four crystal structures of COG1576 members have been determined and deposited in the Protein Data Bank: YbeA from *E. coli* (PDB file 1ns5) (J. Benach, J. Shen, B. Rost, R. Xiao, T. Acton, G. Montelione, and J.F. Hunt, unpubl.), YydA from *Bacillus subtilis* (PDB 1t00) (A.P. Kuzin, W. Edstrom, S.M. Vorobiev, R. Shastry, L.-C. Ma, R. Xiao, T. Acton, G. T. Montelione, L. Tong, and J.F. Hunt, unpubl.), TM0844 from *Thermotoga maritima* (PDB 106d) (Badger et al. 2005), and SAV0024/SA0023 from *Staphylococcus aureus* (PDB 1vh0) (Badger et al. 2005). The proteins display an unusual knotted structure. All SPOUT proteins studied so far crystallize as homodimers, and this is probably the oligomeric state of YbeA in solution (Mallam and Jackson 2007). Details about these proteins are otherwise sparse.

As mentioned above, YbeA and its orthologs are closest relatives of m^1G methyltransferases from the TrmD (COG0336) family, and comparisons between crystal structures give DALI Z-scores >11 (Tkaczuk et al. 2007). Thus, findings from structural, biochemical, and mutagenesis studies on TrmD-AdoMet (Ahn et al. 2003; Elkins et al. 2003) can, with caution, be extrapolated to YbeA. Superimposition of YbeA onto the TrmD-AdoMet homodimer suggests that there are near-identical AdoMet-binding pockets in these two proteins. Conserved YbeA residues and a surface of negative charge are evident at the putative AdoMet site, and this site is located adjacent to a surface of positive charge that could interact with the backbone of the

