Characterization of tRNA^{Cys} processing in a conditional *Bacillus subtilis* CCCase mutant reveals the participation of RNase R in its quality control

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We generated a conditional CCCase mutant of *Bacillus subtilis* to explore the participation *in vivo* of the tRNA nucleotidyltransferase (CCA transferase or CCCase) in the maturation of the single-copy tRNA^{Cys}, which lacks an encoded CCA 3' end. We observed that shorter tRNA^{Cys} species, presumably lacking CCA, only accumulated when the inducible Pspac : cca was introduced into an *rnr* mutant strain, but not in combination with *pnp*. We sequenced the tRNA 3' ends produced in the various mutant tRNA^{Cys} species to detect maturation and decay intermediates and observed that decay of the tRNA^{Cys} occurs through the addition of poly(A) or heteropolymeric tails. A few clones corresponding to full-size tRNAs contained either CCA or other C and/or A sequences, suggesting that these are substrates for repair and/or decay. We also observed editing of tRNA^{Cys} at position 21, which seems to occur preferentially in mature tRNAs.

Altogether, our results provide *in vivo* evidence for the participation of the *B. subtilis* cca gene product in the maturation of tRNAs lacking CCA. We also suggest that RNase R exoRNase in *B. subtilis* participates in the quality control of tRNA.

Received 17 September 2009

Revised 3 March 2010

Accepted 31 March 2010

INTRODUCTION

tRNAs are synthesized as precursors that undergo post-transcriptional modification, such as 5' and 3' processing, addition of the 3' terminal CCA sequence and nucleotide editing. The CCA trinucleotide is a key feature of all mature tRNAs as it is the site of aminoacylation and transpeptidation reactions. In prokaryotes and eukaryotes, maturation of the 5' end of tRNAs is generally performed by the endoRNase RNase P (Mörl & Marchfelder, 2001; Schurer *et al.*, 2001), although in the archaeon *Nanoarchaeum equitans*, which lacks RNase P, the tRNA 5' end is the transcription start point (Randau *et al.*, 2008). Processing of the 3' terminus may vary from organism to organism and even between some tRNAs in the same organism. Processing of the 3' terminus of tRNAs in eukaryotes involves an endonucleolytic cleavage (Dubrovsky *et al.*, 2004; Mörl & Marchfelder, 2001; Schurer *et al.*, 2001), with exonucleolytic processing as an alternative pathway. In *Escherichia coli*, different endo- and exoRNases participate in different

combinations as needed for the particular arrangement of tRNA genes in operons (Li & Deutscher, 1996; Li *et al.*, 2002; Ow & Kushner, 2002; Mohanty & Kushner, 2007, 2008). In *Mycoplasma genitalium*, the only identified exoRNase is RNase R, which is suggested to process the 3' end of tRNAs and to also have degradative functions (Lalonde *et al.*, 2007).

In *Bacillus subtilis* both exonucleolytic and endonucleolytic pathways participate in the maturation of tRNAs (Pellegrini *et al.*, 2003; Wen *et al.*, 2005). In contrast to what happens in *E. coli*, where all tRNAs have an encoded CCA terminus, 30 % of *B. subtilis* tRNA genes do not encode the CCA end. EndoRNase RNase Z has been shown to process the 3' end of tRNAs not containing an encoded CCA in *B. subtilis* (Pellegrini *et al.*, 2003), while tRNAs with an encoded CCA are processed exonucleolytically mainly by RNase PH (Wen *et al.*, 2005).

Maturation of the tRNAs lacking an encoded CCA requires the post-transcriptional addition of CCA to the 3' end. The enzymes involved in the addition of CCA, ATP(CTP):tRNA nucleotidyltransferases (CCases), belong to a nucleotidyltransferase superfamily and are highly conserved (Bralley *et al.*, 2005). In some organisms a single CCCase modifies the 3' end of damaged or immature tRNAs in a template-

Abbreviations: CCCase, ATP(CTP):tRNA nucleotidyltransferase (or CCA transferase); PNPase, polynucleotide phosphorylase.

A supplementary table of sequences from cloned 3' ends of tRNA^{Cys} is available with the online version of this paper.

independent manner. Interestingly, it has been shown that in *Aquifex aeolicus* (Tomita & Weiner, 2001), *Synechocystis* sp., *Deinococcus radiodurans* (Tomita & Weiner, 2001), *Bacillus halodurans* (Bralley *et al.*, 2005), *Bacillus clausii* (Neuenfeldt *et al.*, 2008) and *Geobacter sulfurreducens* (Bralley *et al.*, 2009) there are two enzymes, one of which adds the CC dinucleotide and one that adds the terminal A. In *B. subtilis* there is only a single gene coding for CCCase (Raynal *et al.*, 1998). In *E. coli*, where all tRNA genes encode a 3' CCA, growth is impaired in a mutant lacking CCCase (Zhu & Deutscher, 1987), suggesting that tRNA 3' end repair is an important process.

