

# The universal YrdC/Sua5 family is required for the formation of threonylcarbamoyladenine in tRNA

Basma El Yacoubi<sup>1</sup>, Benjamin Lyons<sup>1</sup>, Yulien Cruz<sup>1</sup>, Robert Reddy<sup>2</sup>, Brian Nordin<sup>2</sup>, Fabio Agnelli<sup>2</sup>, James R. Williamson<sup>2</sup>, Paul Schimmel<sup>2</sup>, Manal A. Swairjo<sup>2</sup> and Valérie de Crécy-Lagard<sup>1,\*</sup>

<sup>1</sup>Department of Microbiology and Department of Microbiology and Cell Science, University of Florida, P.O. Box 110700, Gainesville, FL 32611-0700 and <sup>2</sup>Department of Chemistry and Department of Molecular Biology, The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037, USA

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## ABSTRACT

**Threonylcarbamoyladenine (t<sup>6</sup>A) is a universal modification found at position 37 of ANN decoding tRNAs, which imparts a unique structure to the anticodon loop enhancing its binding to ribosomes *in vitro*. Using a combination of bioinformatic, genetic, structural and biochemical approaches, the universal protein family YrdC/Sua5 (COG0009) was shown to be involved in the biosynthesis of this hypermodified base. Contradictory reports on the essentiality of both the *yrdC* wild-type gene of *Escherichia coli* and the *SUA5* wild-type gene of *Saccharomyces cerevisiae* led us to reconstruct null alleles for both genes and prove that *yrdC* is essential in *E. coli*, whereas *SUA5* is dispensable in yeast but results in severe growth phenotypes. Structural and biochemical analyses revealed that the *E. coli* YrdC protein binds ATP and preferentially binds RNA<sup>Thr</sup> lacking only the t<sup>6</sup>A modification. This work lays the foundation for elucidating the function of a protein family found in every sequenced genome to date and understanding the role of t<sup>6</sup>A *in vivo*.**

## INTRODUCTION

Found in all sequenced genomes to date, the YrdC (COG0009) family has been ranked in the top 10 conserved hypothetical proteins to be considered as high priority targets for experimental study (1). The crystal structures of two *Escherichia coli* COG0009 family members YrdC and YciO (2,3) reveal an  $\alpha/\beta$  twisted open-sheet structure with a positively charged cleft in its

center that was proposed to form a nucleic-acid-binding site (2). Binding to double-stranded RNA (including tRNA) was experimentally verified for the *E. coli* YrdC protein (YrdC<sub>Ec</sub>) (2). The study of the COG0009 encoding gene from *Saccharomyces cerevisiae*, *SUA5*, preliminarily addressed the function of this gene family. A mutant allele, *sua5-1* (Ser107 → Phe replacement) was identified as a suppressor of a translation initiation defect in the leader region of the *cyc1-1019* allele of the iso-1-cytochrome *c* encoding gene *CYC1*. In a strain carrying the *cyc1-1019* allele, initiation was shown to occur at an aberrant up-stream and out of frame ATG (due to a single base-pair substitution) and resulted in expression of ~2% of the normal Cyc1 protein amount. Interestingly, suppression did not occur at the transcriptional level as observed for the other *sua* suppressors identified in the same screen (4). A strain carrying a *sua5* null mutation *sua5::LEU2* displayed pleiotropic phenotypes that included; slow growth, cytochrome *a.a3* deficiency, decreased cytochrome *b* levels and inability to grow on lactate or glycerol (4). The Sua5 protein was implicated in post-transcriptional modulation on gene expression but its biochemical function remained uncharacterized (4). In humans, the YrdC ortholog (YrdC<sub>H</sub>) was identified as a retinoblastoma binding protein 10 (RBBP10) interactor and a possible role in translation was proposed based on its high and ubiquitous expression profile in several tissues including those in which high levels of protein synthesis occurs (5). The YrdC<sub>H</sub> ortholog was also identified as a new ischemia/reperfusion-inducible protein (IRIP) that indirectly regulates the activity of a variety of transporters probably through its interaction with RSC1A1, a modulator of membrane transport with unclear mode of action (6).

In *E. coli*, a small deletion of 12 nucleotides including the initiation codon of *yrdC* ( $\Delta yrdC$ ) was found to

\*To whom correspondence should be addressed. Tel: +1 352 392 9416; Fax: +1 352 392 5922; Email: vcrecy@ufl.edu

Present addresses:

Brian Nordin, ActivX Biosciences, Inc. 11025 North Torrey Pines Road, La Jolla, CA 92037, USA.

Manal A. Swairjo, Burnham Institute for Medical Research, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA.

suppress the temperature-sensitive phenotype caused by *prfA1* (an allele of the *prfA* gene encoding release factor 1). These mutations were found to be inseparable and a function in ribosome maturation was suggested for *yrdC* based on the observation that the double mutant displayed slightly higher amounts of 17S rRNA, an immature form of 16S rRNA (7,8).

In summary, members of the COG009 family seem to act as cellular regulators, with pleiotropic effects often linked to translation that vary with the experimental system. Overall the role at the molecular level of the YrdC/Sua5 proteins remained unclear. The essentiality of the gene family also remained an un-resolved issue, as it appeared to vary with the organism considered and the approach utilized to construct mutations. In *E. coli*, no *yrdC* deletion mutant was obtained in the systematic single-gene knockout mutant collection, whereas the *E. coli* homolog *yciO* was successfully deleted in the same study (9). Also, a *yrdC* mutant could be isolated in a *prfA1* background, but the two mutations were not dissociable, suggesting again that gene *yrdC* might be essential in wild-type *E. coli* (8). However, conflicting results have been reported when an insertion of a Tn5-araP<sub>BAD</sub> construct within the first 15 bp of *yrdC<sub>Ec</sub>* did not lead to lethality on rich medium supplemented with glucose (10).

In yeast, even though it was isolated as a suppressor, the *sua5::LEU2* allele was dissected away from the original mutation, and deletion of the gene was successfully achieved in homozygous backgrounds suggesting that the gene was not essential (4). However, in the yeast systematic gene deletion collection, a single *SUA5* copy could be deleted in the diploid background BY4743, and no deletion was obtained in the haploid background BY4741 (11).

N<sup>6</sup>-threonylcarbamoyl adenosine (t<sup>6</sup>A) is an anticodon-loop modification found at position 37 of tRNAs decoding ANN codons. Although considerable biochemical and biophysical information exists on the function of this hypermodified base (12–21), the t<sup>6</sup>A biosynthesis pathway has only been partially biochemically characterized and shown to be an ATP-dependent process requiring threonine and carbonate although none of the genes involved in its biosynthesis have been identified (22).

In the present study we show by combining a comparative genomic approach with experimental validation that the YrdC/Sua5 family is involved in t<sup>6</sup>A biosynthesis. We also investigated the essentiality of this gene family in two biological systems, *E. coli* and yeast. In light of the role of this gene family in the formation of the critically important tRNA modification t<sup>6</sup>A<sub>37</sub>, hypotheses to explain some of the pleiotropic effects observed in previous studies are presented.

## MATERIALS AND METHODS

### Bioinformatics

Analysis of the COG009 family was performed in the SEED database (23). We also used the Blast tools and resources at NCBI (24). Annotations for paralog families were made using physical clustering on the chromosome

when possible, by building phylogenetic trees using the ClustalW tool (25) integrated in SEED or by deriving specific protein motifs (<http://espript.ibcp.fr/ESPrIPT/ESPrIPT/>).

### Strains and growth conditions

All *E. coli* and *S. cerevisiae* strains used in this study are listed in Table 1. Bacteria were routinely grown on LB medium (BD Diagnostics Systems) at 37°C unless otherwise stated. Bacterial growth media were solidified with 15 g/l agar (BD Diagnostics Systems) for the preparation of plates. Transformation and P1 transductions were performed following standard procedures (26). The sensitivity to P1 phage of all recipient strains used was verified. Ampicillin (Amp, 100 µg/ml), Kanamycin (Km, 50 µg/ml), L-Arabinose (Ara, 0.02–0.2%), were used when appropriate. Bacterial chromosomal DNA was prepared using the Qiagen kit DNeasy<sup>®</sup> Tissue Kit and yeast chromosome DNA was prepared using Blood & Tissue Kit (Qiagen, Valencia, CA).

Yeast strains were routinely grown on YPD (DIFCO Laboratories) at 30°C unless otherwise stated. Synthetic minimal, with or without Agar, SD base or SD base Gal/Raf with or without dropout (-uracil, -ura; -leucine, -leu; -histidine, -his) were purchased from Clontech (Palo Alto, CA) and prepared as recommended by the manufacturer. Glucose (Glu, 2%), Glycerol (Gly, 4%), 5-fluoro-orotic acid (5-FOA, 0.1%) and G 418 (300 µg/ml) were used when appropriate. Transformations were carried out as described in the pYES-DEST52 manual (Invitrogen, Carlsbad, CA).

### Cloning and strains constructions

All plasmids constructed are listed in Table 1. Sequences of all primers used are given in Table 2. The *E. coli* wild-type genes *yrdC<sub>Ec</sub>* (NP\_417741) and *yciO<sub>Ec</sub>* (NP\_415783) were amplified by PCR from genomic DNA prepared from strain *E. coli* K12 MG1655. In a similar fashion the wild-type gene *ywlC<sub>Bs</sub>* (NP\_391576) was amplified from *Bacillus subtilis* 168 genomic DNA and the wild-type yeast gene *SUA5* (NP\_011346) was amplified from strain BY4743 genomic DNA. The wild-type gene *yrdC<sub>Mm</sub>* (NP\_987306) was amplified from *Methanococcus maripaludis* S2 genomic DNA. For subcloning in pBAD24, all forward primers (ol1) contained an *NcoI* sequence and the reverse primers (ol2) contained a *HindIII* sequence, while the reverse primer to amplify *SUA5* contained a *PstI* site, and are BADEcyrdCol1/BADEcyrdCol2; BADEcyrciOol1/BADEcyrciOol2; BADBsywlCol1/BADBsywlCol2; BADMmyrdCol1/BADMmyrdCol2; BADsua5ol1/BADsua5ol2. PCR fragments were first inserted into pCR2.1 TOPO (Invitrogen) and transformed into Top 10 cells (Invitrogen) according to the manufacturer protocol. Sequences were verified before subcloning into the appropriate sites of pBAD24 (27).