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15603799_DM1022_Dagm1	MANOT TAWOTRAND	NUM TICE ENHOPIN	F	FET TIST DACKPCKNA		TERTIFORE	AMA ACCK-	
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15599199-BA4004-Beaser	MPT PT TAWO CPMP	PWVERCHOUW	IDART.S	T FT MAT PT NTPCKNA		WARTTROBE	AMEN DUODC-	
15640967-VC0951-Vibcho	METOTTAVC	KWVFFCFOFVPP	EDUDMD	LEIMSTTACERCKNA		TARTIONE	AMIA AVERC-	
16122818-YPO2605-Yernes	METOLWAVETEMP	DWVOTEFTISYTRE	FPKDMP	FELADTPACKRCKNA		TKRTLENDE	T.M. AVCKN-	
17546914-RSC2195-Ralsol	MOLT TWAWERKMP	BWTEDGEADVAKE	MPDELR	TELRISTKPEORSCSP		TANTIMOTIA	ARTEAAL PKC-	
15793423-NMA0418-Neimen	MNITTYTAVETKMP	RWVDEAVABYAK	FGRDVA	YALKATKPENRGACV		NAAOGMAADE	RINEATPOG-	
19703798-FN0463-Fusnuc	MNINITCICKIKD	KYTNDETABESK	MTSEVS	LNITELKEYNKED		NTNISTER	EUKOISKS-	
15674116-L185851-Laclac	MATKLVVVCKLKE	KYLKDELADWVK	MGTMLP	LEIIDLADENIPDNA	SEK	EAEALKKROC	KUSRIOAG-	
15675944-SPY2215-Strpvo	MEVELICVCKLKE	RYLKDEISEMOK	LSRFCO	FEMIFLTDERTPDKA	SFA	ONOLIMSKEA	DR HKKIGER-	
15902041-SP2238-Strone	MERKVVTVGKLIKE	KYLKDELABYSKI	ISRFAK	FEMIPLSDEKTPDKA	SES	ENOKILEIDE	OR SKIADR-	
15896772-CAC3536-Cloace	MNITLITVGKLKE	KYLKDAVNEYAK	LOKYCK	LNIIBLODEKTPEKA	SLK	EEKLIKENEG	KUSSIKDN-	
15925729-SA0023-Staaur	MKITILAVGKIKE	KYWKOALAEYEKI	LGPYTK	IDIIEVPDEKAPENM	SDK	EIEOVKEKEG	ORTHAKIKPO-	
16799398-LIN0321-Lisinn	MNIOIVTVCKLKE	KYLVOCIACYLK	LSAYAK	VSIVEVPDEKAPEVL	SDA	EMKOVKDKEG	VRILAKIPDD-	
16081075-BS YYDA-Bacsub	MNINIVTICKLKE	KYLKOCIEEYTKE	LSAYAK	IDIIELPDEKAPENL	SDQ	DMKIIKDKEG	ORTISKISPD-	
15616569-BH4007-Bachal	MNISIISVGKLKE	KYLKOCIAEYTKI	LGAYAK	IELIEVPDEKAPEQL	SDT	EMEHVKOKEG	ERILAKLHPD-	
15805646-DR0619-Deirad	MRLHLITVGEPKL	AYARSGWDEYEKI	LRRYHK	VQVSRVSG		KTQQADS	EAIIIIKAAGK	
15643607-TM0844-Themar	ARVRIAVICKL-D	GFIKEGIKHYEKH	LRRFCK	PEVLEIKRVHRG		SIEEIVRKET	EDLTNRILPG-	
15828725-MYPU_2540-Mycpul	MKINIISVGTLSK	EF-QVIFDDMIK	(INFYSN	VNLIKIKEFKSN	1	NKDLIIKNET	MATTEKIPKN-	
13357738-UU180-Ureure	MMIKIISVGKIKQ	KAFVDLINDYLK	INHYLK	CQEIVVSDEPEPVQI	SNK	SLEQIKSKEA	SKUFKNINQN-	
16127663-CC3433-Cauvib	MKHTILTVGKLGR	MVEAQLALDYAS	ATAS-GRALALGP	VDILEVEAR P		GKAABA	EVLRPHLEG	
13473414-MLL4004-Meslot	MKISVHAVGRMKA	GPERELADRYFE	FAKSGP	AVGLEFAGITEIAEG	RSQ	SAIERORDEG	SRLQAQLQPG-	
17986495-BMEI0211-Brumel	MRVSVFAVGRMKS	GPERELVERYFD	FAKAGP	PLGLEFAGVSEIPES	RGQ	TAQLRKAEEA	DREHEALDNAK	SGGAKSGGTS
15890025-AGC5037-Agrtum	MRISIFAVGRIKS	GPEKDLAARMIE	LAKTGP	AIGLEFSRVIEVGES	RAS	NAETRKREDA	AMLEKHLADG-	
15966917-SMC03781-Sinmel	MRIGLIFAVGRIKA	GPEKDLAGRYLD	FAKAGP	AVGLELARVVETAES	RAA	NAETRKREDA	GQLEKALADG-	
1p9p.TrmD	WWGIISLF	PEMFRAITDY	GVTGRAVKNGLLS	IQSWSPRDFTHDRHR	TVDDRPYGGG	PGMLMMVQPL	RDAIHAAKAAA	.G
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16128619-Ybea-Escol	70 . II G	80		110 .C. EC .	120 S		140 .R .	150 . <u>M.R.</u>
16128619-YbeA-Esccol	70 .L G NRIVTLDIPG	80 KPWDTPOLAAELI	0 100	110 .C. EC . IGCPECISPACKAAA	120 S -EQSWSLSAL -EQSWSLSAL	130 . L TLPHPTVRVL TTPHPTVRVL	140 .R . VAESLYRAWSI	150 .Y.R. TTNHPYHRE-
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-HT0033-Haeinf	70 .L G NRIVTLDIPG NRIVTLDIPG GKVVTLDIPG	80 KPWDTPOLAAEL KPWDTPOLANEL	0 100	110 .C. EC. . ICCPECISPACKAAA ICCPECISPACKAAA ICCPECISPECKAAA	120 S -EQSWSLSAL -EQSWSLSAL -EQSWSLSAL	130 .I TLPHPIVRVL TLPHPIVRVL TLPHPIVRVV	140 VADSLYRAWST VADSLYRAWST VADSLYRAWST	150 .Y.R. TTNHPYHRE- TTNHPYHRE- TTNHPYHRE-
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-H10033-Haeinf 15603788-DM1923-Pasmul	70 .L G NRIVTIDIPG GKVVTIDIPG GKVVTIDIPG	80 KPWDTPOLAAEL KPWDTPOLANEL KPWTTPOLAEOL KPWTTPOLAEOL	0 100 ERWKLDC-RDVSLL ERWKQDC-RDVSLL EAWKNDC-RDVCLL	110 .C. EC	120 S -EQSWSLSAL -EQSWSLSAL -EQSWSLSPL -EQSWSLSPL	130 .I TIPHPIVRVI TIPHPIVRVI TIPHPIVRVV TIPHPIVRVV	140 VABSLYRAWST VABSLYRAWST VABSLYRAWST VABSLYRAWST VABSLYRAWST	150 .Y.R. TINHPYHRE- TINHPYHRE- TINHPYHRE- TMNHPYHRE-
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-H10033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xv1fas	70 NRIVTLDIPG NRIVTLDIPG SRIVTLDIPG SRIVTLDIPG PLAVTLDVKG	80 KPWDTPOLAAE KPWDTPOLAAE KPWTTPOLAEO KPWTTFOLAEO KPWTTFOLAEO KSINSFOLAKRMI	0 100 I. I. I. I. I. RWKLDG-RDVSLL RWKQDG-RDVSLL IAWKNDG-RDVCLI TWWKNDG-RDLSLL EWWGLC-RNLVDL	110 .C. EC ICCPECISPACKAAA ICCPECISPACKAAA ICCPECISAECKAAA ICCPECISAECKAAA ICCPECISOEVINIS	120 S -EQSWSLSAL -EQSWSLSAL -EQSWSLSPL -EQSWSLSPL -EQSWSLSPL -NERWSLGPL	130 .L.II TLPHPLVRVL TLPHPLVRVL TLPHPLVRVV TLPHPLVRVV TLPHPLVRVV	140 VAESLYRAWST VAESLYRAWST VAESLYRAWST VAESLYRAWST VAESLYRAWST VVEOLYRAATT	150 .Y.R. TTNHPYHRE- TTNHPYHRE- TTNHPYHRE- TMNHPYHRE- LTNHPYHREK
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-HI0033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xylfas 15599199-PA4004-Pseaer	70 .I G NRIVTLDIPG RRIVTLDIPG SRIVTLDIPG PYAVTLDVKG ERVVTLEVEG	80 KPWDTPOLANEU KPWDTPOLANEU KPWTTPOLAEU KSUNSEOLATOU KSUNSEOLAKEM RPWSTEOLAREU	00 100 I ERWKLDG-RDVSLI ERWKDG-RDVSLI IAWKNDG-RDVCLI TVWRNDG-RDLSLI HWRGLG-RNLVGI ERWRLDA-RTVNIM	110 .C. C. . ICCPEGISPACKAAA ICCPEGISPACKAAA ICCPEGISPECKAAA ICCPEGISPECKAAA ICCPEGISPECKAAA ICCPEGIAPEVCARS	120 -EQSWSLSAN -EQSWSLSAN -EQSWSLSAN -EQSWSLSPI -NERWSLSPI -NERWSLSPI -EORWSLSPI	130 II.I.I.I.I II.PHPLVRVL II.PHPLVRVL II.PHPLVRVV II.PHPLVRVV II.PHMLVRLT II.PHMLVRLT	140 VAESIYRAWSI VAESIYRAWSI VAESIYRAWSI VAESIYRAWSI VVEOIYRAMTI VCEOIYRAATI	150 .Y.R. TTNHPYHRE- TTNHPYHRE- TTNHPYHRE- TMNHPYHRE- LINHPYHRGK LINHPYHRGK