Defective RNA molecules are quickly degraded and this process has been generally termed quality control (Cairrao *et al.*, 2003; Li *et al.*, 2002). It has been shown in *E. coli* that polyadenylation by poly(A) polymerase and polynucleotide phosphorylase (PNPase) activity promotes degradation of defective tRNA^{TP} (Li *et al.*, 2002). However, PNPase and RNase II have been shown to be incapable of processing through stem-loop structures (reviewed by Grunberg-Manago, 1999), while RNase R can process through these structures (Cheng & Deutscher, 2005; Cairrao *et al.*, 2003).

In this study we examined the different steps of maturation and decay of tRNA^{Cys} in *B. subtilis*. We analysed the effect of a conditional CCCase mutant in combination with RNases on growth and on the processing and degradation of tRNA^{Cys}. We identified for the first time in *B. subtilis* a tRNA editing event occurring at position 21 of the D arm of the tRNA^{Cys}, during processing of tRNA^{Cys-Leu}. We also determined that in a conditional CCCase mutant, shorter tRNA^{Cys} species accumulated only when RNase R was also absent, suggesting that this exoRNase has a principal role in the quality control of tRNA^{Cys}, and possibly also other tRNAs, in *B. subtilis*.

METHODS

Bacterial strains. The *B. subtilis* prototroph PY79 (Youngman *et al.*, 1984) was used as the wild-type strain and for the generation of all mutant derivatives. Mutant strains were constructed by transformation of PY79 with genomic DNA from the different strains carrying the genes of interest substituted with antibiotic-resistance markers. Strains employed in this study were $\Delta pnpA::Cm$ (Wang & Bechhofer, 1996) and $\Delta rnr::spc$ (Oussenko *et al.*, 2005). Pspac::cca was constructed by cloning the 3' region of *ypjH*, located upstream from *cca*, into pMutin2 (Vagner *et al.*, 1998). This fragment was amplified from *B. subtilis* PY79 chromosomal DNA using oligonucleotides 2247 (5'-ACAACAGAA-TTCGTCGTCATCCATGTGTCCAAC-3') and 2248 (5'-AGTAAGG-GATCCTGATCACTCCGGTCTGTCTAAATC-3'). The PCR product was cleaved with *EcoRI* and *BamHI* using the sites contained in the amplification oligonucleotides. Transformants were selected on LB plates containing erythromycin (1 μ g ml⁻¹), IPTG (1 mM) and X-Gal (1 μ g ml⁻¹). Integration was confirmed by PCR using oligonucleotides 2361 (5'-AAGGGCATTAAACGACGAA-3') for the *erm* cassette and 2248, and also with 2361 and GOG56 (5'-GAGAGAAGATCTCTT-GCCGCCAAAATAATCAA-3') for the *cca* gene. In this strain, expression of *cca* and *birA* are under the control of the Pspac promoter. The conditional mutation generated was only viable if *birA*, an essential gene (Kobayashi *et al.*, 2003), was ectopically expressed.

birA was amplified using oligonucleotides 2245 (5'-AATAAGGATC-CACATAGAGGAGTGGCTGAAGACAT-3') and 2246 (5'-AATCTG-GATCCCAAGCCAAAAACCTTCCGTTAC-3') and cloned as a *BamHI* fragment in pX (Kim *et al.*, 1996). The pX::*birA* construct was integrated at *amyE*. The Cm^r cassette in pX::*birA* was later exchanged for a Tet^r cassette using plasmid pCm::Tc (Steinmetz & Richter, 1994). To obtain higher repression of Pspac::cca this strain was then transformed with plasmid pMAP65, which carries *lacI*^q (Petit *et al.*, 1998; Yansura & Henner, 1984).

Growth of bacteria and isolation of RNA. To compare growth of the different mutants, single colonies were inoculated into LB medium (without IPTG but adding 1% xylose) and cultures were grown overnight at 37 °C. These were used to inoculate LB medium (with or without IPTG at 1 mM) starting at a cell density of 5 Klett units (Klett–Summerson colorimeter with red filter). Cell density was measured every hour and doubling time was determined during exponential growth. For RNA isolation *B. subtilis* strains were grown at 37 °C in LB medium and aliquots were withdrawn during the exponential phase (100 Klett units). Total RNA was generally isolated using RNeasy mini-columns, as described by the manufacturer (Qiagen). For the experiments shown in Fig. 1, RNA was isolated using the protocol reported by Varshney *et al.* (1991). As this protocol is for *E. coli*, we made some modifications to use it with *B. subtilis*. The cell pellet was resuspended in 0.3 ml of 0.3 M sodium acetate, pH 8.8, 10 mM EDTA and 3 mg lysozyme ml⁻¹ and was incubated at 37 °C for 10 min. The next steps followed the protocol of Varshney *et al.* (1991), but with all solutions at pH 8.8. Samples were treated with DNase as described by the manufacturer (Invitrogen) and RNA was reprecipitated as above.