For subcloning into pYES, the PCR amplified products corresponding to the *yrdC<sub>Ec</sub>*, *yciO<sub>Ec</sub>*, *yrdC<sub>Mm</sub>*, *ywlC<sub>Bs</sub>* genes were first cloned into the entry vector pENTR/D-TOPO (Invitrogen), using

Table 1. Strains and plasmids used

| Strain name            | Relevant characteristics   | Reference                      |
|------------------------|--|--------------------------------|
| Yeast strains          |  |                                |
| BY4741                 | <i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i> (S288C)   | (59)                           |
| BY4743                 | <i>MATa/MATα his3-Δ1/his3-Δ1 leu2-Δ0/leu2-Δ0 lys2-Δ0/lys2-Δ0 ura3-0/ura3-0</i>   | (59)                           |
| VDC5118                | YGN63 transformed with plasmid pYES-DEST52; Ura <sup>+</sup> , Leu <sup>+</sup>  | This study                     |
| VDC5119                | YGN63 transformed with plasmid pYES-DEST52; Ura <sup>+</sup> , Leu <sup>+</sup>  | This study                     |
| VDC5120                | YGN63 transformed with plasmid pBY135; Ura <sup>+</sup> , Leu <sup>+</sup>   | This study                     |
| VDC5121                | YGN63 transformed with plasmid pBY135; Ura <sup>+</sup> , Leu <sup>+</sup>   | This study                     |
| VDC5122                | YGN63 transformed with plasmid pBY136; Ura <sup>+</sup> , Leu <sup>+</sup>   | This study                     |
| VDC5123                | YGN63 transformed with plasmid pBY136; Ura <sup>+</sup> , Leu <sup>+</sup>   | This study                     |
| VDC5124                | YGN63 transformed with plasmid pBY137; Ura <sup>+</sup> , Leu <sup>+</sup>   | This study                     |
| VDC5125                | YGN63 transformed with plasmid pBY137; Ura <sup>+</sup> , Leu <sup>+</sup>   | This study                     |
| VDC5126                | YGN63 transformed with plasmid pBY138; Ura <sup>+</sup> , Leu <sup>+</sup>   | This study                     |
| VDC5127                | YGN63 transformed with plasmid pBY138; Ura <sup>+</sup> , Leu <sup>+</sup>   | This study                     |
| VDC5300                | Derivative of BY4743; <i>SUA5/sua5-Δ1::LEU2</i>  | This study                     |
| VDC5309                | Derivative of BY4741 transformed with pYES-DEST52:: <i>yrdC<sub>Ec</sub></i> ; Ura <sup>+</sup>  | This study                     |
| VDC5416                | Derivative of VDC5309; <i>sua5-Δ1::LEU2</i> ; Ura <sup>+</sup> Leu <sup>+</sup>  | This study                     |
| VDC5417                | Derivative of VDC5309; <i>SUA5</i> ; Ura <sup>+</sup> Leu <sup>+</sup>   | This study                     |
| VDC5440                | Derivative of VDC5416 after FOA treatment; <i>sua5-Δ1::LEU2</i> ; Ura <sup>-</sup> Leu <sup>+</sup> (cured of pYES-DEST52:: <i>yrdC<sub>Ec</sub></i> ), clone 14     | This study                     |
| VDC5441                | Derivative of VDC5416 after FOA treatment; <i>sua5-Δ1::LEU2</i> ; Ura <sup>-</sup> Leu <sup>+</sup> (cured of pYES-DEST52:: <i>yrdC<sub>Ec</sub></i> ), clone 15     | This study                     |
| YJN63                  | <i>MATα CYC I cyc7-67 ura3-52 leu2-3,112 cyh2 sua5::LEU2</i>   | (4)                            |
| YMH13                  | <i>MATα cyc1-1019 cyc7-67 ura3-52 leu2-3,112 cyh2</i>  | (4)                            |
| <i>E. coli</i> strains |  |                                |
| BL21(DE3)              | F <sup>-</sup> <i>ompT hsdSB(rB<sup>-</sup>, mB<sup>-</sup>) gal dcm</i> (DE3)   |                                |
| <i>E. coli</i> TOP10   | F <sup>-</sup> <i>mcrA Δ(mrr<sup>-</sup> hsdRMS<sup>-</sup> merBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (Str<sup>R</sup>) endA1 nupG</i> | Invitrogen                     |
| MG1655                 |  | Coli Genetic Stock Center (60) |
| NM1100                 | IN( <i>rrnD<sup>-</sup>rrnE</i> )1, <i>rph-1</i> , att PL <sub>C1857</sub> Red αβγTet <sup>R</sup>   |                                |
| VDC5173                | Derivative of NM1100 Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup>/pBAD24::yrdC<sub>Ec</sub></i> ; Km <sup>R</sup> /Amp <sup>R</sup>                                      | This study                     |
| VDC5174                | Derivative of NM1100 Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup>/pBAD24::yrdC<sub>Ec</sub></i> ; Km <sup>R</sup> /Amp <sup>R</sup>                                      | This study                     |
| VDC5217                | MG1655 transformed with plasmid pBY125; Amp <sup>R</sup>   | This study                     |
| VDC5218                | MG1655 transformed with plasmid pBY125; Amp <sup>R</sup>   | This study                     |
| VDC5219                | MG1655 transformed with plasmid pBY126; Amp <sup>R</sup>   | This study                     |
| VDC5220                | MG1655 transformed with plasmid pBY126; Amp <sup>R</sup>   | This study                     |
| VDC5221                | MG1655 transformed with plasmid pBY128; Amp <sup>R</sup>   | This study                     |
| VDC5222                | MG1655 transformed with plasmid pBY128; Amp <sup>R</sup>   | This study                     |
| VDC5223                | MG1655 transformed with plasmid pBY130; Amp <sup>R</sup>   | This study                     |
| VDC5224                | MG1655 transformed with plasmid pBY130; Amp <sup>R</sup>   | This study                     |
| VDC5227                | MG1655 transformed with plasmid pBAD24; Amp <sup>R</sup>   | This study                     |
| VDC5228                | MG1655 transformed with plasmid pBAD24; Amp <sup>R</sup>   | This study                     |
| VDC5241                | MG1655 transformed with plasmid pBY166; Amp <sup>R</sup>   | This study                     |
| VDC5333                | MG1655 transformed with plasmid pYC101.1; Amp <sup>R</sup>   | This study                     |
| VDC5333                | MG1655 transformed with plasmid pYC101.4; Amp <sup>R</sup>   | This study                     |
| VDC5335                | MG1655 transformed with plasmid pYC102.3; Amp <sup>R</sup>   | This study                     |
| VDC5336                | MG1655 transformed with plasmid pYC102.3; Amp <sup>R</sup>   | This study                     |
| VDC5337                | MG1655 transformed with plasmid pYC103.1; Amp <sup>R</sup>   | This study                     |
| VDC5337                | MG1655 transformed with plasmid pYC103.2; Amp <sup>R</sup>   | This study                     |
| VDC5339                | MG1655 transformed with plasmid pYC104.2; Amp <sup>R</sup>   | This study                     |
| VDC5340                | MG1655 transformed with plasmid pYC104.4; Amp <sup>R</sup>   | This study                     |
| VDC5342                | MG1655 transformed with plasmid pBY167; Amp <sup>R</sup>   | This study                     |
| VDC5350                | Derivative of VDC5217 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC5173; Km <sup>R</sup> , Amp <sup>R</sup>          | This study                     |
| VDC5351                | Derivative of VDC5219 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC5173; Km <sup>R</sup> , Amp <sup>R</sup>          | This study                     |
| VDC5354                | Derivative of VDC5217 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC5173; Km <sup>R</sup> , Amp <sup>R</sup>          | This study                     |
| VDC5355                | Derivative of VDC5219 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC5173; Km <sup>R</sup> , Amp <sup>R</sup>          | This study                     |
| VDC5358                | Derivative of VDC5223 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC5173; Km <sup>R</sup> , Amp <sup>R</sup>          | This study                     |
| VDC5359                | Derivative of VDC5223 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC517; Km <sup>R</sup> , Amp <sup>R</sup>           | This study                     |
| VDC5362                | Derivative of VDC5242 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC5173; Km <sup>R</sup> , Amp <sup>R</sup>          | This study                     |
| VDC5363                | Derivative of VDC5242 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC5173; Km <sup>R</sup> , Amp <sup>R</sup>          | This study                     |
| VDC5366                | Derivative of VDC5333 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC5173; Km <sup>R</sup> , Amp <sup>R</sup>          | This study                     |
| VDC5367                | Derivative of VDC5333 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC5173; Km <sup>R</sup> , Amp <sup>R</sup>          | This study                     |
| VDC5370                | Derivative of VDC5337 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC5173; Km <sup>R</sup> , Amp <sup>R</sup>          | This study                     |
| VDC5371                | Derivative of VDC5337 obtain from P1 transduction of the Δ <i>yrdC<sub>Ec</sub>-I::Km<sup>R</sup></i> allele of VDC5173; Km <sup>R</sup> , Amp <sup>R</sup>          | This study                     |
| Plasmids               |  |                                |
| pBN200                 | pET21a:: <i>yrdC<sub>Ec</sub></i>  | This Study                     |
| pBN204                 | pYES:: <i>SUA5</i> ; <i>URA3</i>   | This study                     |
| pBY125                 | pBAD24:: <i>ywlC<sub>Bs</sub></i> cloned as <i>NcoI/XhoI</i> fragment; Amp <sup>R</sup>  | This study                     |
| pBY126                 | pBAD24:: <i>yrdC<sub>Ec</sub></i> cloned as <i>NcoI/XhoI</i> fragment; Amp <sup>R</sup>  | This study                     |



Table 1. Continued

| Strain name | Relevant characteristics   | Reference  |
|-------------|--|------------|
| pBY128      | pBAD24:: <i>yciO<sub>Ec</sub></i> cloned as <i>NcoI/XhoI</i> fragment; Amp <sup>R</sup>  | This study |
| pBY130      | pBAD24:: <i>SUA5</i> cloned as <i>NcoI/PstI</i> fragment; Amp <sup>R</sup>   | This study |
| pBY135.1    | pYesDEST52:: <i>yrdC<sub>Ec</sub></i> ; Amp <sup>R</sup> <i>URA3</i>   | This study |
| pBY136.1    | pYesDEST52:: <i>yciO<sub>Ec</sub></i> ; Amp <sup>R</sup> <i>URA3</i>   | This study |
| pBY137.1    | pYesDEST52:: <i>ywlC<sub>Bs</sub></i> ; Amp <sup>R</sup> <i>URA3</i>   | This study |
| pBY138.1    | pYesDEST52:: <i>yrdC<sub>Mm</sub></i> ; Amp <sup>R</sup> <i>URA3</i>   | This study |
| pBY140      | pCR2.1::Up <i>yrdC<sub>Ec</sub></i> ; Amp <sup>R</sup> (999 base pairs of upstream region of <i>yrdC<sub>Ec</sub></i> cloned in pCR2.1)  | This study |
| pBY141.6    | pCR2.1::Up <i>yrdC<sub>Ec</sub></i> ::Dn <i>yrdC<sub>Ec</sub></i> ; Amp <sup>R</sup> (999 base pairs of upstream and downstream region of <i>yrdC<sub>Ec</sub></i> cloned in pCR2.1) | This study |
| pBY146.1    | pGEM-T easy:: Up <i>yrdC<sub>Ec</sub></i> ::Dn <i>yrdC<sub>Ec</sub></i> ; Amp <sup>R</sup>   | This study |
| pBY152.1    | pGEM-T easy::Up <i>yrdC<sub>Ec</sub></i> ::Km <sup>R</sup> ::Dn <i>yrdC<sub>Ec</sub></i> ( <i>ΔyrdC<sub>Ec</sub>-1</i> ::Km <sup>R</sup> cassette)                                   | This study |
| pBY166      | pBAD24:: <i>ycdC<sub>Mm</sub></i> ; Amp <sup>R</sup>   | This study |
| pBY167      | pBAD24:: <i>ycdC<sub>Mm</sub></i> ; Amp <sup>R</sup>   | This study |
| pYC101.1    | Derived from site directed mutagenesis of pBY126; <i>yrdC<sub>Ec</sub></i> <sup>Arg110→Ala</sup> ; Amp <sup>R</sup>  | This study |
| pYC101.4    | Derived from site directed mutagenesis of pBY126; <i>yrdC<sub>Ec</sub></i> <sup>Arg110→Ala</sup> ; Amp <sup>R</sup>  | This study |
| pYC102.2    | Derived from site directed mutagenesis of pBY126; <i>yrdC<sub>Ec</sub></i> <sup>Arg52→Ala</sup> ; Amp <sup>R</sup>   | This study |
| pYC102.3    | Derived from site directed mutagenesis of pBY126; <i>yrdC<sub>Ec</sub></i> <sup>Arg52→Ala</sup> ; Amp <sup>R</sup>   | This study |
| pYC103.1    | Derived from site directed mutagenesis of pBY126; <i>yrdC<sub>Ec</sub></i> <sup>Lys50→Ala</sup> ; Amp <sup>R</sup>   | This study |
| pYC103.2    | Derived from site directed mutagenesis of pBY126; <i>yrdC<sub>Ec</sub></i> <sup>Lys53→Ala</sup> ; Amp <sup>R</sup>   | This study |
| pYC104.2    | Derived from site directed mutagenesis of pBY126; <i>yrdC<sub>Ec</sub></i> <sup>Lys50→Ala</sup> ; Amp <sup>R</sup>   | This study |
| pYC104.4    | Derived from site directed mutagenesis of pBY126; <i>yrdC<sub>Ec</sub></i> <sup>Lys50→Ala</sup> ; Amp <sup>R</sup>   | This study |

