PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins

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ABSTRACT

In this report, we demonstrate that exonucleolytic turnover is much more important in the regulation of sRNA levels than was previously recognized. For the first time, PNPase is introduced as a major regulatory feature controlling the levels of the small noncoding RNAs MicA and RybB, which are required for the accurate expression of outer membrane proteins (OMPs). In the absence of PNPase, the pattern of OMPs is changed. In stationary phase, MicA RNA levels are increased in the PNPase mutant, leading to a decrease in the levels of its target *ompA* mRNA and the respective protein. This growth phase regulation represents a novel pathway of control. We have evaluated other ribonucleases in the control of MicA RNA, and we showed that degradation by PNPase surpasses the effect of endonucleolytic cleavages by RNase E. RybB was also destabilized by PNPase. This work highlights a new role for PNPase in the degradation of small noncoding RNAs and opens the way to evaluate striking similarities between bacteria and eukaryotes.

Keywords: MicA RNA; *ompA*; PNPase; RybB RNA; small RNA

INTRODUCTION

Small noncoding RNAs (sRNAs) have very important regulatory roles, and many sRNAs can be induced by changes in the environmental conditions (Storz et al. 2005). MicA and RybB RNAs are stationary phase regulators and belong to the emerging group of sRNAs that control outer membrane permeability. RybB RNA controls the expression of two outer membrane proteins, OmpC and OmpW, while MicA RNA specifically controls the expression of OmpA, an abundant outer membrane protein that has an important structural role and is involved in pathogenesis (Guillier et al. 2006). Both sRNAs act as antisense RNA and bind the 5' UTR of their target mRNAs, inhibiting translation. Moreover, MicA triggers the decay of *ompA* mRNA in an Hfq-dependent manner (Rasmussen et al. 2005; Udekwu et al. 2005; Johansen et al. 2006). Recent studies have described their transcriptional regulation and established MicA and RybB RNAs as new members of the σ^E -regulon (Figueroa-Bossi et al. 2006; Johansen et al. 2006; Papenfort et al. 2006; Udekwu and Wagner 2007).

However, nothing is known about the post-transcriptional control of either MicA or RybB RNA, and this can be determinant in controlling the levels of these sRNAs.

For the sRNAs that have been analyzed, endonucleolytic processing seems to be an important regulatory event. RNase E has been suggested to be the main endonuclease, although others like RNase III can also be involved (Wassarman et al. 1999). RNase II and PNPase are major *Escherichia coli* 3'–5' exonucleases, and a double mutant is not viable (Donovan and Kushner 1986; Régnier and Arraiano 2000). PNPase is a phosphate-dependent exonuclease that acts as a trimer (Pruijn 2005). The recent available structure of RNase II highlighted the mechanism of degradation of this hydrolytic enzyme (Amblar et al. 2006, 2007; Frazão et al. 2006; Zuo et al. 2006). Homologs of these exonucleases exist in eukaryotic exosomes (a multiprotein complex of exonucleases), and they were shown to be essential in the processing of noncoding RNAs (Houseley et al. 2006). Some sRNAs can also require 3' exonucleolytic trimming to become functional, and exonucleases can play essential roles in RNA quality control (Li et al. 1998, 2002; Cairrão et al. 2003). Recently, it was demonstrated that bacterial exonucleolytic degradation can also proceed in the 5'–3' direction (Mathy et al. 2007).

In this work, we have investigated the post-transcriptional regulation of MicA RNA. We have shown that PNPase mediated degradation can be an essential regulatory

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feature in the expression of a small RNA, and as a result affect its target mRNA with consequences at the protein level. The stabilization of the MicA RNA in the PNPase mutant is higher than the one obtained in an RNase E mutant. Furthermore, we have also shown that PNPase controls the levels of RybB, another chromosomal encoded sRNA also controlling outer membrane protein expression. Here, we demonstrate that exonucleolytic turnover is a much more important feature in the control of sRNA levels than was previously recognized.

RESULTS

PNPase regulates *ompA* expression and MicA RNA levels

We have previously shown that exonucleolytic digestion is very important in the control of *ompA* transcript levels (Andrade et al. 2006). We have found that the levels of *ompA* mRNA were drastically reduced in the absence of PNPase (Fig. 1A), even though it is possible to observe a band using much higher amounts of total RNA (Andrade et al. 2006). The observed differences in *ompA* expression could be related to changes in the amount of the regulatory

MicA RNA. To test this, we have analyzed its expression in different ribonuclease deficient strains. MicA RNA levels were shown to be up-regulated (a threefold increase) in the absence of PNPase and were not altered by any of the other exonucleases analyzed (Fig. 1A). In addition, overexpression of PNPase from a plasmid complemented the *pnp* mutant and reverted MicA RNA levels to wild-type (wt) condition (Fig. 1B). The above results implicate PNPase in the control of MicA RNA concentration in the cell.