Northern blot and RT-PCR analysis. RT-PCR analysis of polyadenylation and cloning of 3' ends from tRNA was done as previously described (Campos-Guillén *et al.*, 2005). The gene-specific primer used for PCR was 1347 (5'-TCCAAAGGCGGCATAGCCAAG-3'), derived from the sequence of the *trnD-cys* region. A 200 ng sample of total RNA was ligated to 200 ng of a hybrid RNA–DNA anchor oligonucleotide [pUUUAACCGCATCCTTCTCT (RNA shown in italics); Dharmacon RNAi Technologies, Thermo Scientific] in 20 μ l using 40 units of T4 RNA ligase (Amersham Pharmacia Biotech). Two microlitres of the ligation reaction mixture was the template for reverse transcription-PCRs using the Superscript one-step RT-PCR system (Invitrogen), as outlined by the manufacturer. The primers for the reverse transcription reaction were as follows: oligonucleotide *a*, 5'-AATTCCAAGAATTCGAGAAGGATGCGGTTAAA-3'; oligonucleotides *b* and *c*, same as oligonucleotide *a* but with three and eight additional T residues at the 3' end, respectively. RT-PCR products were cloned into pGEM-T Easy (Promega). The clones obtained were analysed to select those with inserts of different sizes and these inserts were sequenced.

RT-PCR to detect expression level of the *cca* mRNA was carried out on total RNA treated with DNase I (Invitrogen) using oligonucleotides 5'-TCAAGTAGAACGGCTGTTTCAG-3' and 5'-GGTTTTCTCGTT-TATGATAAAA-3' (SuperScript One-Step RT-PCR with Platinum *Taq*, Invitrogen). PCRs were carried out to verify absence of DNA (Platinum PCR SuperMix, Invitrogen). rRNA was used to normalize the quantity of RNA in the reactions.

In this work, we developed a simple, rapid RT-PCR mismatch amplification editing assay to verify the changes found during sequencing of tRNA clones, principally for A21G editing. The principle of this assay involves the insertion of single-nucleotide mismatches at the 3' end of the annealed forward primer, so that *Taq* polymerase is unable to extend the primer. Thus, the absence or presence of the specific RT-PCR product reveals a variation from the wild-type DNA sequence. Forward primers for editing detection are shown schematically in Fig. 4. Primer 1 corresponds exactly to the sequence of the

Fig. 1. (a) Amplification of a 400 bp internal fragment of *cca* by RT-PCR: results of a representative RT-PCR experiment showing amplification of *cca* mRNA from the wild-type strain (lane 1), and from the Pspac:: *cca* strain grown in the absence of IPTG (lanes 2, 3, independent RNA samples) or in the presence of IPTG (lane 4). (b–d) Analysis of tRNA^{Cys} maturation by Northern blotting in wild-type and exonuclease mutant strains. The presence (+) or absence (–) of each exonuclease is indicated. The lack of or addition of IPTG for induction of *cca* expression is indicated as (+) or (–) CCCase. Sizes (nt) of the marker bands (Ambion, Decade Marker) in panels (b) and (c) are indicated, while in panel (d) the marker is a tRNA^{Cys} synthesized without the CCA sequence. In panel (d), lanes 5–6 and 7–8 show the results of two independent experiments. Putative CCA-less tRNA^{Cys} is indicated with an arrow. The intensity required to observe the CCA-less tRNA makes the synthetic CCA-tRNA^{Cys} used as marker show as a very thick band, so panel (e) shows the first three lanes from panel (d) at lower intensity, to reveal that the synthetic CCA-less tRNA^{Cys} migrates as a discrete band.

relevant region in tRNA^{Cys} (5′-GGCGGCATAGCCAAGTGGTAA-3′) with A at the 3′ end. Primers 2 and 3, with T and G, respectively, at the 3′ end, allowed the detection of A21U or A21G editing processes. We also introduced another nucleotide alteration near the 3′ end (primer 4) to detect both G17A and A21G editing (5′-GGCGGCATAGCCAAGTaGTA-3′), and primer 5 was used to detect G17A editing (5′-GGCGGCATAGCAAGTa-3′). The primer used for reverse transcription was 5′-TGGAGGCGACACCCGATT-3′. In each PCR, a specific forward primer, 1–5 as described above, and the primer used for reverse transcription (RT primer in Fig. 4) were used for editing detection. These primers generated a PCR product of 74 bp, the length of mature tRNA^{Cys}. To detect the A21 editing process, 1 µg RNA from *B. subtilis* PY79 was used for RT-PCR. The RT-PCR conditions were 50 °C (40 min), 94 °C (2 min), and 15 cycles of 94 °C (30 s), 60 °C (10 s), 72 °C (10 s), with a final 1 min chain elongation. The products were visualized in 2% agarose gel.

Northern blot analysis of tRNA was done as previously described (Reuven *et al.*, 1997; Varshney *et al.*, 1991) and following the recommendations from the NorthernMax Protocol (Ambion). tRNAs were separated on 15% denaturing polyacrylamide gels. The probe used to detect tRNA^{Cys} was 5′-GGTTTTCAGACCTCTGCCTTAC-3′ (complementary from nucleotides 18 to 40 of the mature tRNA^{Cys}), which was 5′-end-labelled using T4 polynucleotide kinase (Invitrogen) and [γ -³²P]ATP. To control for RNA loading in Northern blot analyses of

tRNA^{Cys}, membranes were stripped and probed with an oligonucleotide derived from the sequence of the 5S rRNA gene, 5′-GGAACGGG-TGTGACCTCTTCGCCATCATCA-3′. Decade markers from Ambion and a tRNA^{Cys} synthesized without CCA (5′-GGCGGCAUAGCCA-AGUGGUAAGGCAGAGGUCUGCAAACCUUUAUCCCCGGUUC-GAAUCCGGGUGUCGCCU-3′; Dharmacon RNAi Technologies, Thermo Scientific) were used as size markers for the Northern blots. Quantification of radioactivity in bands on Northern blots was done with a Storm 860 PhosphorImager instrument (Molecular Dynamics) and the digital image was analysed with Image Quant (Bio-Rad).