YcEcyrdCol3/YcEcyrdCol2; YcEcyoiOol1/YcEcyoiOol2; YcMmyrdcol1/YcMmyrdCol2; YcBsywlCol1/YcBsywlCol2; then sequenced before recombination into pYES-DEST52 (carrying the *URA3* gene for selection on –ura media) (Invitrogen) using the Gateway LR Clonase Enzyme Mix according the manufacturer protocol. Plasmids were transformed into yeast strains according to the manufacturer protocol (Invitrogen, Carlsbad, CA). Transformants were screened on SD -ura plates and checked for presence of the orthologous gene *in trans* by PCR using the gene-specific primers used for cloning.

For cloning into pRS313 (28), primers CENupsua5.o11 and CENdnsua5.o12 were used to amplify the *SUA5* gene from yeast as well as 272 bp of upstream region and 124 bp of downstream region (Up*SUA5*Dn). The upstream region was designed based on previous data showing that the presence of a 190 bp fragment upstream of *SUA5* is sufficient to drive gene expression (4). The PCR product was cloned into pCR-Blunt TOPO (Invitrogen, Carlsbad, CA) yielding pBY168.6. After checking by sequencing, the Up*SUA5*Dn was cloned into pRS313 using the *EcoRI* restriction site flanking the insert in pCR-Blunt and the *EcoRI* site of pRS313. The resulting plasmid, pBY176.3 (pRS313::Up*SUA5*Dn), allows *SUA5* to be expressed under its native promoter and was used for complementation purposes.

For construction of pBN204, the yeast *SUA5* gene was amplified using forward primer BN1 and reverse primer BN2 from plasmid pM249 (4). The forward primer included the yeast initiation sequence AAUA and a *BamHI* site. The reverse primer included an *XhoI* site. These sites were used to ligate this DNA fragment into pYES2 (*URA3*) (Invitrogen) to generate pBN204. For construction of pBN200, *yrdC<sub>Ec</sub>* was amplified from genomic DNA using primers BN3 and BN4 before cloning into

pET21b (Novagen, Inc., Madison, WI) using *NdeI* and *XhoI*.

#### Construction of the *E. coli ΔyrdC<sub>Ec</sub>-1*::Km<sup>R</sup> allele

Fragments upstream (908 bp) and downstream (991bp) of *yrdC<sub>Ec</sub>* were PCR amplified from *E. coli* MG1655 genomic DNA using US71\_o11 and UP979\_o12, and DS1561\_o11 and DS2552\_o12, respectively. For subcloning purposes, DS1561\_o11 and DS2552\_o12 contained *NotI* and *XhoI* restriction sites respectively. The *yrdC<sub>Ec</sub>* upstream fragment (Up*yrdC<sub>Ec</sub>*) was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). The resulting plasmid pCR2.1::Up*yrdC<sub>Ec</sub>* (pBY140) was checked for orientation. The *yrdC<sub>Ec</sub>* downstream fragment (Dn*yrdC<sub>Ec</sub>*) was digested with *NotI* and *XhoI* and ligated into pBY140 digested with the same enzymes to give plasmid pBY141.6. The Upstream::Downstream fragment from pBY141.6 was then subcloned into pGEM-T easy vector (Promega) using the *ApaI* and *SacI* sites, and the resulting plasmid pBY146.1 was then digested with the *EcoRV* site present between the upstream and the downstream fragments and used to clone the Km<sup>R</sup> cassette (containing FRT sites) that had been amplified from pKD4 (29) using the FRTK<sub>n</sub>\_o11 and FRTK<sub>n</sub>\_o12 primers. The resulting plasmid pGEM-T easy::Up*yrdC<sub>Ec</sub>*::Km<sup>R</sup>::Dn*yrdC<sub>Ec</sub>* (pBY152.1) was then used as a template to generate by PCR the Up*yrdC<sub>Ec</sub>*::Km<sup>R</sup>::Dn*yrdC<sub>Ec</sub>* deletion cassette used for the replacement of *yrdC<sub>Ec</sub>*. Induction of λ recombination functions and preparation of NM1100/pBAD24::*yrdC<sub>Ec</sub>*, NM1100/pBAD24::*yciO<sub>Ec</sub>*, and NM1100/pBAD24 competent cells was done as described in (30). After electroporation, cells were plated on LB Km supplemented with 0.02% arabinose. Km resistant colonies were re-isolated twice and checked for gene replacement using Km-specific primers [k1 and k2, (29)] in

**Table 2.** Primers used in this study

| Primer name         | Primer sequence                                |
|---------------------|--|
| BADBSywlCol1        | CCATGGAAACGAAAAGATGGTTT                        |
| BADBSywlCol2        | AAGCTTTTCAGCGAATCACTCTTCCTCC                   |
| BADEycyiOol1        | CCATGGGCCAGTTTTTTTATATTCAT                     |
| BADEycyiOol2        | AAGCTTTTATAAGAAAAGGCTTCACATCAC                 |
| BADEyrdCol1         | CCATGGATAATAACCTGCAAAGAGA                      |
| BADEyrdCol2         | AAGCTTTTACCCCTGTGCGAAACAGTTC                   |
| BADMmyrdCol1        | CCATGGAAACTTTCGAGATTTTCAGAAA                   |
| BADMmyrdCol2        | AAGCTTTTATTCATTTACTGCTTTTAAAATCTC              |
| BADsua5ol1          | CCATGGACCTTGGACGACATTTTTT                      |
| BADsua5ol2          | CTGCAGTTAAAACTGTATACAATTATTTGCAG               |
| YcMmyrdcol1         | CACCATGAAAACCTTTCGAGATTTTCAGAAAGT              |
| YcMmyrdCol2         | TCATTATTCATTTACTGCTTTTAAAATCTC                 |
| YcBsywlCol1         | CACCATGAAAACGAAAAGATGGTTTGTG                   |
| YcBsywlCol2         | TCAGCGAATCACTCTTCCTCCGG                        |
| YcEycyiOol1         | CACCATGAGCCAGTTTTTTTATATTCATC                  |
| YcEycyiOol2         | TCATTATAAGAAAAGGCTTCACATCAC                    |
| YcEyrdCol1          | CACCATGAATAATAACCTGCAAAGAGA                    |
| YcEyrdCol3          | TCATTACCCCTGTGCGAAACAGTTCA                     |
| CENupsau5.ol1       | GGTACCTATTTTCGTTGAAAAATTCAGCGC                 |
| CENupsau5.ol2       | GGTACCGTATGGGCGACTTTTCGTAT                     |
| BN1                 | CGGCGCTCAAUAATGTACCTTGGACGACATTTTTTGGCA        |
| BN2                 | CGACCAATGCTCGAGTTAAAACTGTATACAATTATTTGCAGC     |
| BN3                 | CAG CCG CAT ATG AAT AAT AAC CTG CAA AGA GAC GC |
| BN4                 | GCC TCG CTC GAG CCC CTG TCG AAA CAG TTC ACC CG |
| US71_ol1            | ACA ATG CCC TGC TAT GGC TG                     |
| US979_ol2           | GTT ATT ATT CCG CCG AAA CCG                    |
| DS1561_ol1          | TTA GCG GCC GCG GAC AGG GGT AAC ATA ATG G      |
| DS2552_ol2          | TTA CTC GAG GCG ATG CTG ACG AAA ACT CG         |
| FRTKno11            | GTGTAGGCTGGAGCTGCTC                            |
| FRTkno12            | CTTAGTTCCTATTCGGAAGTTC                         |
| sua5::Leu.ol1       | TTT CGT TGA AAA ATT CAG GC                     |
| sua5::Leu.ol3       | GGG CGA CTT TTC GTA TAT ACA                    |
| Upleu2_ol1          | AGA TCT CAC ACA GGG GCG CTA TCG C              |
| Dsleu2_ol2          | AGA TCT TAT AAA GTT TAT GTA CAA ATA TCA        |
| Chksua5leuol4       | TTG CCA ATA CAA CAT AAC CG                     |
| Chksua5leuol5       | GTT ATT GCT CAT CAG CAG TA                     |
| EcyrdCLys50Ala.ol1  | ACTGTTGGAGTTAGCGCAGCGTCCGGTTG                  |
| EcyrdCLys50Ala.ol2  | CAACCGGACGCTGCGCTAACTCCAACAGT                  |
| EcyrdCLys56Ala.ol1  | CAGCGTCCGGTTGATGCGGGGCTGATTTTAATC              |
| EcyrdCLys56Ala.ol2  | GATTAATAATCAGCCCCGATCAACCGGACGCTG              |
| EcyrdCArg52Ala.ol1  | GAGTTAAAACAGGCGCCGGTTGATAAGGGGC                |
| EcyrdCArg52Ala.ol2  | GCCCCTTATCAACCGGCGCCTGTTTTAACTC                |
| EcyrdCArg110Ala.ol1 | CTGTTGACGGGCGCGTTTGATTCGCTTGCTG                |
| EcyrdCArg110Ala.ol2 | CAGCAAGCGAATCAAACGCGCCCGTCAACCAG               |
| Leucassol3          | AGT CAT CGA ATT TGA TTC TG                     |

combination with upstream and downstream primers (UP71\_ol1 and DN2552\_ol2) as described in the text.

