

Ytql from *Bacillus subtilis* has both oligoribonuclease and pAp-phosphatase activity

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Received April 2, 2007; Revised and Accepted May 25, 2007

ABSTRACT

Oligoribonuclease is the only RNase in *Escherichia coli* that is able to degrade RNA oligonucleotides five residues and shorter in length. Firmicutes including *Bacillus subtilis* do not have an Oligoribonuclease (Orn) homologous protein and it is not yet understood which proteins accomplish the equivalent function in these organisms. We had previously identified oligoribonucleases Orn from *E. coli* and its human homolog Sfn in a screen for proteins that are regulated by 3'-phosphoadenosine 5'-phosphate (pAp). Here, we identify Ytql as a potential functional analog of Orn through its interaction with pAp. Ytql degrades RNA oligonucleotides *in vitro* with preference for 3-mers. In addition, Ytql has pAp-phosphatase activity *in vitro*. In agreement with these data, Ytql is able to complement both *orn* and *cysQ* mutants in *E. coli*. An *ytql* mutant in *B. subtilis* shows impairment of growth in the absence of cysteine, a phenotype resembling that of a *cysQ* mutant in *E. coli*. Phylogenetic distribution of Ytql, Orn and CysQ supports bifunctionality of Ytql.

INTRODUCTION

Degradation of RNA is an important factor in the regulation of gene expression. Impairment of regulation of mRNA stability was implicated in the pathogenesis of cancer, inflammatory diseases and Alzheimer's disease (1). Enzymes involved in RNA degradation fall into two major classes: endoribonucleases, which cleave RNAs internally and exoribonucleases, which degrade RNAs from the ends. The phylogenetic distribution of endo- and exoribonucleases (2–4) in genomes clearly shows that different species vary considerably with respect to the number and variety of the RNases they harbor. Interestingly, two ribonucleases that are essential in *Escherichia coli* do not have homologous counterparts in *Bacillus subtilis*: RNase E (5) and oligoribonuclease (6).

Belonging to the degradosome, RNase E is widely believed to be the enzyme initiating mRNA decay (7).

Oligoribonuclease, Orn, is the only known exoribonuclease that is essential in *E. coli* (6). The essentiality in *E. coli* is due to its unique ability to degrade RNA oligonucleotides with a length of 5 nt and shorter (8–10), and oligonucleotides of these lengths were shown to accumulate in a conditional *orn*-mutant (6). We would like to introduce the term 'nanoRNA' here to distinguish these extremely short oligonucleotides from the longer microRNAs. We chose the term nano in reference to its roots: Nano originates from the Greek word nanos, which means dwarf. Micro on the other hand descends from the Greek word mikros, which means small. Nano is therefore used in this context simply to articulate 'smaller than' micro.

A recent study reveals the structural basis for the constraints preventing RNase II as a member of the RNR exoribonuclease family from degrading oligonucleotides shorter than 5 nt (11–14). Another member of this exoribonuclease family, RNase R was shown to processively degrade RNA in a 3' to 5' directed manner until a di- or trimer remains which cannot be degraded further by this enzyme (12). This size limit is therefore likely to be common at least among the members of this important family of exoribonucleases. This highlights the importance of enzymes that have the ability to degrade nanoRNA and thus bring the degradation of RNA to completion. Absence of an oligoribonuclease in Firmicutes is in contrast to its general presence in Gram-negative prokaryotic genomes as well as in eukaryotic genomes (3). This prompted us to question which enzyme could functionally replace oligoribonuclease in these organisms.

We had recently discovered an unexpected link between sulfur- and RNA metabolism: oligoribonuclease binds to 3'-phosphoadenosine 5'-phosphate (pAp) and is sensitive to micromolar amounts of the nucleotide (15). pAp is generated in sulfur assimilation and was implicated in the molecular mechanism of lithium's action in the treatment of bipolar disorder due to strong inhibition of pAp-phosphatase by lithium (16). The interaction between pAp and oligoribonuclease was documented for *E. coli*

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oligoribonuclease, Orn and its human homolog, Sfn (15). The purpose of this work was to explore whether the conserved interaction between pAp and oligoribonucleases could be exploited to identify a functional analog of Orn in *B. subtilis*. Surprisingly, the protein identified by this route, YtqI, points to the existence of an even closer link between sulfur- and RNA-metabolism in this organism: YtqI can degrade both nanoRNA and pAp *in vitro*. The pAp-degrading activity of YtqI is similar in magnitude to that of CysQ, the pAp-phosphatase from *E. coli*. Consistent with its *in vitro* activities, YtqI can replace both Orn and pAp-phosphatase (CysQ) in *E. coli*.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

Escherichia coli strains were grown in LB or MOPS minimal medium (17) containing 40 µg/ml of amino acids as indicated, K-phosphate at 2 mM, vitamin B1 at 0.0005%, biotin at 0.001% when needed, glycerol at 0.4%, glucose or arabinose as indicated. *Bacillus subtilis* was grown in minimal medium (18). Ampicillin (100 µg/ml), kanamycin (25 µg/ml) or erythromycin (1 µg/ml) was added for plasmid maintenance or to select for chromosomal marker. Anhydrotetracycline (Atc) was added at 250 ng/ml for induction of P_{LtetO-1}.

To test growth in the absence of cysteine (Figure 7), overnight cultures grown in MOPS minimal medium containing all amino acids were washed twice with medium lacking cysteine before inoculation into medium containing cysteine (100 µM) or lacking this amino acid.

All experiments were performed in accordance with the European regulation requirements concerning the use of Genetically Modified Organisms (level 1 containment, agreement n°2735).

The plasmid for expression of his-tagged YtqI under control of the arabinose-inducible promoter P_{ara} (pUM412) was constructed as follows: Primer UM175 and UM176 were used to PCR-amplify *ytqI* from *B. subtilis* 168 chromosomal DNA. The EcoRI, XhoI digested fragment was used to replace the EcoRI/XhoI fragment of pUM407 coding for Orn leaving the region coding for the C-terminal his-tag and the ribosomal-binding site intact.