RESULTS

Generation of a CCCase conditional mutant

Since 30% of tRNAs from *B. subtilis* lack an encoded CCA at the 3′ end (Table 1), we expected that disruption of *cca* would be lethal or would considerably affect cell growth. In fact, a previous report included *cca* as one of the essential *B. subtilis* genes (Kobayashi *et al.*, 2003). We generated a conditional CCCase mutant using the integrative plasmid pMUTIN, so that *cca* would be under the control of the

Table 1. Summary of tRNAs in *B. subtilis*

Anticodons in bold type indicate tRNAs for which all isotypes lack an encoded CCA 3' end.

Isotype	Anticodon	With CCA	Without CCA	Total*
Ala	GGC	4	1	6
	TGC		1	
Gly	GCC	4		7
	TCC	3		
Pro	TGG	3		3
Thr	GGT		1	5
	TGT	2	2	
Val	GAC		1	5
	TAC	4		
Ser	GCA	1		5
	TGA	2		
	GCT	2		
Arg	ACG	4		7
	CCG		1	
	CCT		1	
	TCT		1	
Leu	GAG		1	8
	CAG	1		
	TAG		2	
	CAA		1	
	TAA	1	2	
Phe	GAA	2	1	3
Asn	GTT	3	1	4
Lys	TTT	4		4
Asp	GTC	4		4
Glu	TTC	1	5	6
His	GTG	2		2
Gln	TTG	1	3	4
Ile	GAT	3		3
Met	CAT	6		6
Tyr	GTA	2		2
Cys	GCA		1	1
Trp	CCA	1		1

*Total number of tRNAs decoding triplets for a given amino acid.

inducible Pspac promoter. Given that immediately downstream from *cca* lies the essential gene *birA*, we placed *birA* under the control of a xylose-inducible promoter (pX) at the *amyE* locus. Disruption of *cca* with pMUTIN was obtained only in a strain carrying pX::*birA*. Disruption was accomplished successfully, as indicated by PCR analysis of putative mutant strains, and there was a substantial reduction of *cca* RNA expression in the mutants. *cca* levels were measured by amplification of a 400 bp fragment, internal to *cca*, by RT-PCR. Densitometric analysis of the PCR bands showed a reduction of approximately 80 % in the level of *cca* transcript in the conditional mutant in the absence of inducer as compared with the wild-type strain (Fig. 1a, compare lane 1 with lanes 2 and 3). In the presence of IPTG the level of the RT-PCR product increased at least

fourfold as compared with the wild-type strain (Fig. 1a, lane 4). The conditional Pspac::*cca* mutant exhibited slower growth than the wild-type in the absence of the IPTG inducer (an increase in doubling time from 40 to 49 min, Table 2), and after induction with IPTG it exhibited the wild-type rate of growth (a doubling time of 39 min, Table 2). Additionally, since tRNAs in general are stable we expected that only after sustained growth would an effect of tRNA depletion be observed. We looked at possible changes in the slope of the growth curve after several passages (inoculating fresh medium with inocula from exponential-phase cultures), but growth was steadily maintained even after 15–20 such passages (data not shown). In the genome of *B. subtilis* the tRNA^{Cys} is present in a single copy and lacks an encoded CCA 3' end, while for some of the tRNAs that do not encode a CCA, there are tRNA isotypes that do (Table 1). For others, however, there is no obvious redundancy to compensate for tRNAs not encoding a terminal CCA. We focused on the tRNA^{Cys} to study the effect of reduced expression of *cca* on tRNA maturation.

Effect on growth of a conditional *cca* mutant in combination with exoRNase mutations

We expected that if the reduced expression of *cca* caused a shortage of aminoacylated tRNAs this would have a strong effect on growth but, as stated above, the mutant exhibited only a 20 % increase in doubling time when grown in the absence of IPTG, and a growth rate identical to that of the wild-type strain when grown with IPTG (see Table 2). The relatively small effect on growth of the conditional CCase mutation might be explained by the fact that there is presumably a substantial amount of CCase still present in

Table 2. Growth rates of strains lacking CCA nucleotidyl-transferase and/or RNases

Strain	Doubling time (min)*
Wild-type	40
$\Delta pnpA::cm$	44
$\Delta rnr::spc$	42
$\Delta pnpA::cm \Delta rnr::spc$	58
Pspac:: <i>cca</i> (–IPTG)	49
Pspac:: <i>cca</i> (+IPTG)	39
Pspac:: <i>cca</i> $\Delta pnpA::cm$ (–IPTG)	54
Pspac:: <i>cca</i> $\Delta pnpA::cm$ (+IPTG)	53
Pspac:: <i>cca</i> $\Delta rnr::spc$ (–IPTG)	50
Pspac:: <i>cca</i> $\Delta rnr::spc$ (+IPTG)	43
Pspac:: <i>cca</i> $\Delta pnpA::cm \Delta rnr::spc$ (–IPTG)	54
Pspac:: <i>cca</i> $\Delta pnpA::cm \Delta rnr::spc$ (+IPTG)	49