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-HI0033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xylfas 15599199-PA4004-Pseaer 15640967-VC0951-Vibcho	70 RIVTIDIE 	80 KPWDTPOLAAED KPWDTPOLAAED KPWTTPOLAED KPWTTFOLAED KSLNSEOLAKM RPWSTFOLARED KRWDTESLAVCD	00 100 I RWKLDG-RDVSLI RWKQDG-RDVSLI LAWKNDG-RDVCLI TVWKNDG-RDLSLI HWRGLG-RNLVBI SWKLDG-RTVNIM SSWKLDG-RDVSII	110 .C. C. IGCPICISPACKAAA IGCPICISPACKAAA IGCPICISPECKAAA IGCPICISAECKAAA IGCPICISAECKAAA IGCPICIAPECKAAA	120 EQSWSLSAI EQSWSLSAI EQSWSLSAI EQSWSLSPI NERWSLSPI EQRWSLSPI DQSWSLSPI	130 TLPHPLVRVL TLPHPLVRVL TLPHPLVRVV TLPHPLVRVV TLPHPLVRLT TLPHPLVRLT TLPHPLVRVV	140 VAESIYRAWSI VAESIYRAWSI VAESIYRAWSI VAESIYRAWSI VVEQIYRAATI VGEQIYRAATI VGEQIYRAWSI	150 .Y.R. TTNHPYHRE- TTNHPYHRE- TTNHPYHRE- TMNHPYHRE- LTNHPYHRK- LSGHPYHR- TNHPYHRE-
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-H10033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xylfas 15599199-PA4004-Pseaer 15640967-VC0951-Vibcho 16122818-YP02605-Yerpes	70 I.I.I.IG NRIVTIDIEG SRIVTIDIEG SRIVTIDIEG PYAVTIDVKG ERVVTIDVKG NRIVTIDIEG NRIVTIDIEG	80 KPWDTPOLANED KPWDTPOLANED KPWTTPOLACU KSLNSEOLAKRMI RFWSTEOLARED KRWDTESLAVOU TPWETPOLACUT	00 100 	110 G. EG . TGCPECISPACKAAA TGCPECISPECKAAA TGCPECISPECKAAA TGCPECISPECKAAA TGCPECIAPEVCARS TGCPECIAPEVCARA TGCPECIAPEXCAAA	120 S EQSWSLSALI EQSWSLSPLI EQSWSLSPLI NERWSLSPLI DQSWSLSPLI EQSWSLSPLI EQSWSLSPLI	130 TLPHPLVRVLI TLPHPLVRVLI TLPHPLVRVV TLPHPLVRVV TLPHPLVRVV TLPHPLVRVI TLPHPLVRVVI	140 VARSLYRAWSI VARSLYRAWSI VARSLYRAWSI VARSLYRAWSI VROLYRAWSI VGEOLYRAWSI VGEOLYRAWSI VARSLYRAWSI VARSLYRAWSI	150
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-HI0033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xy1fas 15599199-PA4004-Pseaer 15640967-VC0951-Vibcho 16122818-YP02605-Yerpes 17546914-RSC2195-Ralsol	70 IC.IC NRIVIDIPG SRIVIDIPG SRIVIDIPG PYAVIDVKG PYAVIDVKG NRIVIDIPG CRIVIDIPG CRIVIDIPG	80 KPWDTPOLAE KPWDTPOLAE KPWTTPOLAE KPWTTFOLAEO KRWTFOLATOH KSLNSEOLAKEM RPWSTFOLAE KRWDTFELAVOH KRWDTFELAVOH KRWDTFELAVOH KRWDTFELAVOH KRWDTFELAVOH KRWDTFELAVOH KRWDTFELAVOH	00 100 	110 .C. EC. . IGCPEGISPACKAAA IGCPEGISPACKAAA IGCPEGISPECKAAA IGCPEGISPECKAAA IGCPEGIAPEVCARS IGCPEGIAPACKAAA IGCPEGIAPACKAAA IGCADGIDPALKARA	120 S -EQSWSLSAL -EQSWSLSAL -EQSWSLSPL -EQSWSLSPL -DQRWSLSPL -EQSWSLSPL -EQSWSLSPL -EQSWSLSPL -EQSWSLSPL -HMIMRLSSL	130 .I. I. I. I. I TLPHPIVRVII TLPHPIVRVVI TLPHPIVRVV TLPHPIVRVII TLPHPIVRVII TLPHPIVRVII TLPHPIVRVII TLPHPIVRVII	140 VAPSIVRAWST VAPSIVRAWST VAPSIVRAWST VAPSIVRAWST VVBOLTRAATT VGEOTYRAMST VAPSIVRAWST VAPSIVRAWST VAPSIVRAWST	150
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-HI0033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xylfas 15599199-PA4004-Pseaer 15640967-VC0951-Vibcho 16122818-YP02605-Yerpes 17546914-RSC2195-Ralsol 15793423-NMA0418-Neimen	70 I.I.I.I.G NRIVTLDIPG SRIVTLDIPG SRIVTLDIPG PYAVTLDVKG PYAVTLDVKG NRIVTLDIPG NRIVTLDIPG NRIVTLDIPG AFLVVLDERG	80 KPMDTPOLANEL KPMTTPOLANEL KPMTTPOLANEL KSINSEOLAKRM KSINSEOLAKRM KRNDTESLAVOL TPMETPOLAOLL KDLTTAALADAH KDLTTAALADAH	100 100 100 100 100 100 100 100	110 .G. EG. . IGCPEGISPACKAAA IGCPEGISPACKAAA IGCPEGISPACKAAA IGCPEGISAECKAAA IGCPEGIAPACKAAA IGCPEGIAPACKAAA IGCPAGIAPACKAAA IGCADGUDPALKARA IGCADGUDPALKARA	120 S -EQSWSISAI -EQSWSISAI -EQSWSISPI -EQSWSISPI -EQRWSISPI -EQSWSISPI -EQSWSISPI -HMIARLSSI -MMIRLSSI	130 TLPHPLVRVL TLPHPLVRVV TLPHPLVRVV TLPHPLVRVV TLPHPLVRVU TLPHPLVRVU TLPHPLVRVU TLPHPLVRVU TLPHPLVRVL TLPHGMVRVL	140 VAESIYRAWSI VAESIYRAWSI VAESIYRAWSI VAESIYRAWSI VCEOIYRAWSI VCEOIYRAWSI VAESIYRAWSI VAESIYRAWSI VAESIYRAWSI VAESIYRAWSI TAEOIYRAWSI	150
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-H10033-Haeinf 15603788-FM1923-Pasmul 15836940-XF0338-Xylfas 15599199-FA4004-Pseaer 15640967-VC0951-Vibcho 16122818-YP02605-Yerpes 17546914-RSC2195-Ralsol 15793423-NMA0418-Neimen 19703798-FN0463-Fusnuc	70 	80 KPMDTPOLABEL KPMDTPOLABEL KENDTPOLABOL KENTFOLABOL KSLNSEOLAKEN KSLNSEOLAKEN KRNDTESLAVOL TPWETPOLAGOL TPWETPOLAGOL KAPTSVELABEL KAPTSVELABEL	00 100 1 ERWKLDG-RDVSLL ERWKQDG-RDVSLL ERWKQDG-RDVSLL HWRGLG-RDVSLL ERWKQDG-RDVSLL ERWKQDG-RDVSLL ERWKQDG-RDVSLL ERWKQDG-EHVCEV SWRQNG-EHVCEV SWRQNG-ESINGL	110 	120 -EQSWSLSAII -EQSWSLSAII -EQSWSLSAII -EQSWSLSPII -NERWSLSPII -DQSWSLSPII -DQSWSLSPII -HMIARLSSII -RMMRLSSII -MKKKFSHF	130 	140 VAESLYRAWSI VAESLYRAWSI VAESLYRAWSI VAESLYRAWSI VEOLYRAATI VGEOLYRAWSI VAESLYRAWSI VAESLYRAWSI TAEOLYRAWSI TEOLYRAWSI TEOLYRAWSI	150
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-HID033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xy1fas 15599199-PA4004-Pseaer 15640967-VC0951-Vibcho 16122818-YF02605-Yerpes 17546914-RSC2195-Ralsol 15793423-NMA0418-Weimen 19703798-PN0463-Fusnuc 15674116-L185851-Laclac	70 I. . G. NRIVIDIPG GRIVIDIPG SRIVIDIPG SRIVIDIPG PYAVIDVKG NRIVIDIPG NRIVIDIPG NRIVIDPG AFLVVADERG AFLVVADERG DQLAIDADG	80 KPMDTPOLAABU KPMTPOLANBU KPMTPOLANDU KPMTFOLATOU KSLNSEOLAKRM KRNDTBOLAKU KRNDTBOLAVOU TPMETPOLAOAU KADTSVELABU KADTSVELABU KATSVELABU KEINSENKSKI KEINSENKSKI	00 100 	110 G. EG. J J. IGCPEGISPACKAAA IGCPEGISPACKAAA IGCPEGISPACKAAA IGCPEGISPECKAAA IGCPEGIAPEVCARS IGCPEGIAPACKAAA IGCADCHTPALKARA IGCADCHTPALKARA IGCADCMTDRLKOCA IGCSIGISKEVYQRS	120 jSj =CoSWSLSAL =COSWSLSAL =COSWSLSPL =COSWSLSPL =COSWSLSPL =COSWSLSPL =HMIMRLSSL =HMIMRLSSL =HMIMRLSSL =DMILKISHF =DMILSFGRM	130 TIPHPINKVL TIPHPINKVL TIPHPINKVL TIPHPINKVL TIPHPINKVL TIPHPINKVL TIPHPINKVL TIPHONKVL TIPHONKVL TIPHONKVL TIPHONKVL	140 	150