The conditional *E. coli orn* mutant (strain UM341) uses the anhydrotetracycline (Atc)-inducible promoter P_{LtetO-1} (19) together with a Tet-repressor (TetR) to ensure tight control in the absence of Atc. This strain was created by introducing the P_{LtetO-1} promoter in front of *orn* together with a cassette coding for TetR and a kanamycin selection marker (Km^R). Two PCR fragments were amplified: PCR1 amplified P_{LtetO-1}, *km^R* and the transcription terminator T₀ from pZE21-MCS1 (19) using primer UM153 and UM156, PCR2 amplified *tetR* including its constitutive promoter P_{N25} and terminator T₁ from chromosomal DNA of DH5αZ1 (19) using primer UM155 and UM154. pZE21-MCS1 and DH5αZ1 were kindly provided by Hermann Bujard. The outside primers UM155 and UM156 and equimolar amounts of PCR fragments 1 and 2 were used to perform overlapping PCR.

The obtained PCR fragment was then cloned into pGEMT-Easy (Promega) by TA cloning followed by sequencing using primer UM172 and UM173. A verified clone was used as template for PCR amplification using primers UM155 and UM156. The obtained PCR fragment was transformed into CF10230 to create the *orn* mutant by lambda Red-assisted recombination according to the protocol of Yu *et al.* (20). CF10230 is a *nic+* derivative of DY329 (20) that was kindly supplied by Michael Cashel (Cashel, M., unpublished data). Mutants were verified by confirmation of the 5' site of integration into the chromosome by means of PCR using primers UM158 and UM159 yielding a 432 bp fragment, as well as the 3' site of integration using primer UM160 and UM161, yielding a 446 bp fragment.

The *cysQ* mutant we used here has been described before (15). We will refer to *cysQ* mutant 1 as UM285 from now on. UM285 has a replacement of the complete coding DNA sequence (CDS) for CysQ by *km^R*.

The *ytqI* mutant strain (BSF66) was part of the European/Japanese effort to inactivate the whole gene set of *B. subtilis* 168 and has an insertion of pMUTIN2MCS after the codon for amino acid 108 (21).

pAp-agarose binding

pAp-agarose-binding experiments were performed as described previously (15). Two hundred milliliter cultures of *B. subtilis* 168 or a protease-deficient mutant, DB430 (22) were grown in minimal medium containing 1.5 mM MgSO₄ at 37°C to an OD₆₀₀ between 1.6 and 1.8. Cells were harvested and washed once with 50 mM NaPO₄ pH 8.0, 300 mM NaCl before freezing. Frozen pellets were resuspended in 2 ml pAp-agarose buffer (50 mM HEPES, pH 7.5, 10 mM CaCl₂, 50 mM KCl) containing 100 mM NaCl, 0.4 mM phenylmethylsulfonyl fluoride (PMSF) and 130 µg/ml lysozyme. After incubation for 45 min on ice, the cells were opened using a Fastprep apparatus (Bio101). Blocking with agarose beads, incubation with pAp-agarose, elution, PAA electrophoresis and identification of proteins was done exactly as described before (15).

Purification of his-tagged YtqI and activity assays

YtqI was purified from a 200 ml culture of MG1655 carrying pUM412 according to the his-tag purification protocol described previously (15).

Activity assays determining nanoRNase activity were performed using custom-made RNA oligo 5-mers or 3-mers (5'Cy5-CCCC3' or 5'Cy5-CCC3') as substrates in reactions containing 50 mM HEPES, pH 7.5, 5 mM MnCl₂, 1.6–3.4 µM substrate. At intervals, 4.5 µl reaction aliquots were taken and stopped by adding to an equal volume of sample buffer (4× TBE, 100 mM DTT, 16% glycerol, 20 mM EDTA) and frozen at –20°C. For analysis of the reaction products, 1.5 or 2.5 µl of samples were applied to PAA gel electrophoresis on a 22% SDS-PAA gel containing 2× TBE and run in 2× TBE. Fluorescent RNA oligos were visualized using a Molecular Dynamics STORM 860 in 650-nm long-pass filter mode. Quantification of the data was done by calculating the percent of fluorescence of each band at a

given time point relative to the total fluorescence of the same time point. Separating reaction products on 22% SDS-PAA gels, we observed a reverse migration phenomenon. This effect can be accounted for by the fact that cyanine dyes have a lower net negative charge than nucleic acids: thus, removing nucleotides will reduce the charge relative to the mass of the oligonucleotide and cause it to shift up instead of down.

Inhibition of YtqI-catalyzed degradation of RNA 5-mers (5'Cy5-CCCC3') by pAp was performed in 30 μ l reactions containing 3 μ M substrate RNA 5-mers, 9 μ g YtqI and pAp as indicated. Reactions were incubated for 30 min at 37°C. Relative activities were assigned as conversion of the substrate into monomers. The activity in the reaction without pAp was set to 100%.

Assays determining degradation activity on a longer substrate were done using a custom-made RNA 24-mer (5'CACACACACACACACACACA3') that was 5'-end labeled with [γ -³³P]ATP. This oligonucleotide was labeled using the MirVana Probe and Marker Kit (Ambion) in a 20 μ l reaction containing 100 pmol oligo, 6.7 pmol [γ -³³P]ATP (20 μ Ci), 90 pmol ATP and 1 μ l T4 Polynucleotide Kinase. Incubation was done for one hour at 37°C. The reaction was stopped by the addition of 2 μ l of 10 mM EDTA and incubation at 95°C for 2 min. The reaction mixture was purified from the unincorporated nucleotides using NucAway spin columns (Ambion) according to the instructions of the supplier. Three microliter of the labeled RNA (~800 000 c.p.m.) were used in a 20 μ l reaction containing 5 mM MnCl₂, 50 mM HEPES pH 7.5 and 3 μ g enzyme; incubation was for 30 min at 37°C after which the reaction was stopped by the addition of 20 μ l loading buffer and incubation for 3 min at 95°C. An aliquot of 5 μ l of the samples were resolved on a 20% PAA, 7 M Urea gel containing 2 \times TBE that was ran in 2 \times TBE. Labeling of the decade-marker was done with [γ -³³P]ATP (125 μ Ci, 1.7 pmol) as suggested by the manufacturer including purification from unincorporated nucleotides. One-fourth of the total volume of labeled decade-marker was used per gel; this amount corresponded to ~800 000 c.p.m. A previously characterized nuclease (with DNase and RNase activity) from *B. subtilis*, YhaM (23) served as positive control in experiments on RNA 24-mers. This protein was purified employing a C-terminal his-tag. HEPES was replaced by Tris pH 8.0 in the reaction containing YhaM as this enzyme is less active in HEPES.

pAp degradation was assayed in 20 μ l reactions containing 6 mM pAp, 2 mM MnCl₂, 50 mM HEPES (pH 7.5) at 37°C. Reactions were started by the addition of 1 μ g YtqI. Aliquots of 4.5 μ l were taken as indicated and mixed with 0.5 μ l 100 mM EDTA before resolving them by polyethyleneimine (PEI) thin-layer chromatography with 0.8 mM LiCl as solvent. Authentic pAp and AMP were used as migration standards. Accumulation of reaction products was estimated after visualization by UV.