*Doubling times were measured during exponential growth at 37 °C, and are the mean of at least three experiments. In the case of the conditional Pspac::*cca*-carrying strains, absence (–) or addition (+) of IPTG is indicated in the left column.

the conditional mutant. In addition, CCA repair might take place via the participation of enzymes that are normally involved in tRNA degradation. We therefore focused initially on PNPase, an enzyme with exonuclease and poly(A) polymerase activities (Mohanty & Kushner, 2000), and we also examined the effect of the absence of RNase R, an enzyme that degrades strong stem-loop structures (Cairao *et al.*, 2003). We observed that the *cca* mutation in combination with either *pnpA* or *rnr* affected growth more than either mutation alone (Table 2). For example, the combination of *cca* and *pnpA* increased the generation time from 39 min to 53 min. However, we also observed a surprising phenotype: growth was improved when IPTG was added to the medium for the Pspac:: *cca rnr* but not for the Pspac:: *cca pnpA* strain (Table 2). This result was observed consistently, and what was more intriguing was that growth actually improved somewhat following induction of *cca* in the triple mutant Pspac:: *cca pnpA rnr*.

The growth defects of the combined *cca* and exonuclease mutants could result from an additive effect of two unrelated enzymic activities, but the fact that some recovery was observed when *cca* was induced in the *rnr* background but that there was no recovery in the *pnp* strain when *cca* was induced suggests that there is a physiological link between the CCCase and PNPase activities. Further work is necessary to substantiate this observation.

CCA-less tRNA^{Cys} is produced in a CCCase mutant

The only gene for tRNA^{Cys} is located at the distal end of the *trnD* operon, eight nucleotides upstream from tRNA^{Leu}. Downstream from tRNA^{Leu} is the presumed transcription terminator, consisting of a stem-loop structure followed by four U residues. tRNA^{Cys} does not encode a 3' end CCA, and therefore CCA addition would be expected to be dependent on a functional *cca* gene.

Northern blot analysis was performed to examine whether the level or length of the 3' end of tRNA^{Cys} was affected by the absence of CCCase. Interestingly, we observed no change in the amount of mature tRNA^{Cys} in the uninduced Pspac:: *cca* mutant as compared to either the wild-type or the IPTG-induced Pspac:: *cca* strain (Fig. 1b, compare lanes 1, 5 and 6).

We also looked at the effect that the absence of exoRNases PNPase and RNase R would have on the maturation and/or degradation of tRNA^{Cys}. We therefore examined the effects of decreased *cca* expression in mutants lacking these exonucleases (Fig. 1b, c). Interestingly, accumulation of a shorter tRNA^{Cys} species could be observed as a band running slightly ahead of the mature tRNA when the *cca* mutant was combined with a mutation in *rnr* (Fig. 1c, lanes 3 and 5). The shorter species was not detectable in combination with the *pnpA* mutation (Fig. 1c, compare lanes 1 and 2). Quantitative analysis of data from three

independent experiments showed that the shorter tRNA^{Cys} species represented around 12 % of total tRNA^{Cys} detected by Northern blotting. The level of the apparently mature tRNA^{Cys} did not change significantly with or without IPTG addition or when RNase R was present. As expected, the shorter tRNA^{Cys} species was not detected when the Pspac:: *cca rnr* strain was grown with IPTG (Fig. 1c, lanes 3 and 4). A similar result was observed in the *cca* mutant when RNase R and PNPase were simultaneously absent (Fig. 1c, lanes 5 and 6). To give support to our suggestion that the tRNA species migrating faster than the mature tRNA^{Cys} could be the CCA-less tRNA^{Cys}, we used a synthetic CCA-less tRNA^{Cys} as a molecular marker (Dharmacon RNAi Technologies, Thermo Scientific) in a Northern blot (Fig. 1d, e). From this experiment, we observed that the shorter tRNA^{Cys} species that accumulated in the absence of RNase R had the same mobility as the marker and presumably represents tRNA^{Cys} lacking the CCA end. We conclude from these experiments that the residual CCCase produced in the conditional mutant in the absence of induction is not enough to process all transcribed tRNA^{Cys}, and the shorter CCA-less tRNA^{Cys} species accumulates when RNase R is absent. The fact that the CCA-less tRNA^{Cys} species accumulated in an *rnr* background suggests that it is normally degraded in an RNase R-dependent pathway.