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-HI0033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xylfas 15599199-PA4004-Pseaer 15640967-VC0951-Vibcho 16122818-YP02605-Yerpes 17546914-RSC2195-Ralsol 15793423-NMA0418-Neimen 19703798-FN0463-Fusnuc 156774116-L185851-Laclac	70 I.I.I.I.G. NRIVTDIPG SRIVTDIPG SRIVTDIPG PYAVTDVKC PYAVTDVKC NRIVTDIPG NRIVTDIPG NRIVTDIPG NSYNILDIEG NSYNILDIEG NSYNILDIEG DQLAIDAIOS	80 KPMDTPOLANEU KPMTPOLANEU KPMTPOLANEU KSINSEOLAKRM KSINSEOLAKRM KRNDTSELAVOL TPMETPOLACOL KOLTTAALADAL KOLTTAALADAL KEINSENKAKII KLMSSEVADFVI KOFFSELTSELI	100 100 100 100 100 100 100 100	110 .G. IEG. J J. IGCPEGISPACKAAA IGCPEGISPACKAAA IGCPEGISPACKAAA IGCPEGISAECKAAA IGCPEGIAPACKAAA IGCPEGIAPACKAAA IGCADGIDPALKARA IGCADGIDPALKARA IGCADGIDPALKARA IGCASNOVSKNVKNSV IGCSIGISKEVYQRS IGCSIGISIKKVASI	120 	130 	140 	150 TTHEPHRE- TTHEPHRE- TTHEPHRE- TTHEPHRE- LTHEPHRE- LTHEPHRE- TTHEPHRE- TOHEPHRE- TOHEPHRE- SNNIKYHK- NRESAWHK- TOSEPHK-
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-HI0033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xylfas 15599199-PA4004-Pseaer 15640967-VC0951-Vibcho 16122818-YP02605-Yerpes 17546914-RSC2195-Ralsol 15793423-NMA0418-Weimen 19703798-FN0463-Fusnuc 15675944-SPY2215-Strpyo 15902041-SP2238-Strpne	70 	80 KPMDTPOLANEL KPMTPOLANEL KPMTPOLANEL KSLNSEOLAKRI KSLNSEOLAKRI KSLNSEOLAKRI KKNDTBELAVOL TPWETFOLAOOL TPWETFOLAOOL KDITTALADA KSLNSENJAEHL KEINSENJAEHL KOFFSETFELI KUTFSEEFSKO	00 100 1 IRWKLDG-RDVSLI RWKQDC-RDVSLI RWKQDC-RDVSLI HWRGLG-RNLVGI SWKLDC-RDVSLI RWKQDC-RDVSLI RWKQDC-RDVSLI RWKQDC-RDVSLI CWQREC-CDVALI CKAEVYCTCNLVGV SNLKNICISSINGI CKAEVYCTCNLVGV SCVTVKCYSTITGI ETSIKGFSTITGI	110 	120 	130 .E	140 VABSLYRAWSI VABSLYRAWSI VABSLYRAWSI VABSLYRAWSI VABSLYRAWSI VABSLYRAWSI VABSLYRAWSI VABSLYRAWSI TABOLYRAWSI LEOVYRWBA TYBOLYRAWSI TEOLYRAPHI VEOLYRAPHI VEOLYRAPHI	150
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-HID033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xy1fas 15599199-PA4004-Pseaer 15640967-VC0951-Vibcho 16122818-YF02605-Yerpes 17546914-RSC2195-Ralsol 15793423-NMA0418-Weimen 19703798-FN0463-Fusnuc 15674116-L185851-Laclac 15675944-SFY2215-Strpyo 15902041-SF2238-Strpne 15896772-CAC3536-Cloace	70 I.I.I.I.I.I.I.I.I.I.I.I.I.I.I.I.I.	80 KPMDTPOLANED KPMTPOLANED KPMTPOLANED KPMTFOLATOU KSLNSEOLAKEM KSLNSEOLAKEM KSLNSEOLAKEM KSLNSEOLAKEM KSLNSEOLAKEM KATSVELAEHU KSLNSEVADFM KOFFSETFSELI KTFSEEFSKOU KJFSEEFSKOU	100 100 1 RRWKLDG-RDVSLL RRWKDG-RDVSLL SWKNDG-RDVSLL SWKNDG-RDVSLL SWKLDG-RDVSLL RRWKLDA-RTVNLM SWKLDG-RDVSLL RRWKQDC-RDVSLL RRWKQDC-RDVSLL RRWKQC-EHVGSV SWKLNGISSINGI KAREVYCTGNVGV SGVTVKCYSTITGI DEFSIKGFSTLTGI DDLGVRENSSIDGV	110 	120 jSj =CoSWSUSAI, =COSWSUSAI, =COSWSUSAI, =COSWSUSAI, =COSWSUSAI, =COSWSUSAI, =COSWSUSAI, =NILMRUSSI, =DKLIKUSAI, =DKLIKUSAI, =NILVSSECH, =NILVSSECH, =NILVSSECH,	130 	140 	150
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-HI0033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xylfas 15599199-PA4004-Psear 15640967-VC0951-Vibcho 16122818-YP02605-Yerpes 17546914-RSC2195-Ralsol 15793423-NMA0418-Neimen 19703798-FN0463-Fusnuc 1567416-L185851-Laclac 15675944-SP72215-Strppo 15902041-SP2238-Strpne 15925729-SA0023-Staaur	70 ICIC NRIVIDIPG SRIVIDIPG SRIVIDIPG PYAVIDVKC PYAVIDVKC NRIVIDIPG NRIVIDIPG CRIVADDERC CRIVADDERC 	80 KPMDTPOTAAED KPMTPOTAAED KPMTPOTAAED KPMTFOTATOL KSINSECTAKEM KSINSECTAKEM KSINSECTAKEM KSINSECTAKEM KNDTESTAF KUTTSAETADAE KATSSETSAET KMTSSECTAETA	100 1 IRWKLDG-RDVSLL RWKQDG-RDVSLL 2000	110 	120 	130 	140 	150 I.J.W.R.J. TTHEPYHRS- TTHEPYHRS- TTHEPYHRS- LTHEPYHRS- LTHEPYHRS- TTHEPYHRS- TOHEPYHRS- SINIKYHK NRCSAVHK SRCEPYHK SRCEPYHK SRCEPYHK
16128619-YbeA-Esccol 16764018-STM0641-Saltyp 16272008-HI0033-Haeinf 15603788-PM1923-Pasmul 15836940-XF0338-Xylfas 15599199-PA4004-Pseaer 15640967-VC0951-Vibcho 16122818-YP02605-Yerpes 17546914-RSC2195-Ralsol 1579342-SNM0418-Neimen 19703798-FN0463-Fusnuc 15675944-SPY2215-Strpyo 15902041-SP2238-Strpne 15896772-CAC3536-Cloace 15925729-SA0023-Staaur 16799398-LIN0321-Lisinn	70 I.I. G. NRIVTIDIPG GKVVTIDIPG 	80 KUMDTPOLANEL KUMDTPOLANEL KUMTTEOLATOL KUMTTEOLATOL KUNTEOLAKUM RUNSEOLAKUM RUNSEOLAKUM KUNSEOLAKUM KUNSEULAEHU KUNSEULAEHU KUNSEULAEHU KUNSEULAEHU KUNSEULAEHU KUNSEULAEHU KUNSEULAEHU KUNSEULAEHU KUNSEULAEHU KUNSEULAEHU	00 100 II.EWKLDG-RDVSLI RWKQDC-RDVSLI RWKQDC-RDVSLI EWRDG-RDVSLI HWRGLG-RNIVEL EWRGLG-RNIVEL RWKQDC-RDVSLI RWKQDC-RDVSLI RWKQDC-RDVSLI RWKQDC-RDVSLI RWKQDC-RDVSLI SWKLG-EHVGSV INLKNIGISSINEI KAEVYCTGHLVEV SGVTVKCYSTITEI DELGVRCNSSIDEV VQRMTQCSDFVBV RLATYCKSKVAEV	110 .G. IEG. J I. IGCPEGISPACKAAA IGCPEGISPACKAAA IGCPEGISPACKAAA IGCPEGISPACKAAA IGCPEGIAPEVCARS IGCPEGIAPEVCARS IGCPEGIAPACKAAA IGCPEGIAPACKAAA IGCSIGISSEVVRSV IGCSIGISSEVVRSV IGCSIGISSEVVRSA IGCSIGISSEVVRSA IGCSIGISSEVVRS IGCSIGISSAVLARA IGCSIGISSAVLARA	120 iSi ZoswSJSAI ZoswSJSAI ZoswSJSPI NERWSJCPI DOSWSJSPI ZOSWSJSPI ZOSWSJSPI ZOSWSJSPI DOSWSJSPI DOSWSJSPI DOSWSJSPI DOSWSJSPI DOSWSJSPI DISTR DISTR DISTR NILKTSH NILSVSPGRI NILSVSPGRI NIZJSPSCA	130 .E.I.I.I.PHPINRVLI TIPHPINRVLI TIPHPINRVLI TIPHPINRVLI TIPHPINRVLI TIPHPINRVLI TIPHPINRVLI TIPHPINRVLI TIPHONRIV TIPHONRIV TIPHONRIV TIPHONRIV TIPHONRIV TIPHONRIV	140 VABSLYRAWSI VABSLYRAWSI VABSLYRAWSI VABSLYRAWSI VGBOLYRAWSI VABSLYRAWSI VABSLYRAWSI VABSLYRAWSI VABSLYRAWSI ILEOLYRAWSI ILEOLYRAWSI VBOLYRAPMI VBOLYRAPMI VBOLYRAPMI VBOLYRAPMI LEOVYRAPRI LEOVYRAPRI LEOVYRAPRI	150
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FIGURE 5. Sequence alignment of *E. coli* YbeA with its orthologs from COG1576 and with *E. coli* TrmD, the closest paralog with known function. Conserved and conservatively substituted residues are shaded. Residues L72 to R154 that are putatively directly involved the methylation reactions are indicated *above* the alignment; the extent of α -helical (tubes) and β -sheet secondary structures (arrows) are shown *below* the alignment.