Expression of his-tagged proteins was monitored by PAA gel electrophoresis followed by staining with Bio-Safe Coomassie stain (BIO-RAD) or by western blot using Anti-His₆ Peroxidase antibodies (Roche) at 1:200 in

1 \times PBS, 1% skim milk, 0.1% Tween and ECL Plus Western Blotting Detection System (GE Healthcare). For these experiments, different expression levels of the P_{ara}-controlled genes were achieved by growing cultures in liquid LB in the presence or absence of arabinose (0.2 or 0.02%).

Phylogenetic analysis

A total of 393 completely sequenced bacterial genomes published before 4 January 2007 (<http://www.ebi.ac.uk/genomes/>) were analyzed for the presence of YtqI, Orn and CysQ orthologous proteins. Orthologs were defined by searching for bi-directional best hits (BBH) (24) based on the following parameters: \geq 40% amino acid similarity and \leq 20% difference in protein length. The phylogenetic tree presented in Figure S1 was constructed from 141 representative species based on 16S rRNA similarity.

RESULTS

Identification of YtqI among the proteins binding to pAp

The conserved interaction of pAp and oligoribonucleases between *E. coli* and human cells encouraged us to ask if we could identify a functional analog of oligoribonuclease in *B. subtilis* among the pAp-binding proteins from this organism. Extracts of *B. subtilis* 168 and a protease minus mutant (DB430) (22) were used in pAp-binding experiments. The protein pattern obtained looked similar for both strains; we therefore present only the data acquired for the wild-type strain. Two major protein bands were visible in the pAp-binding fraction (Figure 1). Analysis of band A by liquid chromatography tandem mass spectrometry (LC-MS/MS) revealed HisIE (SwissProt, O34912) with an overall score of 2080 and 5 identified peptides covering 22.5% of the total mass of the protein. HisIE was identified previously as pAp-binding protein in *E. coli* (15). The second major band gave high scores for two proteins: GuaC (SwissProt O05269), GMP reductase with an overall score of 292 and 4 peptides covering 13% of the total mass, and YtqI (SwissProt O34600) with an overall score of 140 and 4 peptides covering 11.5% of the total mass. YtqI is an unknown protein that belongs to the DHH/DHHA1 family (25). This family of proteins consists of enzymes with phosphoesterase activity, including RecJ. YtqI was therefore our best candidate for a potential functional Orn analog.

YtqI complements an *E. coli* orn mutant

In order to perform complementation experiments, we created a conditional promoter mutant of the essential *orn* gene in *E. coli* (Figure 2). This mutant (strain UM341) uses the anhydrotetracycline (Atc)-inducible promoter P_{LtetO-1} (19) together with a Tet-repressor (TetR) to ensure tight control in the absence of Atc. A growth defect of this mutant was easily observable in cultures lacking Atc grown in LB liquid medium. While growth of the *orn* mutant carrying a plasmid-borne copy of *orn* (pUM408) was not affected by the absence of Atc (42 versus 41 min doubling time for cultures minus and plus Atc,

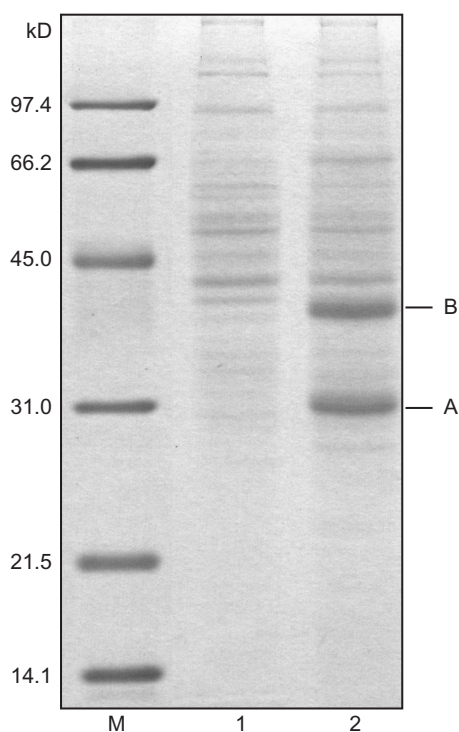


Figure 1. pAp-binding proteins from *B. subtilis*. Shown is a colloidal Coomassie stained SDS-PAA gel separating the pAp-binding fraction of *B. subtilis* extract. M: marker, lane 1: fraction binding to blocked agarose beads (control), lane 2: pAp-binding fraction.

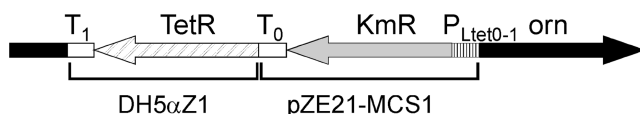


Figure 2. Design of the conditional $P_{LtetO-1}$ *orn* mutant. The $P_{LtetO-1}$ promoter together with a cassette coding for the kanamycin resistance marker (Km^R) and the Tet-repressor (TetR) were inserted directly in front of *orn*. The arrows correspond to the direction of transcription. DH5αZ1 (19) was the source of TetR and terminator T_1 , and plasmid pZE21-MCS1 (19) was the source of the $P_{LtetO-1}$, km^R and terminator T_0 .

respectively), mutants carrying the vector control (pBAD18) had a 1.9-fold longer doubling time when Atc was missing (77 versus 43 min, respectively) and their growth leveled off at an OD_{600} of ~ 0.5 . On plates, a similar effect could be observed in the absence of Atc; transformants of strain UM341 with pBAD18 produced pinpoint-sized colonies that stopped growing, while transformants with the *orn* carrying plasmid were significantly larger after overnight exposure and continued to grow (Figure 3).