Decay intermediates and 3' end variation of tRNA^{Cys} processing products

The data presented above indicated that around 12 % CCA-less tRNA^{Cys} accumulated in the conditional *cca* mutant in combination with *rnr*. This observation led us to examine the tRNA 3' ends of various decay and maturation intermediates produced in the wild-type strain and in various mutants utilized in this study. We used an RT-PCR cloning procedure we had employed previously to study RNA decay products (Campos-Guillén *et al.*, 2005) (see Methods). This strategy is based on the ligation of an adaptor oligonucleotide to the 3' end of total RNA and subsequent PCR amplification with a gene-specific oligonucleotide. The oligonucleotide complementary to the adaptor has specific variations that allow the detection of poly(A)-modified ends. Clones are size-selected to enrich for different-sized intermediates. This strategy allows the detection of rare processing intermediates, as tRNAs are stable and abundant, and otherwise only mature tRNAs would be cloned. We generated a collection of clones that contained fragments of tRNA^{Cys-Leu} of different lengths (Fig. 2). From 150 sequenced clones, 21 corresponded to the mature form (with CCA at position 71). As for the other clones we could distinguish three classes: class 1 (10 clones), corresponding to the full-size tRNA (71 bases), but with an end different from CCA; class 2 (100 clones), corresponding to decay fragments; and class 3 (19 clones), corresponding to precursors that included tRNA^{Cys-Leu} or even the transcription terminator. We found among these three classes of clones that 79 had different 3' end

Summary of observed editing sequenced clones

○ Clones exhibiting no editing	54
● Clones exhibiting editing A21 to G21	85
Clones with changes at other positions	11
Total number of clones sequenced	150

Fig. 2. Compilation of cDNA clones representing the 3' ends of tRNA^{Cys-Leu} from wild-type and exoRNase mutant strains. cDNA clones of different sizes were selected to allow the examination of truncated, mature and immature tRNA^{Cys-Leu} transcripts. The 3' end of each clone is indicated by a vertical line. The numbers indicate the position where the anchor primer was ligated or where nucleotides were added post-transcriptionally. Clones with or without tails are indicated (upper and lower). Coding genes are depicted by rectangles. The double slash to the left indicates that the region shown is part of operon *trnA^D*. The transcription-termination sequence is indicated by the lollipop structure. Positions are numbered from the first base in mature tRNA^{Cys}. The arrow at position 21 indicates the clones exhibiting editing from A to G or U. Sequences of the relevant regions and clones with editing are shown in Supplementary Table S1.

additions, typical of those that have been observed in decaying *B. subtilis* RNAs (Campos-Guillén *et al.*, 2005). However, when comparing the additions produced in the wild-type and the different mutants, we observed heteropolymeric tails only in clones from the wild-type and *cca* strains and not from *pnp* or *rnr* mutants (in any combination).

Class 1 clones, with additional residues -AAA, -CAAAA, -CAAA or -CC, in position 71 of tRNA^{Cys}, were obtained from strains where PNPase, RNase R or CCase were absent (Fig. 2; see also Supplementary Table S1, available with the online version of this paper), but not from the wild-type strain. These additions may perhaps occur as part of a repair mechanism or may represent initiation of decay.

It should be noted here that although we were able to identify species that were longer than the mature tRNA by RT-PCR we did not observe such species in our Northern blots. We suggest that those species, although detectable because of the sensitivity of RT-PCR, are heterogeneous and present in amounts too small to detect by blotting. Taken together, these data appear to reflect a preferential addition of A residues by the machinery responsible for modifying the 3' ends of the RNAs examined in this study, with a lower preference for any of the other three nucleotides. These data strongly suggest that a poly(A) polymerase activity played a significant role in the decay of the tRNA fragments.

Editing of position 21 in tRNA^{Cys}

While sequencing clones of the tRNA^{Cys} 3' ends we observed that there was a change of A to G at position 21 in many of the clones. Given the high frequency of occurrence (56%) and that it was independently obtained from different genetic backgrounds we were confident that we had uncovered an editing process. In order to determine the order of occurrence of the different maturation events of the tRNA^{Cys}, we examined different products, hoping that we could deduce whether such editing occurred before or after the processing at the 3' end and the addition of CCA. Fig. 3 shows a schematic of these results. Most of the clones representing incompletely processed tRNA^{Cys} (containing part of the tRNA^{Leu} or the entire tRNA^{Cys-Leu} 3' trailer region) lacked such modification. Most clones representing a truncated tRNA^{Cys} contained the A21G change. We also obtained 11 clones with changes at other positions. Five truncated tRNA^{Cys} species contained both the A21G and a G17A change, one mature tRNA^{Cys} contained the same change, and four truncated tRNA^{Cys} species contained A44G, A57G, U45G and A20G respectively (Fig. 3, Supplementary Table S1). Since it was possible that the observed changes, including those at A21, were the result of errors during PCR or DNA sequencing, we employed an RT-PCR mismatch amplification editing assay (see Methods) to determine if the editing process, particularly that of position 21, was indeed occurring. This procedure involved the use of PCR primers with bases at

Fig. 3. Summary of 3' tail addition and editing for tRNA^{Cys}. A representative clone from various strains is shown to depict typical 3' tail addition and editing. The arrows indicate the addition at position 71 or the base change. Sequence (a) was obtained from the wild-type strain, (b) from an *rnr::spc* strain, (c) was repeatedly obtained from different strains – *rnr::spc*, *pnpA::cm rnr::spc*, *Pspac::cca pnpA::cm* and *Pspac::cca* strains, (d) was obtained from *rnr::spc* and *Pspac::cca* strains and (e) was obtained from a *Pspac::cca pnpA::cm* strain (see Supplementary Table S1).