RNA substrate (Fig. 6). The C-terminal domain of TrmD, which probably binds the tRNA substrate, is absent from YbeA; in fact, YbeA lacks any structural elaboration and thus appears to be a minimalist member of the SPOUT superfamily.

A model for the interaction between YbeA-AdoMet and Ψ 1915 was generated using the HADDOCK docking program (Dominguez et al. 2003). The proximity between the N3 atom of pseudouridine and the methyl group of AdoMet was used as a spatial constraint and, predictably, places Ψ 1915 into the putative active site of YbeA (Fig. 7). The conserved residues L72, G76, G103, G107, S121, and L127 in YbeA respectively correspond to L87, G91, G113, G117, S132, and L138 in TrmD (Fig. 5). YbeA also possesses a number of conserved residues that are spatially equivalent to important TrmD residues, even though they do not coincide in the primary structure alignments. These include YbeA-R142, which seems comparable to TrmD-R154 and might bind the target base, YbeA-R154, which like TrmD-R24 could be involved in RNA backbone interaction, and YbeA-Y152, which might be analogous to TrmD-L160 and possibly stacks upon and stabilizes the target base (Fig. 7). The specificity of YbeA for a pseudouridine substrate (Ero et al. 2008) suggests that base stacking (Davis 1995) and hydrogen bonding with the N1-position