### Construction of the *sua5-Δ1::LEU2* allele

Primers sua5leu.ol1 and sua5leu.ol3 were used to amplify a 1671 bp fragment encompassing *SUA5*. This fragment was cloned into pGEMT easy (Promega, Madison, WI) yielding pBY154. A *Bgl*III site exists within the amplified fragment and when used deletes about 1000 bp from the amplified fragment, while retaining, 225 bp of homology region on the 5' side (56 of which are the 56 first bp of *SUA5*) and 323 bp of homology on the 3' side. A leucine cassette was amplified from wild-type BY4743 genomic DNA using primers Upleu.ol1 and DNleu.ol2 that contained *Bgl*III sites and ligated into pBY154 after digestion of both vector and insert by *Bgl*III. The resulting plasmid, pBY164 was used as template to amplify a Up::*sua5-Δ1::Dn::LEU2* cassette using sua5leu.ol1 and

sua5leu.ol3 primers. The Up::*sua5-Δ1::Dn::LEU2* PCR product was used to transform yeast cells. Transformants were plated on SD Gal/Raf -leu and verified by PCR. To cure the *URA3* marker containing plasmids (plasmid derived from pYES-DEST52 and pYES2), 10 μl of serial dilutions of yeast liquid cultures grown in SD Gal/Raf -leu were spotted onto two different types of plates. Plates containing SD Gal/Raf -ura -leu medium to check for the presence of expected markers and 5-FOA medium (SD Gal/Raf supplemented with 50 μg/ml of uracil and 0.1% 5-FOA) to select yeast cells that did not retain the *URA3* carrying plasmid [in presence of the *URA3* gene 5-FOA becomes toxic (31)].

### Construction of point mutations in *yrdC<sub>Ec</sub>* by site-directed mutagenesis

Plasmid pBY126 (pBAD24::*yrdC<sub>Ec</sub>*) was used as a template for mutagenesis. Primer pairs EcyrdCLys50Ala.

ol1/EcyrdCLys50Ala.ol2, EcyrdCArg52Ala.ol1/EcyrdCA rg52Ala.ol2, EcyrdCLys53Ala.ol1/EcyrdCLys53Ala.ol2 and EcyrdCArg110Ala.ol1/EcyrdCArg110Ala.ol2, were used to mutate Lysine at position 50, Arginine at position 52, Lysine at position 53 and, Arginine at position 110 into Alanine. Mutagenesis was performed as described in the QuickChanges™ Site Directed Mutagenesis Kit from (Stratagene, La Jolla, CA). All plasmids were checked by sequencing.

### Growth curves

Growth curves were performed using a Bioscreen C MBR (Oy Growth Curves AB Ltd, Finland) at 30° and at maximum shaking. 300 µl of culture was used in each well, and 10 replicates were used for each condition. Yeast cultures were grown on SD Gal/Raf -his to saturation diluted 200 times in SD Gal/Raf -his before loading on the Bioscreen. The growth curves presented are representative of 10 replicates.

### Purification of bulk tRNA

For *E. coli* derivatives, 1 liter of cultures were grown overnight at 37°C in LB media supplemented with ampicillin (50 µg/ml) and arabinose when required, harvested, and then stored at -20°C. The cell pellets were defrosted and resuspended in 20 ml of buffer (10 mM Tris, 10 mM MgCl<sub>2</sub>, pH 7.4). For yeast cultures, 2 l of cell cultures were grown to saturation at 30°C, harvested and pellets immediately resuspended in 20 ml of buffer (10 mM Tris, 10 mM MgCl<sub>2</sub>, pH 7.4). In both cases, the homogenate was treated with buffer-saturated phenol, pH 7.4 (Sigma), the aqueous layer was collected after the phenol extraction step, and the RNA was precipitated with ethanol. The bulk tRNA was then purified on Nucleobond AX-400 columns (Clontech Laboratories, Mountain View, CA) (according to the manufacturer's protocol) and precipitated with isopropanol.

### HPLC separation coupled with electrospray tandem mass spectrometry analysis of digested bulk tRNA

To compare the nucleoside constituents of the bulk tRNA purified from the different strains, samples of each purified bulk tRNA preparation were enzymatically hydrolyzed as described by Pomerantz *et al.* (32). Approximately 100 µg of bulk tRNA was digested with 10 units of Nuclease P1 (Sigma), 0.01 units of Phosphodiesterase I (Sigma) and 3 µl of *E. coli* Alkaline Phosphatase (Sigma P4252) in a total volume of 113 µl. The digested extracts were lyophilized and resuspended in 20 µl of water. The resuspended tRNA degradation extracts were injected in a LC-MS/MS system for separation and identification of the nucleosides. A C18-RP (Gemini 5µ, 250 × 4.6 mm, Phenomenex, Torrance, CA, 14 USA) analytical column was used, with a C18 cartridge to prolong its lifetime. The mobile phase consisted of two solvents: 250 mM of ammonium acetate pH6.0 for solvent A and 40% acetonitrile for solvent B. The gradient used for nucleoside separation was performed as previously described (32). It consisted in an elution gradient of several steps: 0–5% B in 7 min, 5–10% B in 15 min, 25–50%

B in 5 min, 50–75% B in 4 min and stay at 75% B for 3 min, then 75–100% B in 8 min. The column was then washed at 100% B for 7 min and re-equilibrated at 100% A for 10 min. The flow rate used was 1 ml/min. Chromatography was performed at room temperature. The elution of nucleosides was followed by UV detection at 254 nm. The HPLC was coupled to a hybrid triple quadrupole-ion trap (4000 Q-TRAP, Applied Biosystems, Foster City, CA, USA) mass spectrometer equipped with a TurboIonSpray (TIS) interface operated in the positive ion mode. The ESI parameters were: curtain gas (CUR) at 30 psi, the ion source (IS) at 5000V, nebulizer gas (GS1) at 45 psi, TIS gas (GS2) at 45 psi and the TIS probe temperature at 300°C. The information-dependent acquisition (IDA) mode of operation was employed in which a survey scan from *m/z* 100–600 was acquired followed by collision-induced dissociation (CID) of the two most intense ions. Survey and MS/MS spectra for each five IDA cycle were accumulated for 1 s and 3 s, respectively. tRNA extractions and analysis were performed at least twice independently.

### HPLC analysis

After digestion, samples were immediately used for analysis on HPLC. For that, 100 µl of digested sample was added to a glass HPLC micro-vial and run using 250 mM ammonium acetate pH 6 (Organic Solvent A) and 40% Acetonitrile (Aqueous Solvent B). Samples run for 60 min at a flow rate of 1.5 ml/min in a Suppleco Discovery C-18 Reverse Phase column (15 cm × 4.6 mm × 5 µm) (Suppleco 504955) detected by a Waters 2487 Dual Wavelength UV/Vis detector. Samples were injected by a Waters 717 plus Autosampler, and pumped by a Waters 1525 Binary Pump with a Waters Inline Degasser. Analysis was done by Waters Breeze Software v3.20. The method used was a linear gradient consisting of 98% solvent A and 2% solvent B at start with a flow rate of 1.5 ml/min, decreasing to 80% A and 20% B at 60 min with no change in flow rate.

### Preparation of tRNA<sup>Thr</sup>

The plasmid containing the *E. coli* tRNA<sup>Thr</sup><sub>CGU</sub> gene *thrW* was a kind gift of Dr Waas (The Scripps Research Institute). tRNA<sup>Thr</sup><sub>CGU</sub> was overexpressed in MRE600 cells induced with isopropyl-β-D-thiogalactoside (IPTG, 0.5 mM, 37°C, 12 h) in LB-Amp (100 µg/ml). The harvested cells were lysed in buffer (0.3 M NaOAc, pH 5.2, 10 mM EDTA) and subjected to phenol extraction (pH 4.5) and ethanol precipitation. tRNA<sup>Thr</sup> was partially purified from total nucleic-acid extract on a Nucleobond column (Clontech Laboratories, Mountain View, CA) using manufacturer protocols, precipitated with isopropylalcohol (100%, room temperature), washed with ethanol (80%, -20°C), and dissolved in water, at which point it was 80% pure. tRNA<sup>Thr</sup> was further purified on a C4 reverse-phase preparative column pre-equilibrated with buffer A (10 mM sodium phosphate pH 5.5, 1 M sodium formate, 8 mM MgCl<sub>2</sub>) and eluted with a 60-min 0–40% gradient of buffer B (10 mM sodium phosphate pH 5.5, 10% methanol). The tRNA eluted in two equal-sized



peaks. Upon assaying the fractions for threonine acceptance from *E. coli* threonyl-tRNA synthetase using a standard aminoacylation assay as described in (33), both peaks were found to contain tRNA<sup>Thr</sup> at >97% purity. Treatment with base (pH 8.5) of a pooled sample from both peaks yielded the same two separate peaks by analytical reverse-phase chromatography, indicating that the two species do not correspond to different aminoacylation states. Fractions for the two peaks were pooled separately and tRNA was precipitated, washed twice with cold ethanol, and re-dissolved in water. MALDI-TOF mass spectrometry analysis of final tRNA from the two peaks revealed molecular masses of 24 540 and 24 686 Da. The mass difference is accounted for by the molecular mass of carbamoyl-threonine (145.12 Da).

The tRNA<sup>Thr</sup> transcript was synthesized by standard T7 polymerase transcription (34) of a linearized DNA template [pUC18; (35)] containing the *E. coli* tRNA<sup>Thr</sup><sub>CGU</sub> gene *thrW* (kind gift of Dr K. Beebe, The Scripps Research Institute). After phenol-chloroform extraction of the reaction, the RNA was ethanol precipitated, re-dissolved in water and purified by anion-exchange high-performance liquid chromatography on a heated (90°C) preparative DNA-Pac column (Dionex, Sunnyvale, CA) according to standard protocols (34). The single tRNA<sup>Thr</sup> eluant was pooled, ethanol precipitated, re-suspended in water and assayed for threonine acceptance. Final purity was >95% by 10% urea PAGE analysis. All final tRNA samples were prepared for binding studies by heating to 90°C and slow-cooling after addition of 5 mM MgCl<sub>2</sub> to ensure proper folding.

### Overexpression and purification of the *E. coli* YrdC protein (YrdC<sub>Ec</sub>)

*E. coli* BL21(DE3) cells were transformed with a pET21 derivative (Novagen) vector containing the *E. coli* *yrdC* gene (pBN200). The protein was overexpressed by induction with 1 mM IPTG in LB medium containing 100 µg/ml Amp. Cells were harvested and lysed in buffer (20 mM Tris, 500 mM NaCl, 28 mM imidazole, 1 mM β-mercaptoethanol and protease inhibitor cocktail). The protein was purified by Ni-NTA affinity chromatography, dialyzed against phosphate-buffered saline and concentrated using a YM-3 Centriprep filter (Millipore Corp., Billerica, MA).