To evaluate if it was possible to establish a direct correlation between the increasing levels of MicA and the reduced quantity of *ompA* mRNA in the absence of PNPase, we decided to compare *micA* and *pnp* mutants. As expected, a higher concentration of *ompA* mRNA was found in *micA* cells. Moreover, the combined absence of *pnp* and *micA* mutations results in a significant increase in *ompA* transcripts when compared with the *pnp* single mutant (Fig. 1C), revealing a direct role between MicA levels and the reduction of *ompA* mRNA levels in *pnp7* cells. Nevertheless, in the double mutant *pnp micA*, the *ompA* mRNA levels are ~50% of the wild-type levels. This shows that in the absence of PNPase, MicA is probably not the only regulator of *ompA* expression. In fact, it has been recently described that *ompA* is also regulated by RseX (Douchin et al. 2006).

FIGURE 1. PNPase regulates *ompA* and MicA RNA levels. (A) Analysis of *ompA* mRNA and MicA RNA levels in the wild type and exonuclease mutant strains in stationary phase. *ompA* mRNA was analyzed by agarose Northern blot. (Upper panel) Reprobing the same membrane with a 23S-RNA probe. (Lower panel) MicA RNA expression was analyzed with the same RNAs in a polyacrylamide Northern. (B) Northern blot showing that overexpression of PNPase from plasmid pKAK7 complements the *pnp* mutant and changes MicA RNA levels to wild-type amounts. (C) Analysis of PNPase and MicA absence in the levels of *ompA* mRNA in stationary phase cells. (Top) Loading control with a 23S RNA probe. (Bottom) MicA RNA expression was analyzed as an additional control. Full-length transcripts were quantified using a Molecular Dynamics PhosphorImager. The amount of the RNA found in wild type was set as 1. The ratio between each strain and the wild type is depicted. (ND) Nondetectable.

Outer membrane composition is altered in a *pnp* mutant strain

The above results prompted us to analyze if the reduced levels of *ompA* mRNA found in the absence of PNPase had consequences at the protein level. For this purpose, we analyzed total protein extracts from stationary phase cultures of the wild-type and the *pnp* mutant strain by 2D gel electrophoresis (Fig. 2A). When compared with wild type, the spot corresponding to OmpA is less intense in the *pnp* mutant. Thus, the results showed that OmpA levels are reduced in the absence of PNPase and established a parallel between *ompA* mRNA and OmpA protein levels. It was previously reported that PNPase activity was important for cell membrane-associated processes, but the reasons for this were unclear (Nicholson 1999). To further elucidate if the outer membrane composition was being generally affected by PNPase, or rather if it was specific to OmpA, we analyzed the outer membrane protein (OMP) profile of wild-type and *pnp* mutant strains. It is possible to observe a distinct pattern of OMPs between stationary phase cultures of these strains (Fig. 2B). Besides the reduction in OmpA levels, the *pnp* mutant also changed the expression of other membrane proteins.

PNPase is involved in the growth phase regulation of the antisense MicA RNA

We have monitored the degradation of the MicA RNA in wild-type and exonuclease mutant cells in stationary phase.

FIGURE 2. Protein profile of *Escherichia coli* MG1693 (wt) and SK5691 (*pnp7*) strains from stationary phase cultures. (A) Two-dimensional gel electrophoresis (2D-PAGE) analysis of total protein extracts. Proteins with pI values in the range 3.0–10.0 and molecular mass in the range of 10–250 kDa were resolved in 12% SDS-PAGE gels followed by Coomassie G-250 staining; corresponding sections of representative gels show the down-regulation of the OmpA porin in the *pnp7* strain compared with wild type. (Arrow) OmpA spot. (B) Outer membrane protein fraction analysis by urea-SDS-polyacrylamide gel electrophoresis. The positions of the OmpC, OmpF, and OmpA bands are indicated. An *ompA* mutant was used as control.

Strikingly, the *pnp* mutant showed more than a threefold increase (from 8.2–27.5 min) in full-length MicA RNA stability when compared with wild type (Fig. 3A,B). This agreed with the threefold increase in the steady-state levels of MicA RNA. The other two main exonucleases, RNase II and RNase R, did not affect the stability of the MicA RNA. Moreover, PNPase was also shown to be important in the decay of degradation intermediates. Only in this mutant can we observe increased stabilization of these smaller species of RNA (Fig. 3A). Furthermore, PNPase degradation of the MicA RNA was shown to be a stationary-phase-specific mechanism, and exponentially growing cells did not present this regulation (Fig. 3C,D). Neither RNase II nor RNase R was shown to significantly affect the stability of this sRNA, independently of the growth phase. Contrastingly, MicA RNA levels are found to be down-regulated in the absence of PNPase in the exponential phase. However, RNA stability is not significantly changed. This could possibly indicate an indirect role for PNPase in the transcriptional control of MicA RNA in the exponential phase. Altogether, these results showed that post-transcriptional regulation by PNPase is a main feature controlling MicA expression.