the 3' end that do not match the tRNA^{Cys} sequence unless the editing occurs. The presence of these mismatches prevents *Taq* polymerase from extending the primers efficiently. The results of this analysis are shown in Fig. 4. Primers 1 and 2 have A and T, respectively, at their 3' ends. If G is the edited base present at position 21 in

mature tRNA^{Cys} primers 1 and 2 would not be expected to function effectively in PCR with the reverse transcript of tRNA^{Cys} as the template. Lanes 1 and 2 in Fig. 4 show that this is indeed observed. In contrast, primer 3, which corresponds to the sequence containing the A21G edit, functions efficiently in PCR and yields a strong band

Fig. 4. RT-PCR mismatch amplification editing assay. Mismatch primers used in this study are shown above and the reverse primer below the sequence of the tRNA^{Cys}. Letters shown above each mismatch primer indicate the nucleotide alterations introduced at the 3' end to enhance the 3' mismatch effect. Letters shown below the tRNA^{Cys} sequence indicate changes found during sequencing. The agarose gel (shown as a negative image) shows the PCR products obtained with the various mismatch primers. The numbers above each lane indicate the mismatch primer, 1–5, used for PCR. The position of the 74 bp amplification product of tRNA^{Cys} is indicated. Lane M contained DNA size standards (100 bp ladder).

corresponding in size to mature tRNA^{Cys} (Fig. 4). Thus, the A21G edit is present in most mature tRNA^{Cys} molecules. No PCR product was obtained with primers 4 and 5, suggesting that the putative G17A edit is probably a sequencing or PCR artefact.

Regarding the mature tRNA^{Cys} species (containing the CCA end), we obtained both unedited and edited clones, suggesting that complete maturation of the tRNA^{Cys} by CCA addition is not essential for editing at this position but neither is editing essential for maturation.

DISCUSSION

tRNA maturation requires a series of post-transcriptional steps that may include editing of some bases and addition of the CCA trinucleotide at the 3' end, necessary for aminoacylation of tRNAs. In this study, using the single-copy gene tRNA^{Cys} that lacks an encoded CCA, we provide *in vivo* evidence for the participation of *cca* and *rnr* genes in tRNA processing. We also show that the tRNA^{Cys} is edited during its maturation and that its decay pathway is similar to the one described for other *B. subtilis* RNAs.

In *B. subtilis*, *cca* encodes the only gene for the CCase enzyme (Raynal *et al.*, 1998), and given that 30 % of the tRNA genes in *B. subtilis* lack an encoded CCA it is expected to be an essential gene. Of the *B. subtilis* tRNAs that lack an encoded CCA, 11 have anticodons that are not present in other tRNAs (see Table 1), notably those for Arg and Leu, and the single Cys decoding tRNA. All the *E. coli* tRNA genes encode a 3' end CCA, so CCase is thought to have mainly a repair function in that organism (Zhu & Deutscher, 1987). We analysed the growth of strains carrying a conditional *cca* mutation in combination with other exoRNase mutations. A *cca* mutation in combination with mutations in either *pnpA* or *rnr* severely reduced growth, suggesting that the functions of these enzymes converge in some common process. It seems possible that this effect on tRNA metabolism may be responsible, at least in part, for the slower growth of *cca* strains bearing exonuclease mutations as compared to the wild-type.

In contrast to many mRNAs, the secondary structure of tRNAs and their aminoacylation are factors that contribute to their protection against the principal exoRNases. Our results show that a decrease in the expression of *cca* coupled with the removal of RNase R from *B. subtilis* leads to an increase in the amount of a tRNA species that is shorter than mature tRNA^{Cys}, which we suggest is a CCA-less form of tRNA^{Cys}, as it has the same mobility as a synthetic CCA-less tRNA^{Cys} (Fig. 1d, e). The CCA-less tRNA^{Cys} species is no longer observed when CCase is expressed (by addition of IPTG), suggesting that in *B. subtilis* RNase R and CCase are the principal competitors for immature or damaged tRNA^{Cys}. RNase R may be the major exonuclease involved in maintaining quality control of tRNA, a conclusion supported by the results shown in Fig. 1c (lanes 1 and 2), which indicate that PNPase plays no

role in this process. It has been shown that RNase R from *E. coli* is capable of degrading through a stem-loop structure, but that it requires at least seven free nucleotides at the 3' end (Vincent & Deutscher, 2006). Additionally, RNase R is the main enzyme in the poly(A)-dependent pathway for the decay of the *rpsO* mRNA (Andrade *et al.*, 2009). If this is true for *B. subtilis*, RNase R would not be expected to be able to initiate decay unless a few nucleotides were added to the 3' end. We have observed that in fact a few tRNA^{Cys} cDNA clones obtained from *rnr* or *pnpA* mutant strains contained CCAA, CAAA or AAA tails at position 71 (see Supplementary Table S1). PNPase has both 3' to 5' exonuclease and nucleotidyltransferase activity (Mohanty & Kushner, 2000; Yehudai-Resheff *et al.*, 2001) and could be expected to participate in degradation by adding a tail to the tRNAs, but this enzyme is presumably incapable of degrading through strong secondary structure. There may, nevertheless, be another enzyme that adds the required 3' tail, possibly the same enzyme that adds poly(A).