### Measurements of YrdC<sub>Ec</sub> binding to tRNA by Trp fluorescence quenching

We used steady-state techniques to monitor changes in the intrinsic fluorescence of the two tryptophan side chains (at positions 89 and 106) of *E. coli* YrdC upon addition of tRNA as previously described (2). Using a Jobin Yvon FluoroMax-3 spectrofluorometer (Edison, NJ), with a cell compartment kept at a constant temperature of 21°C, the fluorescence signal ( $\lambda_{\text{ex}} = 295$  nm;  $\lambda_{\text{em}} = 345$  nm) of free YrdC (2 µM in 20 mM HEPES, 100 mM NaCl, pH 7.5) was taken. Defined volumes of a concentrated aqueous solution of tRNA were then added in steps to final tRNA concentrations of 0.05–3.50 µM

and the fluorescence signal was taken after a 3-min incubation period at each step to allow for complete binding. To correct for dilution effects, the same titration was repeated with the addition of the same incremental volumes of water. All readings were corrected for buffer absorption without need for inner filter correction (absorbance at 295 nm by tRNA at its highest concentration was less than 0.05). Theoretical curves were fitted to the experimental data and dissociation constants were determined according to the following equation (36):

$$\frac{I}{I_0} = 1 + (f-1) \left( \frac{(K_d + P_0 + N_0) - \text{aqrt}((K_d + P_0 + N_0)^2 - (4P_0N_0))}{2P_0} \right)$$

where  $I_0$  and  $I$  are the corrected fluorescence intensities of protein and protein-tRNA complex, respectively.  $P_0$  and  $N_0$  are the total concentrations of protein and tRNA, respectively,  $f$  is the fluorescence intensity ratio of bound and free protein, and  $K_d$  is the dissociation constant.

### Detection of ATP binding by STD-NMR

NMR experiments were performed at 278K on a Varian INOVA 600 NMR spectrometer equipped with a 5 mm triple resonance inverse detection probe. Samples were prepared in 500 µl of 99% D<sub>2</sub>O PBS buffer at pH 7.5 (uncorrected) containing 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl<sub>2</sub> and 0.02% NaN<sub>3</sub>. Concentration of the NMR sample was 20 µM protein and 1 mM ligand. The STD pulse sequence was implemented as suggested in (37), with minor variations. The excitation train was composed of 50 ms G4 composite pulses with 1 ms delay between pulses. On-resonance and off-resonance irradiations were centered at -1 ppm and 30 ppm, respectively, and the total saturation time was 3 s. Initial FIDs were multiplied by a line broadening function of 1 Hz and zero filled to twice the original data size prior to Fourier transformation. A total of 512 scans with 16 dummy scans were acquired, with a recycle delay of 2 s.

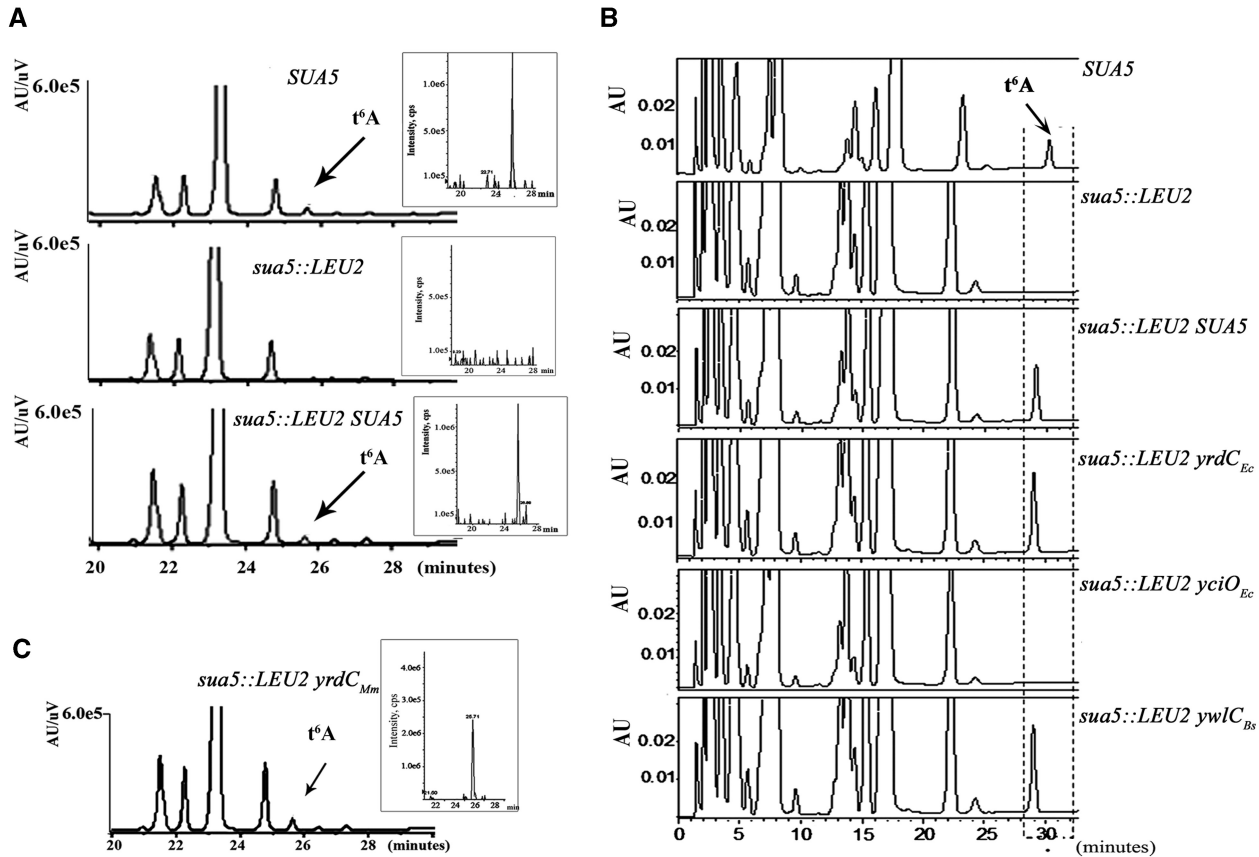
## RESULTS

### Involvement of the Sua5/YrdC family of proteins in t<sup>6</sup>A formation

*Bioinformatic identification.* Analysis of all sequenced tRNAs (38) suggested that all organisms should have a t<sup>6</sup>A biosynthesis pathway. Using the MGDB database (<http://mbgd.genome.ad.jp/>), a list of orthologous families present in *E. coli* K12, *S. cerevisiae*, *B. subtilis* 168, *Buchnera aphidicola* APS, *Wigglesworthia glossinidia*, *Wollbachia endosymbiot mel* and all the mollicutes in the database (16 genomes) was generated. This analysis revealed the existence of 95 orthologous protein families, mostly related to translation (ribosomal protein, tRNA synthetases and tRNA modification). Only nine of these families did not have an experimentally verified function when this work was initiated but many have been







**Figure 2.** (A) LC-MS/MS analysis of yeast tRNA extracted from different strains linking the disappearance of the  $t^6A$  peak to the deletion of *sua5*. UV traces and ion extraction chromatograms for 413  $m/z$  (small windows) are shown for WT (YMH13) upper panels, *sua5::LEU2* (YJN63) middle panels and YGN63 transformed with pBN204 (*SUA5 URA3*) lower panels. (B) Complementation of the  $t^6A$  minus phenotype followed by HPLC analysis of bulk yeast tRNA extracted from various strains. The position of the  $t^6A$  peak was confirmed by running a  $t^6A$  standard and spiking the WT sample (data not shown). Strain YGN63 was transformed with *URA3* plasmids carrying *SUA5* (as a control) or carrying wild-type genes *yrdC<sub>Ec</sub>*, *yciO<sub>Ec</sub>* and *ywC<sub>Bs</sub>*, respectively and the recovery of the  $t^6A$  peak was monitored. (C) LC-MS/MS analysis of yeast tRNA extracted from the *sua5::LEU2 yrdC<sub>Mm</sub>* strain. UV traces and ion extraction chromatograms for 413  $m/z$  (small windows) are shown. Positive and negative controls were run as presented in (A) (data not shown).

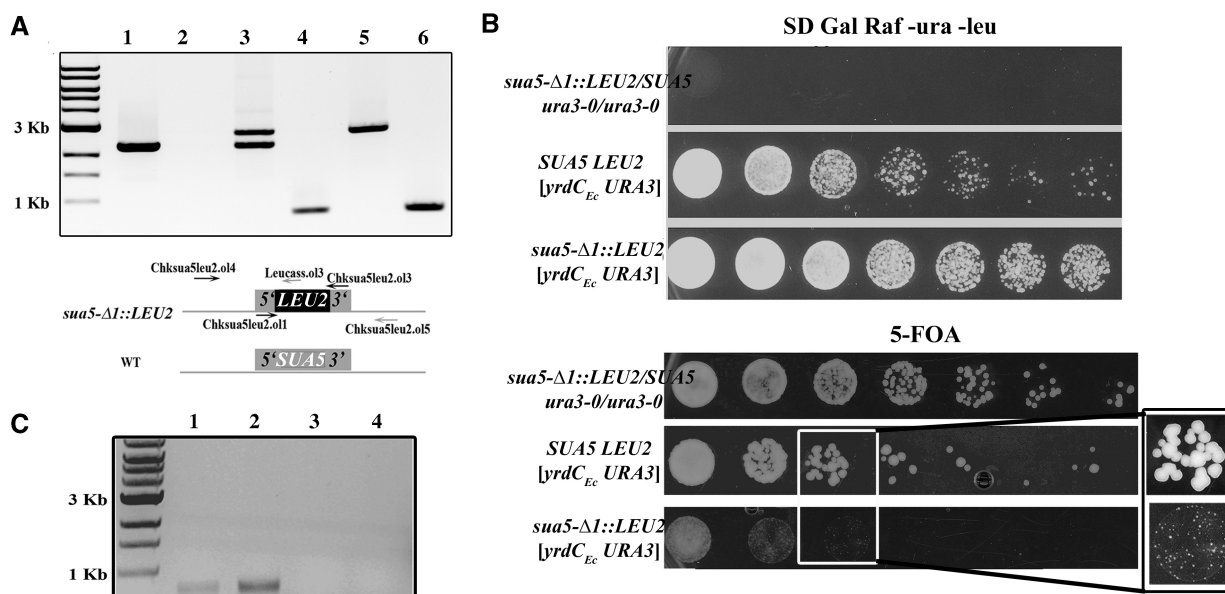
**In vivo experimental validation.** In order to examine the  $t^6A$  phenotype (i.e. presence or absence of  $t^6A$  modification in RNA) of strains lacking members of the *SUA5/yrdC* gene family an existing strain, YJN63 (*sua5::LEU2*) (4), which carries a deletion allele of *SUA5* was analyzed. Bulk tRNA was extracted from both YGN63 and a near isogenic wild-type strain YMH13 (*SUA5*) (4) and processed for LC-MS/MS analysis as described in the ‘Materials and Methods’ section. This analysis revealed that the 25.5 min peak detected on the UV trace in the parent strain, corresponding to the protonated molecular weight of  $t^6A$  ( $MH^+ = 413m/z$ ), disappeared in the strain carrying the *sua5::LEU2* allele (Figure 2A). The  $t^6A$  peak was recovered when this strain was transformed with a plasmid carrying the wild-type *SUA5* gene (plasmid pBN204) confirming that the  $t^6A^-$  phenotype (absence of  $t^6A$  peak) was due to the loss of *SUA5* (Figure 2A).