C-terminally his-tagged YtqI was expressed under control of the arabinose-inducible P_{ara} promoter (plasmid pUM412) for complementation experiments. As seen in Figure 3, *ytqI* expression completely rescued the growth defect of the *orn* mutant on plates lacking Atc. Expression was induced by the addition of 0.2% arabinose. Expression levels of YtqI and Orn were similar under these conditions as judged from Coomassie-stained gels

(data not shown). Complementation could be seen even in the absence of arabinose. As opposed to the expression level of YtqI in the presence of 0.2% arabinose, in the absence of arabinose expression was not visible on a Coomassie-stained protein gel and was below the amount that could be detected by western blotting using Anti-His₆ antibodies (data not shown). We concluded therefore that even low levels of YtqI expression were sufficient for Orn complementation.

YtqI degrades nanoRNA *in vitro*, with 3-mers being a preferred substrate

Purified recombinant YtqI was tested for nanoRNase activity. In the presence of manganese, YtqI was able to degrade nanoRNA 5-mers (Figure 4). The activity in the presence of other ions tested (magnesium, zinc and calcium) was negligible (data not shown). Comparing YtqI- and Orn-catalyzed degradation of nanoRNA 5-mers, we noticed significant differences: The amount of YtqI required for appreciable activity was two orders of magnitude higher than that necessary for Orn-catalyzed activity. In addition, the pattern of degradation products as well as the kinetics of this reaction looked very different. Here, 3-mers were virtually missing and other intermediates (2-mers and 4-mers) accumulated less than in Orn-catalyzed hydrolysis (Figure 4). Therefore, we hypothesized that 3-mers might be a preferred substrate for YtqI and as such they might be hydrolyzed so fast that accumulation could not be observed. We tested this hypothesis by comparing degradation of 3-mers and 5-mers (Figures 4 and 5). We used three times more enzyme in the reaction with 5-mers as substrate in order to obtain appreciable conversion into monomers (Figure 4A) as compared to the reaction on 3-mers (Figure 5A). Turnover numbers for 3-mers were one order of magnitude higher than for 5-mers (1.5 versus 0.14 pmol/ μ g/min). In Figure 5B, we compare the kinetics of the disappearance of different substrates (3-mers or 5-mers) and the appearance of the final reaction product monomers in reactions with equal amounts of YtqI (1.5 μ g). These results clearly document that 3-mers were a much better substrate for YtqI than 5-mers. Moreover, it seems that degradation of 3-mers to 2-mer was the fastest step in catalysis as the 2-mers formed here disappeared considerably slower.

In order to ask whether YtqI degrades specifically nanoRNA or is active on longer substrates as well, we tested degradation of a RNA 24-mer 5'-end labeled with ³³P. Figure 6 shows that activity of YtqI on this substrate was insignificant. The YtqI-catalyzed turnover of 24-mers into monomers could be roughly estimated from this experiment as 0.01 pmol/ μ g/min.

Sensitivity of YtqI to pAp

Binding of YtqI to pAp could point to the following possibilities: (i) activity of YtqI is affected by pAp or (ii) pAp can be a substrate for YtqI. We had reported before that Orn-catalyzed degradation of nanoRNA is highly sensitive to pAp (15). Therefore, we decided to test the possibility (i) first. Unlike what we observed with Orn,

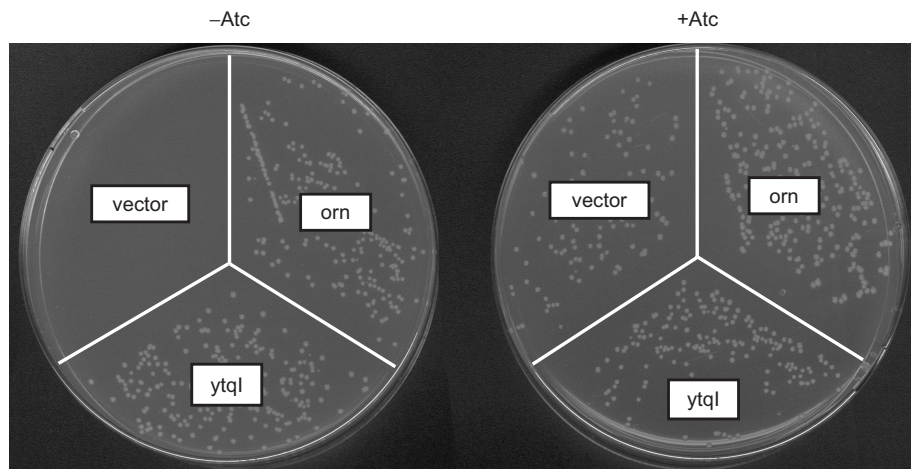


Figure 3. Complementation of the conditional *orn* mutant by expression of YtqI. Transformants of strain UM341 with pBAD18 (vector control), pUM408 (arabinose-inducible *orn*) or pUM412 (arabinose-inducible *ytqi*) were spread on LB plates containing 0.2% arabinose in the presence or absence of anhydrotetracycline (Atc).