FIGURE 6. Surface representations of a model for the YbeA-AdoMet complex. (*A*) Model colored according to the distribution of electrostatic potential from red (-10 kT) to blue (+10 kT). (*B*) Model colored to show sequence conservation in the COG1576 family: deep blue (invariant) through light blue (conserved), green (similar) to yellow and then red (highly variable). A stick representation of AdoMet is shown (nitrogen atoms, blue; oxygen, red; sulphur, yellow).

are probably important determinants for recognition and methylation of the Ψ 1915 target.

There are also obvious differences that could reflect RNA substrate specificities. YbeA-E106 is homologous to TrmD-E116, which is important for target base binding, although E106 is not conserved in the COG1576 family and its side chain is turned away from the active site in YbeA. Surprisingly, there is no obvious YbeA equivalent to TrmD-D169, which was proposed to act as a general base catalyst and deprotonate the N1 atom of guanosine. Possibly a conformational rearrangement is required in YbeA to position another suitable negatively charged residue into the vicinity of the active site.

Interaction of YbeA with the ribosome

Binding of the YbeA dimer to the 70S ribosome (Schuwirth et al. 2005) was investigated computationally using GRAMM (Vakser 1997), which allows rigid body docking with simple criteria for steric and electrostatic compatibility between different kinds of biological macromolecules. A representative structure is shown in Figure 8 from the largest cluster of high-scoring docking solutions that place YbeA active site in the vicinity of the U1915 nucleoside. No attempt has been made to optimize the rigid-body model, and, in the absence of methods to calculate reliable binding energies or conformational changes in protein–RNA complexes, the model should be regarded as preliminary.