To determine whether *sua5* homologs were actual functional orthologs, *E. coli*, *B. subtilis* and *M. maripaludis* *yrdC*-like genes were tested for their ability to complement the  $t^6A^-$  phenotype of the *sua5::LEU2* strain. YGN63 was transformed with *URA3* plasmids carrying the following

wild-type genes: *yrdC<sub>Ec</sub>* (pBY135.1), *yciO<sub>Ec</sub>* (pBY136.1), *ywC<sub>Bs</sub>* (pBY137.1) or *yrdC<sub>Mm</sub>* (pBY138.1). Bulk tRNA was then extracted and analyzed to check for the presence of  $t^6A$  in the transformed strains. All genes with the exception of *yciO<sub>Ec</sub>*, were able to complement the  $t^6A^-$  phenotype of YJN63 (Figure 2B and C).

### Essentiality of *yrdC* orthologs

**Essentiality of *S. cerevisiae* *SUA5* wild-type gene.** In order to clarify the contradictory data on the essentiality of *SUA5*, the replacement of the wild-type allele by a *sua5-Δ1::LEU2* cassette was attempted in three different backgrounds (see ‘Material and Methods’ section for construction details): the diploid, BY4743 (*SUA5/SUA5 ura3Δ0/ura3Δ0 leu2-Δ0/leu2-Δ0*), the haploid BY4741 (*SUA5 ura3Δ0 leu2-Δ0*) and VDC5309 (*SUA5 yrdC<sub>Ec</sub> URA3 ura3Δ0*) which is strain BY4741 transformed with a *yrdC<sub>Ec</sub> URA3* carrying plasmid (pBY135.1).  $Leu^+$  clones were obtained in all three backgrounds, however, when analyzed by PCR, only clones derived from the diploid BY4743 and the merodiploid VDC5309 carried the *sua5-Δ1::LEU2* marker in the right locus, yielding



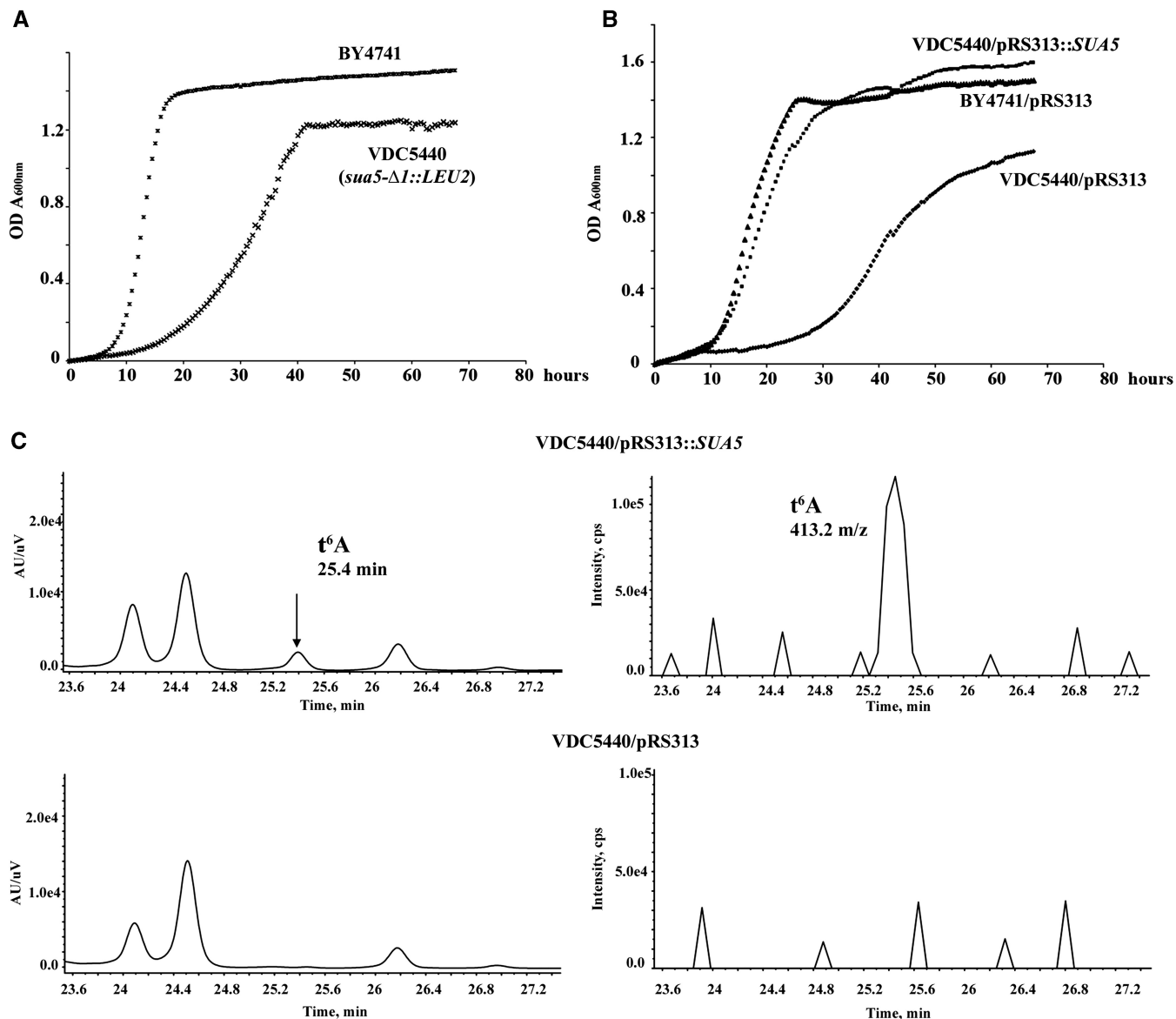
**Figure 3.** (A) PCR amplification to confirm the presence and correct location of the *sua5-Δ1::LEU2* allele in various yeast strains. Lanes 1, 3 and 5: primers Chksua5leu.o14 and Chksua5leu.o15 and lanes 2, 4 and 6: primers Chksua5leu.o14 and Leucass.o13. Strains VDC5417 (BY4741 derivative *SUA5 LEU2 URA3 yrdC<sub>Ec</sub>*) lanes 1/2; VDC5300 (BY4743 derivative (*SUA5/sua5-Δ1::LEU2*) lanes 3/4; VDC5416 (BY4741 derivative *sua5-Δ1::LEU2 yrdC<sub>Ec</sub>*) lanes 5/6. Strains were tested with both primer combinations. In VDC5417 the cassette has inserted ectopically, i.e. Leu<sup>+</sup> and negative in PCR tests using the Chksua5leu.o14/Leucass.o13 primer set. For both VDC5033 and VDC5416 insertion of the cassette at the correct location was confirmed. Expected sizes are: 750 bp for *sua5-Δ1::LEU2* allele at the right locus using the Chksua5leu.o14/Leucass.o13 combination and 2300 bp for the wild-type allele *SUA5* and 2800 bp for the *sua5-Δ1::LEU2* allele using the Chksua5leu.o14/Chksua5leu.o15 primer combination. Annealing positions for primers used are shown at the bottom of panel A. Only relevant genotypes are shown, refer to Table 1 for completeness. (B) FOA curing of pBY135.1 (*URA3 yrdC<sub>Ec</sub>*) from strains VDC5300 (*SUA5/sua5-Δ1::LEU2*), VDC5417 (*SUA5 LEU2 URA3 yrdC<sub>Ec</sub>*) and VDC5416 (*sua5-Δ1::LEU2 yrdC<sub>Ec</sub>*). 20 μl of serial dilutions of saturated cultures of VDC5416, VDC5417 and VDC5300 were replica-plated on SD Gal/Raf -ura -leu and FOA plates and analyzed after 5 days. Strains carrying the *URA3* marker are unable to grow on 5-FOA. VDC5300 was used as a control for auxotrophies. (C) Verification of cured strains by PCR using the plasmid specific primers DEST52.o11 and DEST52.o12: lane 1, VDC5417; lane 2, VDC5416; lane 3 and 4 the corresponding respective FOA treated derivatives.

*SUA5/sua5-Δ1::LEU2* and *sua5-Δ1::LEU2 yrdC<sub>Ec</sub>* derivatives, respectively (Figure 3A). In the haploid background BY4741, ectopic insertion of the cassette occurred yielding *SUA5 LEU2* strains, but no correct insertion of the deletion cassette could be obtained. Thus, *SUA5* could only be disrupted in backgrounds where a functional copy remained present in the genome, suggesting that *SUA5* is an essential gene in yeast, and in agreement with the result of the systematic yeast gene deletion study (11).

In order to further demonstrate the essentiality of *SUA5*, attempts were made to dissociate the *sua5-Δ1::LEU2* allele from the *URA3 yrdC<sub>Ec</sub>* plasmid pBY135.1. If *SUA5* is essential then the plasmid should not be curable from VDC5416 (*sua5-Δ1::LEU2* pBY135.1), while it should be readily lost from VDC5417 (*SUA5 LEU2* pBY135.1). Since the *URA3* allele carried on the plasmid confers sensitivity to 5-FOA, strains were tested for their ability to grow in the presence of this compound. Culture dilutions of VDC5416 were plated on 5-FOA medium and the resulting growth phenotype compared to that of two control strains: VDC5417 carrying an ectopic insertion of the deletion cassette and the heterozygote VDC5300 (*SUA5/sua5-Δ1::LEU2 ura3Δ0/ura3Δ0*). Derivatives from both control strains grew in presence of 5-FOA as expected. After prolonged incubation, 5-FOA<sup>R</sup> derivatives of *sua5-Δ1::LEU2* pBY135.1 were also recovered suggesting that pBY135.1 could be eliminated from this

background (Figure 3B). Both the curing of pBY135.1 and the *sua5-Δ1::LEU2* genotype were confirmed by PCR (Figure 3C) and by plating on SD Gal/Raf agar plates lacking either uracil/leucine or leucine (data not shown). These results showed that the *SUA5* gene is not essential in yeast confirming results of a previous study (4). Further analysis of the growth phenotype of *sua5-Δ1::LEU2* strains confirmed the growth defect phenotypes observed on FOA plates. Growth experiments on YPD show a dramatic decrease in growth rate of cured strain VDC5440 compared to the haploid parent strain BY4741 (Figure 4A). A plasmid carrying *SUA5* under the control of its endogenous promoter region (pBY176.3) complemented both the t<sup>6</sup>A deficiency of VDC5440 and the slow growth phenotype (Figure 4B and C), confirming that it was not due to a secondary mutation. These results show that neither the gene nor the modification is essential in yeast. The failure to obtain the haploid mutant by direct transformation is most likely due to its reduced growth phenotype.