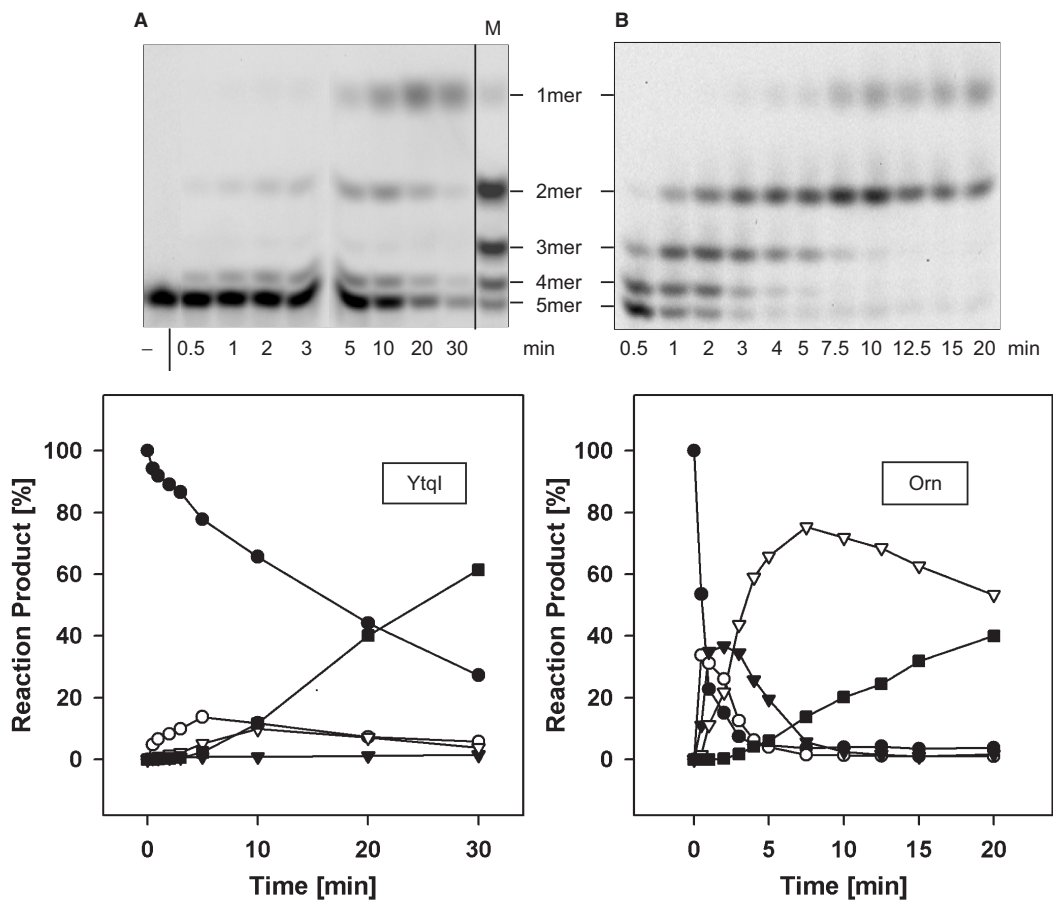


Figure 4. Comparison of YtqI and Orn-catalyzed degradation of nanoRNA 5-mers. Shown are the separation of reaction products on 22% PAA gels (upper panel) and the corresponding quantification (lower panel). Reactions contained 12 μ g YtqI (A) or 0.14 μ g Orn (B) and 1.5 μ M or 2.7 μ M RNA 5-mer (5'Cy5-CCCCC3'), respectively. The minus indicates a control lacking enzyme. M specifies a size marker obtained by Orn-catalyzed reaction. Closed circle: 5-mers, open circle: 4-mers, closed triangle: 3-mers, open triangle: 2-mers, square: 1-mers.

the addition of small amounts of pAp to the YtqI-catalyzed reaction (10, 20 and 50 μ M) did not produce an easily observable effect on degradation of nanoRNA. At 100, 200 or 500 μ M pAp the activity of YtqI based

on the conversion of 5-mer into monomers in 30 min dropped to 28, 4 and 1%, respectively (data not shown). The effect produced by 500 μ M pAp was comparable to the effect seen in the presence of 20 μ M pAp in an

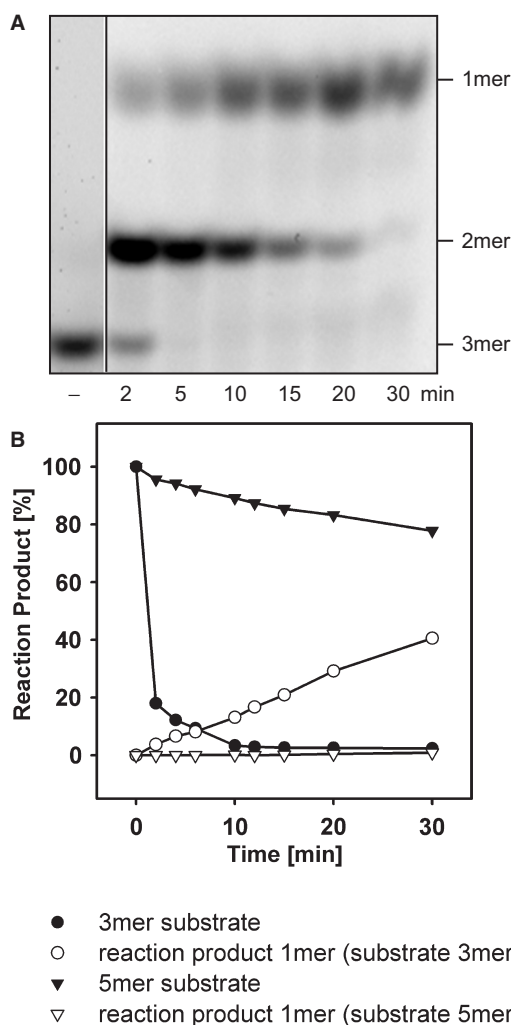


Figure 5. Substrate preference of YtqI for RNA 3-mers. (A) YtqI-catalyzed degradation of RNA 3-mers. A 30 μ l reaction contained 3 μ g YtqI and 3.5 μ M RNA 3-mers (5'Cy5-CCC3'). The minus indicates a control-lacking enzyme. (B) Comparison of YtqI-catalyzed degradation of RNA 5-mers or 3-mers. Shown are the amounts of substrate and reaction product monomer present at times indicated. Numbers indicate fractions of the total amount of fluorescent present in substrate, intermediate and complete reaction products. Reactions contained 2.7 μ M substrate RNA 5-mers or 3-mers and 1.5 μ g YtqI.

Orn-catalyzed reaction with 0.07 μ g Orn and 3 μ M substrate (15).

YtqI is also a pAp-phosphatase

The observed effect of pAp on YtqI-catalyzed degradation of nanoRNA did not exclude the possibility of pAp being a substrate for YtqI. We therefore tested the ability of YtqI to degrade pAp *in vitro*. Remarkably, YtqI was able to degrade pAp to AMP (data not shown). YtqI converted 6 nmol of pAp/ μ g/minute. The pAp-degrading activity of YtqI was similar in magnitude to that of CysQ (33 nmol/ μ g/min), the pAp-phosphatase from *E. coli* (15). Unlike CysQ activity, pAp-degrading activity of YtqI was not affected by either LiCl or CaCl₂ at concentrations of 5 mM (data not shown).