Despite this approximation, several striking features are immediately apparent. First, the YbeA dimer makes contacts with both ribosomal subunits. The 30S interactions are extensive and contribute considerably to the orientation and stability of the YbeA interaction; these include contacts to 16S rRNA nucleotides within and adjacent to the decoding site as well as r-protein S12 (Fig. 8, see the legend for details). The 50S subunit contacts are confined to domain IV of 23S rRNA and are mainly in the loop of hairpin 69; YbeA does not come close to any of the 50S subunit proteins. We repeated the docking on the Thermus thermophilus 70S ribosome structure (Korostelev et al. 2006), and these simulations placed YbeA in essentially the same position and orientation, although additional 16S rRNA nucleotides (G517-C519 and U531) came within 5 Å of the methyltransferase together with broader stretches of r-protein S12 (VVRTSL51, AK56, HNLQEHS80) and rprotein S13 residues KK₁₂₁. The extensive YbeA contacts seen on the 30S subunit match well with the accompanying



FIGURE 7. (*A*) Docking model of YbeA-AdoMet- Ψ 1915 based on (*B*) the TrmD-AdoMet crystal structure. Ligands and side chains of residues potentially involved in the methyl transfer reaction are labeled indicating carbon (gray), nitrogen (blue), and oxygen atoms (red). The backbone of the AdoMet-binding site (conserved between YbeA and TrmD) is indicated in yellow.



FIGURE 8. Interaction of the YbeA dimer with the *E. coli* 70S ribosome. (*A*) View through the E (exit) site showing how the YbeA methyltransferase (green) fits into the interface between the 50S (gray) and 30S ribosomal subunits (sand). The methylation target Ψ 1915 in helix 69 is located adjacent to the AdoMet moiety (red) of YbeA that is visible from this angle. (*B*) View through the A (aminoacyl) site showing the second AdoMet moiety on the YbeA dimer, which appears to be redundant. (*C*) View from above after removal of the 30S head and 50S central protuberance to show the position of YbeA relative the P-site tRNA (magenta) and mRNA (yellow). (*D*) Close up of 23S rRNA helix 69 (blue) showing the methylation target at nucleotide 1915 (light blue stick) and its orientation when flipped (dark blue) into the YbeA active site containing AdoMet (red stick). Residues approaching within 5 Å of YbeA are 16S rRNA nucleotides A1912–U1917 and U1946, and r-protein S12 residues VYT₃₈ and Q₇₄.

study (Ero et al. 2008) showing that YbeA activity requires the 70S ribosome and that the isolated 50S subunit does not function as a suitable methylation substrate.

A second feature of the model is that it offers an explanation for the overall streamlined structure of YbeA and its lack of additional domains. The YbeA structure facilitates the snug fit between the ribosomal subunits where a larger dimer would not be able to access the target. The significance of the dimeric structure is still not fully understood, and we note that the second AdoMet moiety faces out into the solvent at the entry to the A site (Fig. 8B). Another characteristic of the model is that YbeA is positioned where it does not interfere with tRNA binding in the ribosomal P site, but it would occlude tRNA binding in the A site. This might indicate that the physiological substrate is the ribosome initiation complex with fMet-tRNA in the P site. In the top view (Fig. 8C), YbeA can be seen to come close to the anticodon stem of the P-site tRNA and the A-site codon of the mRNA; insufficient data are available at present to assess whether YbeA also contacts these RNAs.

Finally, the model suggests how the methylation reaction might be initiated. The shape complementarity between YbeA and its target site suggests that only a minor change in the rRNA conformation is required to place Ψ 1915 into the active site of the enzyme by swinging out the target base into an unstacked configuration (Fig. 8D). Similar base-flipping mechanisms are used by a variety of nucleic acid modification enzymes such as DNA methyltransferases (Klimasauskas et al. 1994), RNA methyltransferases (Lee et al. 2005), and pseudouridine synthases (Hoang and Ferré-D'Amaré 2001).

Assessment of whether such a baseflipping mechanism is used by YbeA will require a crystallographic study of the methyltransferase bound to the 70S ribosome. Other predictions made from this model, including the YbeA-rRNA contacts, could be checked by more readily available techniques such as footprinting. Despite the need for more experimental data, the YbeA substrate requirements taken together with structural considerations lead us to the unavoidable conclusion that the methyltransferase must interact with both ribosomal subunits. YbeA is, to our knowledge, the first example of an rRNA methyltransferase

that makes simultaneous contact with both subunits.

In closing, we suggest that a more suitable designation for the SPOUT-superfamily member YbeA would be the rRNA large subunit methyltransferase RlmH, consistent with the terminology used for the other rRNA methyltransferase characterized in *E. coli* (Ofengand and Del Campo 2004; Andersen and Douthwaite 2006).

MATERIALS AND METHODS

Cloning and knockout of ybeA

Cloning of the *ybeA* gene and generation of a knockout strain were carried by Saka et al. (2005) and Baba et al. (2006), respectively; the recombinant plasmid and the knockout strain were obtained

from the Keio collection (Baba et al. 2006). Briefly, the full-length *ybeA* gene was cloned on a plasmid under control of the *lac* promoter, and the recombinant YbeA protein is expressed with a C-terminal histidine tag. The *ybeA* gene in *E. coli* K-12 strain BW25113 was replaced with a kanamycin cassette flanked by FRT sites (Flp recombination target) in a one-step, site-specific recombination event creating an in-frame deletion of the entire *ybeA* gene.

The structure of the relevant chromosomal region of the BW25113 $\Delta ybeA$ strain was confirmed by PCR sequencing, and the structure of plasmid pCA24N $ybeA^+$ was verified by a combination of restriction enzyme mapping and sequencing. Restriction endonucleases and other enzymes for DNA manipulations (Fermentas) were used according to the suppliers' recommendations; plasmid DNA was isolated by using a plasmid miniprep kit (Qiagen); *E. coli* transformations were performed by standard methods (Sambrook et al. 1989).

Growth experiments

The growth rates of the wild-type and *ybeA*-knockout strains were determined during exponential growth phase in triplicate cultures at 37° C in rich (LB) liquid medium.(Sambrook et al. 1989). Overnight cultures were diluted to an optical density (A₄₅₀) of 0.01, and growth was monitored spectroscopically every 20 min.