*Essentiality of E. coli yrdC.* To test whether the *yrdC<sub>Ec</sub>* gene is essential or not in *E. coli*, a similar approach as that employed for yeast was followed. Homologous replacement of the wild-type *yrdC* allele by a  $\Delta yrdC_{Ec}$ -1::Km<sup>R</sup> allele was attempted by direct transformation of a *U<sub>pyrdC<sub>Ec</sub></sub>*::Km<sup>R</sup>::D<sub>nyrdC<sub>Ec</sub> PCR generated fragment</sub>



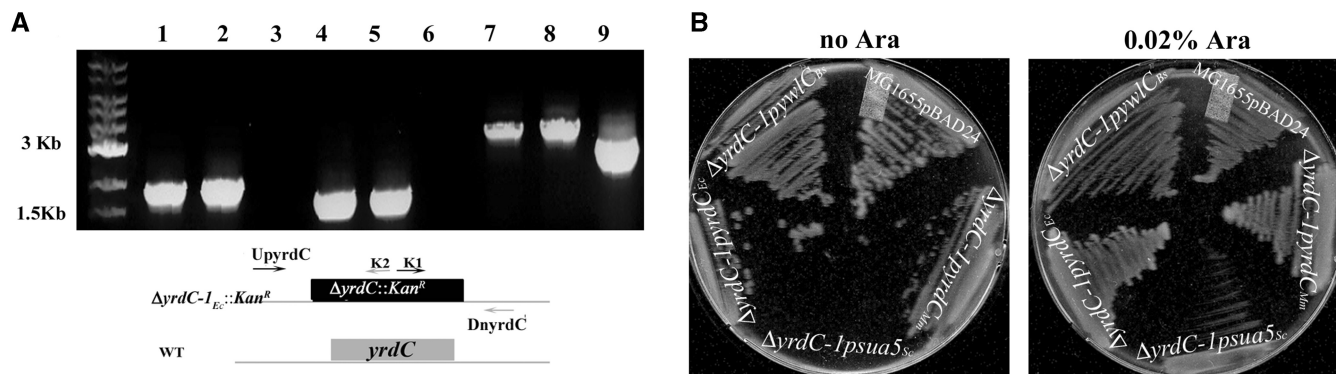
**Figure 4.** (A) Growth phenotype of the VDC5440 (*sua5-Δ1::LEU2 ura3Δ0*) compared to the WT parent BY4741 (B) Growth phenotype of VDC5440 transformed with plasmid pRS313 carrying *SUA5* under the control of its endogenous promoter region (pBY176.3) or empty plasmid control pRS313 on SD Gal/Raf<sup>-</sup>-his. All growth experiments were performed at 30°C. Shown here are representative plots out of ten replications. (C) LC-MS/MS analysis of tRNA extracted from yeast strains showing the absence or presence of the t<sup>6</sup>A peak on the UV trace (left side) and extraction chromatogram (right side) for VDC5440 transformed with pBY176.3 (upper panels) and VDC5440 transformed with pRS313 (lower panels).

into three different backgrounds: *E. coli* NM1100, NM1100/pBAD24 and NM1100/pBAD24::*yrdC<sub>Ec</sub>* (pBY126) (see 'Material and Methods' section), the latter strain contains a functional copy of *yrdC<sub>Ec</sub>* on a plasmid. Km<sup>r</sup> resistant clones with insertion of the marker in the expected location (as verified by PCR) were obtained in NM1100/pBAD24::*yrdC<sub>Ec</sub>* but not in the two other backgrounds suggesting that the chromosomal copy of the gene can only be eliminated if its function is insured by a gene *is trans* (Figure 5A).

The essentiality of *yrdC<sub>Ec</sub>* was further demonstrated by P1 transduction experiments. Strain VDC5173 (NM1100 Δ*yrdC<sub>Ec</sub>-1::Km<sup>R</sup>/pBAD24::*yrdC<sub>Ec</sub>*) was used as donor for*

P1 transduction with the following strains used as recipients: MG1655 transformed with pBAD24 derivatives carrying either functional *yrdC* homologs, *yrdC<sub>Ec</sub>*, *yrwC<sub>Bs</sub>*, *yrdC<sub>Mm</sub>* or *SUA5<sub>Sc</sub>* (pBY126, pBY125, pBY167 and pBY130 respectively) or carrying the non-functional homolog *yciO<sub>Ec</sub>* (pBY128). P1 transductions were carried at least five times in two independent MG1655 backgrounds for each ortholog, Km-resistant colonies were recovered in all backgrounds (with a variation of 120–500 colonies per transduction) except MG1655/pBAD24 and MG1655/pBAD24::*yciO<sub>Ec</sub>* that never yielded any colonies (while non-essential markers were readily transduced in these strains). These data confirm





**Figure 5.** (A) PCR amplification to confirm the presence of the  $\Delta yrdC_{Ec}-I::Km^R$  allele in kanamycin-resistant NM1100/pBAD24::yrdC<sub>Ec</sub> derivatives. Two independent  $Km^R$  strains (VDC5173, lanes 1, 4 and 7 and VDC5174 lanes, 2, 5 and 8) obtained from transformation of NM1100/pBAD24::yrdC<sub>Ec</sub> with the PCR generated  $\Delta yrdC_{Ec}-I::Km^R$  cassette and the NM1100/pBAD24::yrdC<sub>Ec</sub> background strain (lanes, 3, 6 and 9) were subjected to PCR analysis using the primer combinations UpyrdC/K2 (lanes 1, 2 and 3), DsyrdC/K1 (lanes 4, 5 and 6) and UpyrdC/DnyrdC (lanes 7, 8 and 9). The expected sizes for the UpyrdC/DsyrdC amplicons in WT and mutant strains are 2340 and 3113 bp respectively, DsyrdC/K1 and UpyrdC/K2 should give no amplification for the NM1100 control. (B) Growth phenotype of MG1655  $\Delta yrdC_{Ec}-I::Km^R$  P1 transductants. Strains were streaked on LB Amp plates supplemented or not with 0.02% arabinose and incubated 24 h at 37°C. MG1655/pBAD24 was used as control.

that yrdC<sub>Ec</sub> is an essential gene in the *E. coli* strains MG1655 and NM1100, and supports the result obtained in yeast, namely that *SUA5*, yrdC<sub>Ec</sub>, yrdC<sub>Mm</sub>, ywI<sub>C<sub>Bs</sub> are functional orthologs but yciO<sub>Ec</sub> is not. The pBAD construct places the genes under the control of an arabinose inducible promoter and among the orthologs, *SUA5<sub>Sc</sub>* was the least proficient in replacing the yrdC<sub>Ec</sub> gene. In the absence of the inducer, the *E. coli* strain carrying  $\Delta yrdC_{Ec}-I/pBY130$  (pBAD24::*SUA5*) showed a delayed growth phenotype not observed for strains carrying the three other genes (Figure 5B).</sub>

Analysis of bulk tRNA extracted from all four classes of P1 transductants obtained showed that all contained t<sup>6</sup>A indicating that these orthologs complement both the essentiality and tRNA modification phenotypes (data not shown).

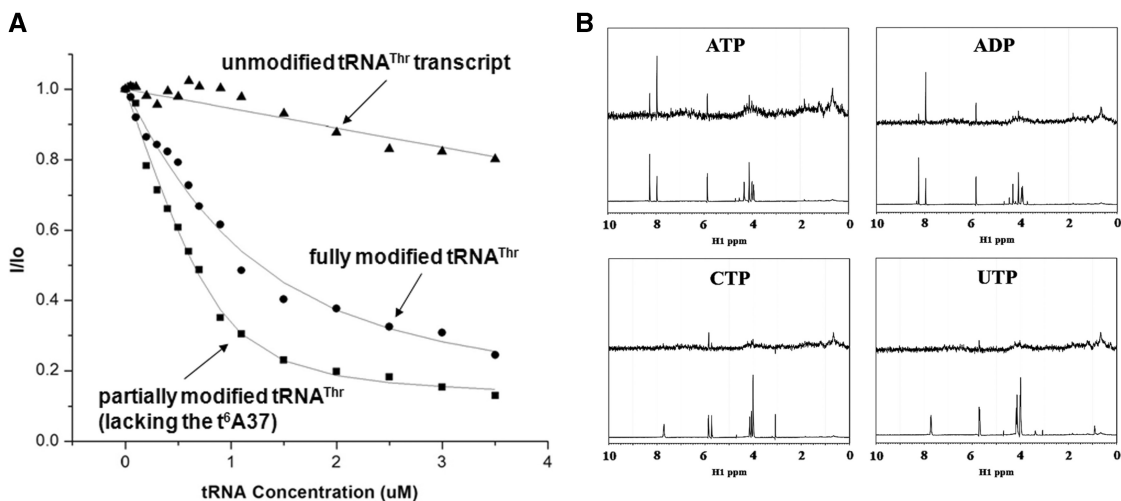
### Direct involvement of YrdC in t<sup>6</sup>A biosynthesis

**Preferential binding of YrdC<sub>Ec</sub> to apomodified tRNA<sup>Thr</sup>.** To investigate whether members of the YrdC/Sua5 family act directly on tRNA in t<sup>6</sup>A biosynthesis, YrdC<sub>Ec</sub> binding to a tRNA species known to contain t<sup>6</sup>A in *E. coli*, tRNA<sup>Thr</sup>, was tested. Three forms of *E. coli* tRNA<sup>Thr</sup><sub>CGU</sub> were generated; two *in vivo* expressed forms and one *in vitro* transcribed form that lacked all post-transcriptional modifications. As seen previously by other investigators (40), overexpression of tRNA<sup>Thr</sup> produced two forms that differ by the exact molecular mass of carbamoyl-threonine suggesting that they differ by the presence or absence of this modification at nucleotide 37. Both forms were aminoacylated with ThrRS to similar degrees indicating that they were properly folded, functional tRNAs. Fluorescence quenching data show that YrdC binds to fully modified tRNA<sup>Thr</sup> and to partially modified tRNA<sup>Thr</sup> (lacking only the threonyl-carbamoyl modification) with estimated dissociation constants of  $0.62 \pm 0.13$  and  $0.11 \pm 0.05 \mu M$ , respectively, indicating high binding affinity (Figure 6A). Only weak and

apparently non-specific binding between YrdC and unmodified *in vitro* transcript of tRNA<sup>Thr</sup> could be detected. These results suggest that YrdC is involved in t<sup>6</sup>A biosynthesis by interacting directly with tRNAs, consistent with the previously reported binding of YrdC to total tRNA (2).

**Binding of YrdC<sub>Ec</sub> to ATP.** t<sup>6</sup>A biosynthesis in crude enzyme preparations has been shown to require ATP (22) and the Sua5<sub>St</sub> ortholog from *Sulfolobus tokodaii* has been recently found to bind and hydrolyze ATP (41). To investigate whether YrdC<sub>Ec</sub> can specifically bind ATP, we screened various nucleotides for their potential to bind YrdC by saturation-transfer difference NMR (STD-NMR) spectroscopy. A strong STD-NMR signal was seen with ATP and ADP but not with CTP and UTP, indicating adenine-specific binding (Figure 6B), and pointing to the presence of one or more ATP-binding sites in YrdC<sub>Ec</sub>.