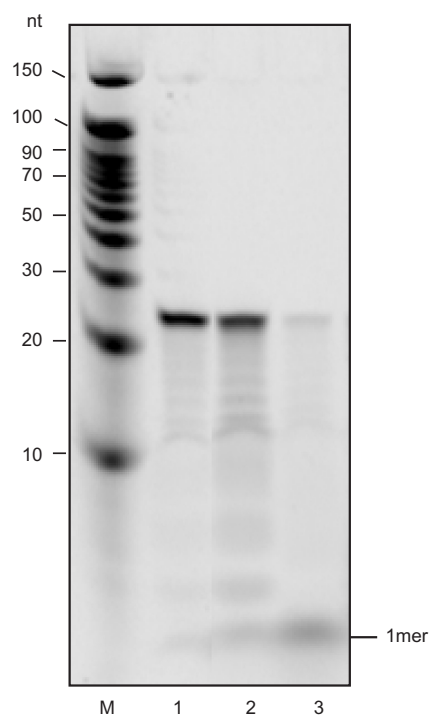


Figure 6. Activity of YtqI on RNA 24-mers. Reactions containing 5'-³³P-labeled RNA 24-mers (5'CACACACACACACACACACACA CA3') were incubated for 30 min at 37°C. M, decade marker; 1, no enzyme control; 2, YtqI; 3, positive control (YhaM).

To test if this *in vitro* activity of YtqI has physiological relevance, we asked whether the expression of *ytqI* could complement the *cysQ* mutant phenotype, i.e. the growth impairment of CysQ-lacking cells in the absence of cysteine. Figure 7 shows that complementation could indeed be achieved. Transformants of UM285 (Δ *cysQ*) with the vector control formed very small colonies when plated on medium lacking cysteine (Figure 7A). In liquid medium, growth of the vector control strain was severely affected in the absence of cysteine (Figure 7B). Transformants of UM285 with a plasmid expressing YtqI (pUM412) or CysQ (pUM404) however formed normal size colonies (Figure 7A). In liquid medium, UM285 strains transformed with plasmids expressing YtqI or CysQ were not affected in their growth when omitting cysteine (Figure 7B). A comparison of expression levels of YtqI and CysQ in the presence of 0.02% arabinose showed that CysQ was expressed at a somewhat higher level than YtqI (data not shown).

The ability of *ytqI* to complement a *cysQ* mutant in *E. coli*, prompted us to investigate the phenotype of an *ytqI* mutant (BFS66) in *B. subtilis*. Growth rates of the *B. subtilis* wild type and BFS66 were compared either in the absence or in the presence of cysteine. Doubling times were similar in the presence of cysteine with 42 and 44 min for wild type and the *ytqI* mutant, respectively, but varied considerably in the absence of cysteine with 43 versus 68 min. This phenotype resembled that of a *cysQ* mutant in *E. coli*. The latter seemed however more pronounced as withdrawal of cysteine affected growth more severely (88 versus 203 min) (Figure 7B).

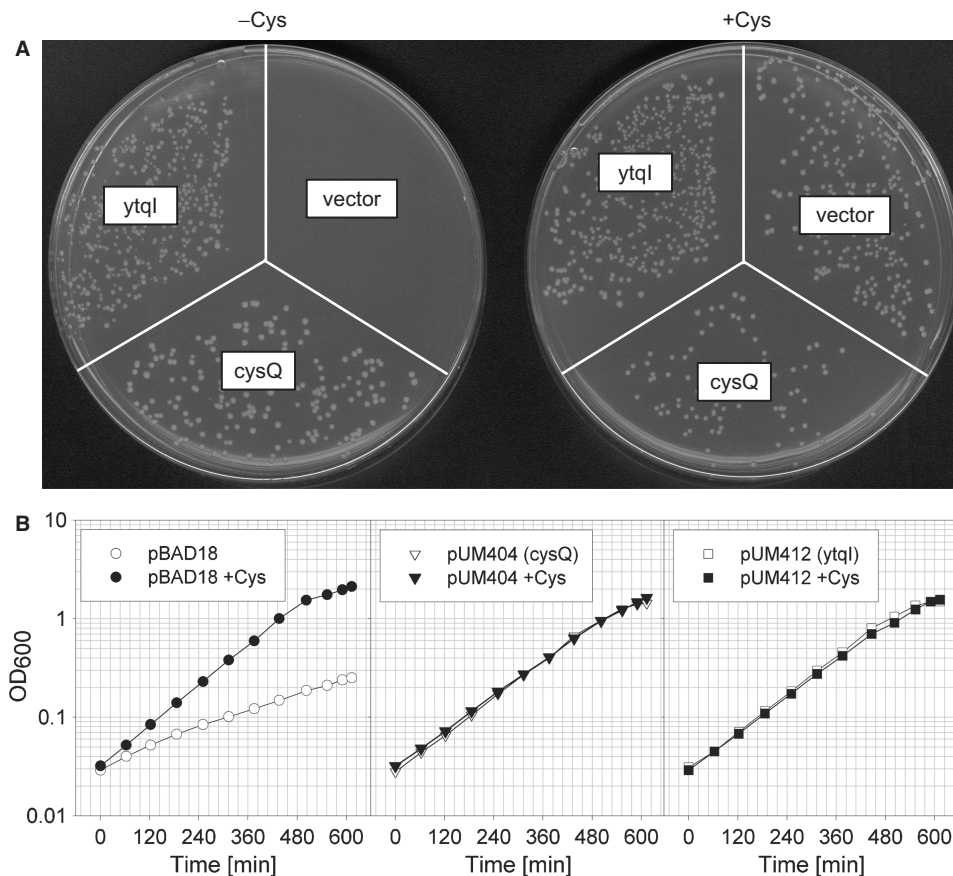


Figure 7. Complementation of the *cysQ* mutant by the expression of YtqI. (A) Transformations of strain UM285 with pBAD18 (vector control), pUM404 (arabinose-inducible *cysQ*) or pUM412 (arabinose-inducible *ytqI*) were spread on MOPS-minimal plates containing 0.4% glycerol and 0.2% arabinose in the presence or absence of cysteine. (B) Growth of strain UM285 carrying the plasmids indicated in MOPS-medium containing 0.4% glycerol and 0.02% arabinose in the presence or absence of cysteine (100 μ M).