Growth competition experiments were performed in duplicate as previously described (Gutgsell et al. 2000; Toh et al. 2008) starting with approximately equal numbers of xylA- and ybeAknockout cells at the same growth phase or, alternatively, starting under the same conditions with wild-type and ybeA-knockout cells. The xylA gene in the growth comparator had been inactivated with the same kanamycin resistance cassette used in the ybeA strain, and, in theory, xylA cells should grow at the same rate as the wild-type strain in rich medium unless expression of the resistance cassette has a biological cost. Growth competition was followed in rich (LB) liquid medium (Sambrook et al. 1989) at 37°C. At the end of every 6-h cycle, cells were diluted to an A₄₅₀ of 0.01 in fresh medium. The relative numbers of ybeA and xylA cells were followed throughout the growth cycles by plating on MacConkey agar supplemented with 1% (w/v) D-xylose (Toh et al. 2008), and also on MacConkey/xylose agar with kanamycin at 50 mg/L, and screening for red and white colonies. In parallel experiments, the relative numbers of wild-type and ybeA cells were estimated by plating on LB agar (where all cells grow) and LB agar with kanamycin at 50 mg/L (only the *ybeA* cells grow).

Analysis of rRNA by MALDI mass spectrometry

Total rRNA was extracted from ribosomal particles isolated from *E. coli* wild-type and *ybeA*-knockout strains. The defined rRNA sequence from G1891 to G1948 in 23S rRNA was isolated by hybridization to a complementary 58-mer deoxyoligonucleotide (Andersen et al. 2004; Douthwaite and Kirpekar 2007). Briefly, 100 pmol of total rRNA were heated with 400 pmol of deoxyoligonucleotide at 85°C for 1 min, followed by slow cooling to 45°C over 2 h. Regions of the rRNAs that were not protected by hybridization were digested away with Mung bean nuclease (NE Biolabs) and RNase A (Sigma), and the rRNA sequence paired to the deoxyoligonucleotide was separated by gel electrophoresis and then extracted. The rRNA sequence of ~58 nt was digested with 20 units of RNase T1 (USB) at 37°C for 3 h in 2 μ L H₂O containing 0.25 μ L of 0.5 M 3-hydroxypicolinic acid. Samples

were dried and resuspended in 1 μ L H₂O prior to analysis by MALDI-MS (Voyager Elite, Perseptive Biosystems) recording in reflector and positive ion mode (Kirpekar et al. 2000). Spectra were analyzed using the program m/z (Proteometrics Inc). Tandem mass spectra were recorded in positive ion mode on a MicroMass MALDI Q-TOF Ultima mass spectrometer as previously described (Kirpekar and Krogh 2001).

Primer extension

A deoxynucleotide primer complementary to the 1925 to 1942 region of *E. coli* 23S rRNA was 5'-end-labeled with ³²P (and in later experiments with ³³P), hybridized to rRNA samples and extended with AMV reverse transcriptase (Finnzymes) as described by Stern et al. (1988). Extension products were run on polyacrylamide/urea gels alongside dideoxy sequencing reactions performed on rRNA from the *ybeA*-knockout strain to ensure read-through past U1915. Gels bands were visualized by phosphorimaging (Typhoon, Amersham Biosciences).

Molecular modeling and docking

The YbeA protein structure was analyzed and visualized with the PyMol molecular graphics system (http://www.pymol.org) and VMD (Humphrey et al. 1996); calculations of the electrostatic potential were carried out with APBS tools. Sequence conservation was mapped onto the model via the COLORADO3D server (Sasin and Bujnicki 2004), using the Rate4Site method, with the JTT substitution matrix and ML model for rate inference. The model of protein–pseudouridine complex was constructed with the docking method HADDOCK (Dominguez et al. 2003).

GRAMM v1.03 (Vakser 1997) was used for rigid-body docking of the E. coli YbeA dimer (crystal structure 1ns5) to the E. coli 70S ribosome (Schuwirth et al. 2005). The PDB files used for the ribosome structure were as follows: 30S subunit, 2AVY and 2I2P; 50S subunit 2AW4 and 2I2T. The truncated anticodon stem-loop structure was aligned with the P-site tRNA from the T. thermophilus 70S ribosome structure, 1GIX (Yusupov et al. 2001). All docking simulations were repeated with the T. thermophilus 70S ribosome structure (Korostelev et al. 2006), with essentially the same result. In all simulations, the high-resolution docking mode was used to generate 1000 alternative orientations that optimized the steric and electrostatic complementarity between the ribosome and YbeA structures. These alternative models were subsequently ranked according to the proximity between the putative active site of YbeA (as inferred from earlier docking analyses of AdoMet and Ψ 1915) and the position of U1915 in the ribosome structure. The ranking was carried out with the FILTREST3D method (M.J. Gajda and J.M. Bujnicki, in prep.; a web server is available at http://filtrest3d.genesilico.pl/filtrest3d/index.html). One hundred top-scoring models (those with the active site of YbeA close to U1915 in the ribosome) were clustered using the MAXCLUSTER method (http://www.sbg.bio.ic.ac.uk/~maxcluster/index.html), and the central conformation from largest cluster was selected as the most preferred orientation.

SUPPLEMENTAL DATA

Data from the ribosome docking simulations are available as supplementary material at http://www.rnajournal.org.

ACKNOWLEDGMENTS

We thank Anette Rasmussen, Michelle O'Connor, Jacob Poehlsgaard, and Finn Kirpekar for help and advice with data interpretation. We are indebted to Jaanus Remme for revealing to us that 70S and not 50S is the substrate for YbeA methylation (see accompanying article Ero et al. 2008). S.D. gratefully acknowledges support from the Danish Research Agency (FNUrammebevilling 272-07-0613), the Nucleic Acid Center of the Danish Grundforskningsfond, and NAC-DRUG under the FP6 Marie Curie Initial Training Networks (to E.P). Support was provided to J.M.B., K.H.K., J.M.K. and E.P. by the Polish Ministry of Science (grant N301 2396 33).

Received May 30, 2008; accepted July 9, 2008.

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