**Essentiality of *E. coli* YrdC conserved residues Arg52 and Lys50.** Arg52 and Lys50 residues are strictly conserved among YrdC proteins but are absent from the YciO proteins (Figure 1B). Both residues are located in the center of a positively charged cleft that is the putative nucleic-acid-binding site, and based upon the crystal structure of the *S. tokodaii* protein Sua5<sub>St</sub>, their side chains (Arg59 and Lys57 using *S. tokodaii* residue numbering) interact with and stabilize an AMP derived phosphate group found bound in the cleft. (41). This suggested that nucleotide binding by YrdC and Sua5 (41) is critical for function. To test the functional significance of these two residues *in vivo*, site-directed mutagenesis was used to construct *E. coli* strain MG1655 carrying pBAD24 plasmids harboring yrdC<sub>Ec</sub> Lys50 → Ala or Arg52 → Ala, and yrdC<sub>Ec</sub> Arg110 → Ala or Lys53 → Ala for control (pYC102.3, pYC104.2, pYC101.1 and pYC103.1, respectively). These were used in P1 experiments as recipient strains as described earlier. More than a hundred clones were



**Figure 6.** (A) YrdC binding to tRNA<sup>Thr</sup> measured by the intrinsic fluorescence quenching assay. Relative Trp fluorescence intensity from YrdC as a function of partially modified *in vivo* expressed tRNA<sup>Thr</sup> lacking the t<sup>6</sup>A37 modification (filled square), fully modified *in vivo* expressed tRNA<sup>Thr</sup> (filled circle) and an unmodified tRNA<sup>Thr</sup> transcript (filled triangle). Sample conditions: 2  $\mu$ M protein, 20 mM HEPES, pH 7.5, 100 mM NaCl, 20°C.  $\lambda_{\text{ex}} = 295$  nm,  $\lambda_{\text{em}} = 345$  nm. (B) STD-NMR spectra of nucleotides in the presence of *E. coli* YrdC. For the nucleotides ATP, ADP, CTP and UTP two traces are shown, where the lower trace is the reference 1D NMR spectrum, and the upper trace is the STD spectrum obtained by irradiation of the aliphatic region of YrdC. Ligand binding is indicated by positive signals in the STD spectrum, which is particularly strong for the H8, H2 and H1 resonances of ATP and ADP in the 6–8 ppm region. Little or no binding is observed for CTP or UTP.

recovered from P1 experiments when the control strain MG1655pyrdC<sub>Ec</sub>, MG1655pyrdC<sub>Ec</sub> Arg110→Ala or MG1655pyrdC<sub>Ec</sub> Lys53→Ala were used as recipients, but no transductants were recovered when the MG1655/pYC104.2 and MG1655/pYC102.3 were used, implying that Arg52 and Lys56 are essential for YrdC<sub>Ec</sub> function. All receiver strains were tested for sensitivity to P1. These results suggest that nucleotide binding is essential for the function of these proteins and are consistent with the known ATP requirement for t<sup>6</sup>A biosynthesis (22).

## DISCUSSION

Our results indicate that the universally conserved YrdC/Sua5 family is involved in the biosynthesis of N<sup>6</sup>-threonyl-carbamoyl adenosine. Deletion of *SUA5* led to the loss of t<sup>6</sup>A in two independent yeast backgrounds. *E. coli* YrdC preferentially bound tRNA<sup>Thr</sup> containing all modifications but t<sup>6</sup>A as compared to *in vitro* (unmodified) transcribed tRNA. Two invariant residues involved in nucleotide binding were characterized, strongly indicating that the role of YrdC/Sua5 in t<sup>6</sup>A biosynthesis is mediated by their ATP binding and hydrolyzing functions, and further suggesting a direct role for this protein in the biosynthetic pathway, since ATP is required for t<sup>6</sup>A formation (22).

The YrdC/Sua5 proteins have been assigned to COG009 and most organisms have only one member of this family. Exceptions include plants and several bacteria that have two homologs. Expression of the second COG009 encoding gene of *E. coli*, *yciO*, failed to complement the t<sup>6</sup>A minus phenotype of yeast strains lacking a functional *SUA5* gene. The two subfamilies can also be distinguished by sequence analysis as residues K50 and R52 are found at the bottom of a positively charged

concave surface in the crystal structure of YrdC and are strictly conserved in the YrdC subfamily but absent in YciO sequences (Figure 1B). These residues are essential for YrdC function as shown by site directed mutagenesis and complementation experiments. Therefore COG009 should be split into two sub-families as the results presented here suggest distinct functions for YciO and YrdC.

Like m<sup>1</sup>G37 and  $\Psi$ 55, t<sup>6</sup>A<sub>37</sub> is one of the few tRNA modifications present in all organisms for which the whole set of tRNA sequences is available (38,42). It is one of the few universal modifications believed to have been present in the last common ancestor (43,44) and every sequenced genome (>600 to date) has a YrdC ortholog.

In this study the essentiality of this gene family has been resolved. In *E. coli*, *yrdC* appears essential, however whether the observed lack of viability is due to the absence of the t<sup>6</sup>A modification still remains to be established. In apparent contradiction to our results, insertion of the P<sub>BAD</sub> promoter within the first 15 nucleotides of *yrdC* was reported to be lethal under repressing conditions, namely on minimal but not rich medium (10). The phenotype on minimal medium was attributed to polar repression of the downstream *aroE* gene (aromatic amino-acid biosynthetic gene), which is essential for growth on minimal medium, whereas on LB neither *aroE* nor *yrdC* was considered essential. However, since expression from the pBAD promoter is not completely repressed by glucose (27,45), expression under pBAD repression may yield sufficient YrdC protein to fulfill essential function(s) in the cell.

In yeast, neither the gene nor the modification is essential, as two *SUA5* deletion strains lacking t<sup>6</sup>A have been constructed [this study and (4)]. However, t<sup>6</sup>A<sup>-</sup> strains display a marked slow growth phenotype [this study and

(4)] and possess defective mitochondria (4). Since mitochondria are considered of prokaryotic origin, this result provides additional evidence that this modification plays an essential role in prokaryotic translation.

The t<sup>6</sup>A modification has been shown to be a determinant for efficient aminoacylation by bacterial Isoleucyl-tRNA synthetase (IleRS), whereas it is not for yeast cytoplasmic IleRS (46,47) and this might be the molecular basis for the requirement of YrdC and Sua5 in bacteria and the yeast mitochondria, respectively.

Four mitochondrial tRNAs contain t<sup>6</sup>A (Lys, Ile, Arg2 and Met) however only one *yrdC/SUA5* homolog has been identified in yeast, and this protein is predicted to be cytoplasmic (48). In the absence of a mitochondrial specific homolog, *Sua5* must also be targeted to the mitochondria, possibly by alternative translational start site usage as has been described for proteins such as yeast Trm5, HTS1, VAS1 and *Arabidopsis thaliana* alanyl-, threonyl- and valyl-tRNA synthetases (49–52).

Strains lacking a member of the YrdC/Sua5 family resulting in a defective pool of tRNAs, will be instrumental in studying the *in vivo* role of this modification. Accurate codon–anticodon recognition is critical in insuring the accuracy of translation and the t<sup>6</sup>A<sub>37</sub> modification has been shown to affect this process *in vitro*, mainly by preventing the formation of U33-A37 across-the-loop base pairing interaction, and allowing cross-strand stacking of A<sub>38</sub> and t<sup>6</sup>A<sub>37</sub> with the first position of the codon (15,16, 18–20). The t<sup>6</sup>A modification is found in seven *E. coli* and 12 *S. cerevisiae* tRNAs, four of which are mitochondrial (38). As the effects of anticodon-surrounding modifications in adjusting the thermodynamic properties of anticodon/codon interactions for cognate and non-cognate recognition by the ribosome are context dependent, it is expected that the absence of t<sup>6</sup>A in these tRNAs will affect translation in several ways. This is reflected by the pleiotropic phenotypes of strains lacking a functional YrdC or Sua5 (4–8). In yeast, for example, the *sua5-1* mutation was isolated as a suppressor of an initiation defect affecting the *CYC1* gene expression (4). *CYC1* is a nuclear gene encoding the iso-1 form of the electron carrier protein cytochrome *c* which is localized to the mitochondria (53). The *cyc1-1019* mutant expresses 2% of the normal amount of iso-1-cytochrome *c* due to a single base-pair substitution that creates an upstream ATG codon that is out of frame with the normal *CYC1* translational start. The *sua5-1* point mutation suppressed this translational defect, restoring Cyc1 levels to 60% of the normal levels (4). In this *sua5* suppressor strain lacking t<sup>6</sup>A (this study), Cyc1 protein expression is possibly due to high levels of frameshift errors during the translation initiated at the upstream and out of frame ATG, thus restoring the original reading frame. The absence of other modifications at position 37 such as Wyeosine or m<sup>1</sup>G has also been shown to drastically increase frame-shifting in yeast (54,55). However, the fact that the Cyc1 protein is also expressed at nearly of wild-type levels in a wild-type *CYC1 sua5::LEU2* strain (4), i.e. in the absence of the aberrant ATG found in the *cyc1-1019* allele, suggests yet another hypothesis. In Eukaryotes, the cytoplasmic initiator tRNA contains a t<sup>6</sup>A at position 37, and is restricted to

recognition of AUG alone, while its prokaryotic counterpart is neither modified nor restricted to recognizing AUG, suggesting that this modification may play an important role in selecting the appropriate translational start site. In the absence of t<sup>6</sup>A, the scanning ribosome could initiate at the natural ATG start codon, but also promiscuously at other ATG or non-ATG codons, as interactions between initiator tRNAs and their codons would be less stringent. This would account for the situation in prokaryotes, where the initiator tRNA is not modified at position 37 and the ribosome can initiate at either ATG, GTG or TTG (56). For yeast, the lack of stringent translational start site selection would lead to several N-terminal variants, including active forms of the Cyc1 protein and would account for the similar levels of Cyc1 activity seen in both the *sua5::LEU2* strain and the *cyc1-1019 sua5-1* suppressor strain.

Finally, other pleiotropic phenotypes linked to the absence of YrdC/Sua5 members have been observed. In *E. coli*, *yrdC* has been described as a possible ribosome maturation factor (RimN) needed for correct processing of 16S ribosomal RNA, and for maintenance of normal amounts of translating ribosomes (7,8). The ribosome maturation phenotype may reflect an indirect consequence of the *yrdC* mutation since tRNA defects due to loss of modification(s) or other cellular malfunctions have been shown to cause ribosome maturation defects (57).

In conclusion, we propose that the diverse phenotypes of YrdC/Sua5 mutant strains are due to the central and primordial role of the t<sup>6</sup>A modification that includes: insuring accurate decoding, maintaining the reading frame, selecting the appropriate translation initiation codon (mainly in Eukaryotes) and, determining recognition and aminoacylation of tRNAs by prokaryotic and mitochondrial IleRSs.

Although a biochemical function for the YrdC/Sua5 proteins (i.e. catalytic activity) aside from tRNA and ATP binding (this study) and ATPase activity (41), has not been determined, appropriately modified tRNA substrates appear critical for interacting with these proteins. We were unable to show threonine binding by YrdC, under ATP binding conditions, nor incorporation of threonine in a tRNA<sup>Thr</sup> transcript in the presence of YrdC (data not shown). Further experimentation using apomodified tRNA as a substrate is needed to determine the role of YrdC in t<sup>6</sup>A biosynthesis. It is also likely that other protein partners interact with YrdC especially since the biosynthesis of t<sup>6</sup>A is proposed to require two ATP-dependent steps (58).

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