Phylogenetic distribution of YtqI

Analysis of the phylogenetic distribution of Orn and YtqI (Supplementary Figure S1), clearly demonstrated that the majority of bacterial species possess only one of the two proteins. YtqI was present in Firmicutes, Bacteroidetes, Chlorobi and in the delta subdivision of Proteobacteria. Orn, however, was present in beta and gamma-Proteobacteria and in Actinobacteria. This distribution points to some anti-correlation: the presence of one of the genes seemed to exclude the presence of the second one (26). The two proteins of different origin might therefore exert the same function. Some Actinobacteria were exceptional in that they had both *orn* and *ytqI*. Cyanobacteria and alpha-proteobacteria had neither Orn nor YtqI.

Figure 8 shows the distribution of YtqI, Orn and CysQ in 393 completely sequenced genomes. This figure shows that most organisms that had YtqI, did not have CysQ or Orn. Whereas the overlap between organisms carrying both Orn and CysQ was considerable (58% of the species having Orn have also CysQ), only 21% of the species having YtqI had also CysQ, and only 11% of species having YtqI carried also Orn. This distribution supports our hypothesis that YtqI might fulfill the function of two proteins, Orn and CysQ.

Interestingly, while Orn was absent in all sequenced archaeal genomes (27), YtqI was represented in 42% of them.

A complete list of genomes investigated and details concerning presence of YtqI, Orn or CysQ orthologs is shown in Table S1.

DISCUSSION

This study was conducted in order to search in the model organism *B. subtilis* for a functional analog of *E. coli* oligoribonuclease, Orn. Encouraged by the observation that the pAp-oligoribonuclease interaction is conserved between *E. coli* and humans, we identified YtqI as potential functional Orn analog through its binding to pAp. The other *B. subtilis* proteins interacting with pAp, HisIE and GuaC, are of known function and were not the focus of this study. It is however noteworthy that the interaction between HisIE and pAp was observed previously using *E. coli* extracts (15), which points to biological relevance of this interaction.

YtqI belongs to the DHH family of phosphoesterases, more specifically to the DHHA1 subfamily (25), some members of which are involved in nucleic acid

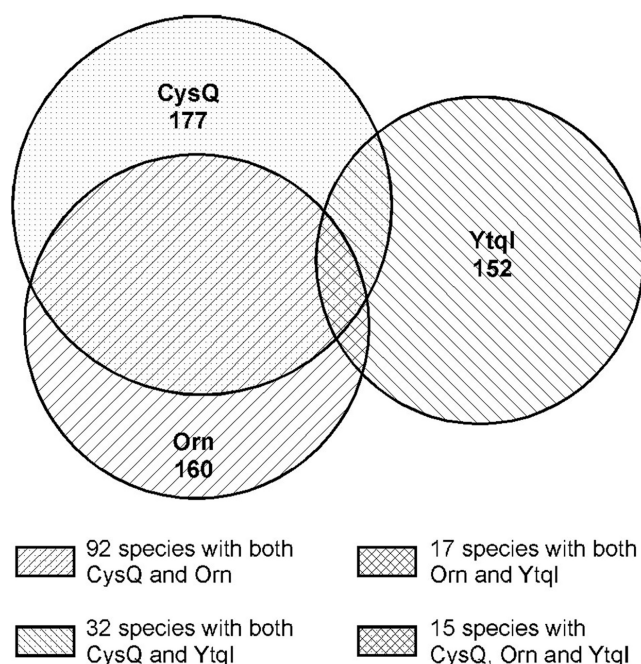


Figure 8. Distribution of CysQ, Orn and YtqI in bacterial genomes.

metabolism. It was therefore a good candidate for a functional Orn analog. YtqI can complement a conditional *orn* mutant in *E. coli* when expressed at similar levels as Orn. This complementation does not require high amounts of YtqI, as expression levels that are below the detection limit of Anti-His₆ antibodies are sufficient. Recombinant YtqI is able to degrade nanoRNA 5-mers *in vitro* in the presence of manganese. Whereas Orn is essential in *E. coli*, YtqI is not essential in *Bacillus*. This points to the existence of at least one more enzyme with the ability to degrade nanoRNA.

The pattern of degradation products on the PAA gel as well as the kinetics of their appearance make it clear that Orn and YtqI employ different mechanisms for the degradation of nanoRNA. 5-mers are not a good substrate for YtqI, they might be degraded in a distributive rather than a processive way. Another obvious difference was the absence of 3-mers from the degradation pattern. One possible explanation for this could be a preferred degradation of 3-mers into 2-mers. When used as substrate, 3-mers are degraded much faster than 5-mers, requiring approximately 10 times less enzyme than 5-mers for complete degradation. We therefore concluded that 3-mers are much better substrates than 5-mers. This *in vitro* result could reflect the intriguing possibility that YtqI acts preferentially on 3-mers *in vivo* and cannot efficiently degrade 5-mers. In this case, the fact that YtqI can complement Orn in *E. coli* could suggest that the accumulation of 3-mers and not 5-mers is the main cause of growth deficiency in *E. coli* lacking Orn. According to the literature (28,29), 90% of RNA degradation in *E. coli* is done hydrolytically, implying a more significant contribution of RNase II and RNase R as compared to PNPase. The relative contribution of RNase II and RNase R is under dispute; RNase II was considered

to be the main contributor to mRNA degradation (30), but this result was questioned by a genome-wide analysis of mRNA levels in a strain deleted for RNase II (31). The end products of degradation catalyzed by RNase II and RNase R differ slightly in size; for RNase II experimental data indicate 3–5-mers (12) or 4–6-mers (11,32) as final product and 4-mers according to the structural model (13), and RNase R leaves 2–3-mers (12,14) or 1–2-mers (11). The size range of fragments produced by RNase R seems to be more suitable for degradation by YtqI than that of oligonucleotides produced by RNase II. The importance of RNase R is increasingly recognized. RNase R has the ability to degrade stable RNA (12,33) and contributes to quality control of rRNA (33). More recently this enzyme was shown to be involved in the degradation of mRNA substrates with extensive secondary structure (27). In addition, RNase R was shown to increase dramatically under different stress conditions (34–36). *Bacillus subtilis* harbors only one member of the RNR family of exoribonucleases, RNase R, which seems to be equally important for the degradation of highly structured RNA as its counterpart in *E. coli* (37). Another requirement for oligonucleotide degradation might also come from systems expressed under the control of cyclic dGMP. Indeed this regulatory molecule is degraded by a phosphodiesterase, which should result in formation of pGpG, a dinucleotide that needs to be further degraded (38).