Our data indicate that presumed tRNA^{Cys-Leu} decay and processing intermediates are extensively modified at their 3' ends. A summary of these modifications is presented in Fig. 3. We found different additions, including CCA or CCAA, CAAA, CAAA, AAA or CC, at position 71 when CCase, RNase R or PNPase enzymes were absent (Supplementary Table S1 and Fig. 3). Depending on the strain from which the cDNAs were obtained, we observed homopolymeric poly(A) tails (obtained from all genetic backgrounds) and heteropolymeric tails containing extensive oligo(A) stretches at the 3' ends of various tRNA fragments. Heteropolymeric tails were never recovered from strains containing *pnp* or *rnr* mutations. While *B. subtilis* apparently lacks a poly(A) polymerase like poly(A) polymerase I of *E. coli* (Raynal *et al.*, 1998), the results presented above and data from our earlier studies (Campos-Guillén *et al.*, 2005) indicate that the organism does contain an activity that catalyses poly(A) tail synthesis. Heteropolymeric tail addition is characteristic of PNPase participation in decay, which appears to target any RNA species (mRNA, rRNA and tRNA) (Mohanty & Kushner, 2000; Yehudai-Resheff *et al.*, 2001; Condon, 2003). It would be interesting to investigate further why none of the 66 clones from an *rnr* background had heteropolymeric tails. We suggest that RNase R is essential to carry out degradation of tRNA, as PNPase cannot degrade through secondary structure, but the observed nucleotide addition of heteropolymeric tails is probably done by PNPase. However, a few tRNA^{Cys} cDNA clones obtained from a double mutant strain (*pnpA rnr*) exhibited truncated polyadenylated products, suggesting that in their absence other poly(A)-dependent exonucleases are able to degrade tRNAs.

Growth of the *B. subtilis* CCase mutant was reduced by only 20 % as compared with the wild-type strain (Table 2). This may be explained by the residual expression of *cca* from a leaky promoter and the fact that tRNAs have a very long half-life. However, we cannot exclude the participation of other enzymes in the repair of the CCA end in

B. subtilis. In other biological systems CCA addition for overlapping tRNA precursor molecules, where the 3' end of one precursor is part of the 5' end of the next tRNA, seems to depend on insertional editing and CCA addition. According to Yokobori & Pääbo (1997), poly(A) polymerase and exoRNases might restore the 3' end in such situations. In organelles, the tRNA nucleotidyltransferase only has to add two nucleotides, C and A, to the 3' end because a high number of plastid tRNA genes encode a C at the 3' end (Mayer *et al.*, 2000; Tomita *et al.*, 1996). *Aquifex aeolicus*, *Deinococcus radiodurans*, some cyanobacteria and recently *Bacillus halodurans*, *Bacillus clausii* and *Geobacter sulfurreducens* have been found to contain separate enzymes for the addition of A and C residues to tRNA *in vivo* (Bralley *et al.*, 2005, 2009; Neuenfeldt *et al.*, 2008; Seth *et al.*, 2002; Tomita & Weiner, 2001, 2002). In *Streptomyces coelicolor* less than a third of the tRNA genes encode the CCA end and the product of SCO3896, the *S. coelicolor* CCase, is essential in this process (Bralley *et al.*, 2006). In *E. coli*, where CCase activity is not essential, PNPase and poly(A) polymerase participate in the repair of the 3' end of tRNAs in the absence of CCase (Reuven *et al.*, 1997). Our results show that in *B. subtilis* tRNA^{Cys} about 90 % of the steady-state RNA seems to be in the mature form when *cca* expression is greatly decreased, as observed in the *rnr* background (Fig. 1c). We think it likely that enough CCase is produced in the uninduced condition, but we cannot rule out the possible existence of an alternative pathway involved in repair.

After the elimination of 5' and 3' sequences in tRNA precursors and CCA addition, further processing steps are essential to obtain a complete and functional tRNA molecule. It is a frequent occurrence during processing that the common bases are replaced by unusual ones or are modified by addition or deletion, apparently to ensure proper folding of the tRNA molecules (Schurer *et al.*, 2001). One interesting result of our studies was the observation of tRNA^{Cys} editing at position 21 of the D arm. The A21G edit was confirmed by RT-PCR mismatch amplification editing assay (Fig. 4). Fig. 2 shows that editing occurred in 96/150 cDNA clones that we analysed, from wild-type and RNase mutant strains. Interestingly, the majority of the clones obtained with CCA, CCAAA, CAAA or AAA tails had the A21G edit; however, we found clones with CCA, CCAAA or AAA tails with no editing (Fig. 2 and Supplementary Table S1). This suggests that editing is not required for addition of the CCA sequence by CCase. Similarly, the CCA sequence itself does not appear to be necessary for editing. These results suggest that these two reactions, CCA addition and editing, function independently of each other, at least for tRNA^{Cys} in *B. subtilis*.

ACKNOWLEDGEMENTS

We thank Africa Islas for technical assistance. This work was supported by CONACYT Grant No. 36950 to G.O.-A. and a fellowship to J.C.-G.

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Edited by: T. Msadek