We previously demonstrated that Orn and Sfn bind pAp, but cannot degrade it, instead pAp is a strong inhibitor of these enzymes (15). YtqI however can degrade pAp *in vitro*; it also complements a *cysQ* mutant in *E. coli*. Both results clearly indicate that YtqI is a pAp-phosphatase. The phenotype of an *ytqI* mutant in *B. subtilis* resembles that of an *E. coli cysQ* deletion: growth is impaired in the absence of cysteine. Withdrawal of cysteine causes doubling times to increase 1.6- or 2.3-fold in an *ytqI* mutant in *B. subtilis* or an *E. coli cysQ* mutant, respectively. The effect of withdrawal of cysteine seems therefore slightly more moderate in *B. subtilis* lacking YtqI than in *E. coli* lacking CysQ. In fact, the difference between *B. subtilis* and *E. coli* in this respect could be somewhat larger, considering the fact that *E. coli* strain MG1655 used in our experiments has a rather leaky *cysQ* mutant phenotype as compared to other strains of *E. coli* (39). One possible explanation for this interspecies difference could be bispecificity of *B. subtilis* protein CysH1. This enzyme has the ability to reduce both PAPS and APS (40) *in vitro*. In addition, expression of *B. subtilis cysH1* can complement an *E. coli* mutant defective for APS kinase encoded by *cysC* (41). This raises the possibility that APS could be reduced directly in *B. subtilis*, which would bypass the requirement for PAPS synthesis and thus pAp accumulation could be unnecessary. The direct reduction of APS is commonly used in plants (42) and was documented for some bacteria including *Mycobacterium tuberculosis* (41), *Pseudomonas aeruginosa* (43) and *Rhizobium meliloti* (44).

The existence of a second enzyme able to hydrolyze pAp could be an alternative explanation for the only partial

Table 1. Bacterial strains, plasmids and primers

	Description	Reference
Strains		
CF10230	nic ⁺ derivative of DY329 (20)	Michael Cashel, unpublished data (15)
UM285	As CF10230 but Δ cysQ, km ^R	(15)
UM341	As CF10230 but <i>orn</i> under control of P _{LtetO-1} , TetR, km ^R	This study (19)
DH5 α Z1	<i>tetR</i> under control of P _{N25} , <i>lacI</i> under control of P _{laciq} , Sp ^R	(19)
BSF66	<i>B. subtilis</i> 168, <i>ytqI</i> ::pMUTIN2MCS, Em ^R	(21)
DB430	<i>B. subtilis</i> 168, deficient in multiple proteases	(22)
Plasmids		
pBAD18	Vector, P _{ara} , pBR replicon, Ap ^R	(45)
pGEM-T Easy	Vector for TA cloning	Promega
pZE21-MCS1	Vector, P _{LtetO-1} , ColE1 replicon, Km ^R	(19)
pUM404	As pBAD18, CysQ with C-terminal his-tag	(15)
pUM408	As pBAD18, Orn with C-terminal his-tag	(15)
pUM412	As pBAD18, YtqI with C-terminal his-tag	This study
Primers		
UM153	5'GATTGCGGCCGCGCTTGGATTCTACCAATAAAA3'	
UM154	5'GCGTTTTTTATTGGTGAGAATCCAAGCGCGGCCGCAATCATAAAAA TTTATTTGCTTTCAGGAAAATTT3'	
UM155	5'GATACACGCAATTGTGGATCAATATAGTCACTGTGAATGGGTGGAAA ATAGCGTCGACGGCGGATTTGTCTACTCAGG3'	
UM156	5'CCGGTCATCTCAAGATCGATCCAAATCAGGTTGTTTTTCATTGGCACTCA TGGTACCTTTCTCCTCTTTAATGAATTCGG3'	
UM158	5'TTCCACATCAGCGTGCATAC3'	
UM159	5'GCAACGAACAGGTCACTATC3'	
UM160	5'CCTGCGCCATCAGATCCTTG3'	
UM161	5'TCAGCGCCAGCTGTTTCATCA3'	
UM172	5'GTGCTGCAAGGCGATTAAGT3'	
UM173	5'CGCCAAGCTATTTAGGTGAC3'	
UM175	5'GGGGAATTCACCATGAAAACAGAATTGATCAGAACCATATC3'	
UM176	5'GGGGCTCGAGCTCGTGTCTTTACATAATGTTTCCAGATC3'	

growth defect of the *ytqI* mutant in the absence of cysteine.

The phylogenetic distribution of YtqI, Orn and CysQ and in particular the anti-correlation is in agreement with the hypothesis that YtqI fulfills the functions of two proteins in *E. coli*, Orn and CysQ.

Until now, RNase R was the only exoribonuclease known in the small genomes of *Mycoplasma* species (3). Noteworthy is therefore the presence of YtqI homologs among the small set of proteins of unknown function in the genomes of *Mycoplasma genitalium* and *Mycoplasma pneumoniae*, where proteins MG371 and MPN140 respectively, are likely to perform the essential function of nanoRNA degradation.

Different species seem to have found different solutions to the same cellular problem, the problem being the degradation of nanoRNA or pAp. Yet another solution to the problem of nanoRNA degradation awaits to be discovered, as cyanobacteria and the alpha division of proteobacteria have neither YtqI nor Orn orthologs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We wish to thank Dr Hermann Bujard for the gift of the anhydrotetracycline tightly regulated system, Dr Michael Cashel for the gift of strain CF10230 Drs Isabelle Martin-Verstraete and Anne-Marie Gilles for helpful discussions. This work was supported by European Union's 6th Framework Program: BioSapiens-Network of Excellence, Grant LSHG CT-2003-503265, section 66010 and by the French Ministry of Research ACI IMPBIO (program Blastsets). Funding to pay the Open Access publication charges for this article was provided by the Institut Pasteur, Unité Génétique des Génomes Bactériens.

Conflict of interest statement. None declared.

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