The role of PNPase on MicA RNA decay is independent of the target *ompA* mRNA

It is known that MicA acts as an antisense RNA, which binds to the 5' UTR of its target *ompA* transcript in an Hfq-dependent fashion, promoting mRNA decay by RNase E (Rasmussen et al. 2005; Udekwa et al. 2005). To clarify if PNPase-mediated degradation of MicA RNA was dependent on the binding of the small RNA to its target *ompA* mRNA, we examined the decay of MicA in the absence of *ompA*. We have generated an *ompA* deletion mutant, and we also constructed the double mutant *pnp ompA*. Results showed that the absence of its target *ompA* mRNA does not significantly affect MicA RNA stability (Fig. 4). Moreover, PNPase-mediated degradation of MicA RNA is shown to be independent of the presence of *ompA* mRNA, as the double mutant *pnp ompA* showed identical stability to the *pnp* single mutant. Consequently, PNPase does not require the previous binding of the MicA RNA to its target *ompA* mRNA for efficient degradation of this antisense RNA.

Polyadenylation participates in the decay of the MicA RNA

Polyadenylation is a major regulatory process controlling gene expression (Régner and Arraiano 2000; Dreyfus and Régner 2002). Polyadenylation of some sRNAs has been described, and polyadenylated RNA is a better substrate for PNPase (Xu and Cohen 1995; Lisitsky and Schuster 1999). To address whether PNPase was acting through a Poly(A)-dependent pathway in the decay of the MicA RNA, we first studied the *pcnB* mutant strain, which is defective in PAP I, responsible for >90% of the polyadenylation in *E. coli* (O'Hara et al. 1995). We have found a slight increase in the half-life in the absence of polyadenylation (Fig. 5), revealing the involvement of PAP I in the MicA RNA degradation pathways. We have also analyzed MicA RNA decay in the *pnp ΔpcnB* double mutant. Results showed that the combined absence of PNPase and PAP I presented about the same MicA RNA stability as found in the single absence of PNPase. These results indicated that polyadenylation by PAP I appears not to be critical for PNPase activity on the MicA RNA turnover.

MicA is more stabilized in the absence of PNPase than in the RNase E mutant

RNase E is an important enzyme controlling sRNA stability (Wassarman et al. 1999; Moll et al. 2003; Morita et al. 2005; Suzuki et al. 2006). Therefore, we have also analyzed the role of RNase E in the degradation of MicA RNA in stationary phase cells. Since RNase E is an essential enzyme, we used the *rne1* thermosensitive allele (Arraiano et al. 1988). Wild-type and *pnp* mutant strains were studied in

FIGURE 3. Comparison of the decay rates of the MicA RNA in the wild-type and exonuclease mutants in stationary and exponential phase. (A) Northern blot of RNA extracted from stationary phase cultures. (C) Northern blot of RNA extracted from exponential phase cultures. Total RNA was extracted from culture samples withdrawn after inhibition of transcription (timepoints are shown in minutes). (*) Nonspecific band that cross-reacts with the antisense MicA-probe. A more stringent washing eliminates this band, while the signal for MicA RNA remains. (B,D) The band corresponding to the full-length MicA RNA was quantified with a PhosphorImager and plotted versus time of extraction (min).

the same conditions. Results shown in Figure 6, A and B, demonstrated that RNase E is involved in the decay of the full-length MicA RNA (a twofold increased stability is observed in the *rne1* mutant when compared with wild type). Comparatively, the *pnp* mutant presented a higher stabilization of this sRNA (more than a threefold increase). Analysis of the double mutant *rne1 pnp* showed the highest stability for MicA RNA (more than a 10-fold increase), greater than any of the stabilities found in the single mu-

tants. This large stability indicates that PNPase and RNase E are major factors involved in the MicA RNA turnover.

The degradosome assembly is not crucial for PNPase-mediated degradation of the MicA RNA

PNPase and RNase E can physically interact in the degradosome, a multiprotein complex involved in mRNA

FIGURE 4. MicA RNA degradation in the absence of the target *ompA* mRNA. (A) Northern blot comparing the importance of *ompA* mRNA in the decay rate with its antisense regulator MicA RNA. Timepoints of sample cultures are shown in minutes after inhibition of transcription. (*) Nonspecific cross-hybridization with the antisense MicA-probe. (B) Full-length MicA RNA stability, after PhosphorImager quantification along time of sample extraction (in min).

FIGURE 5. The role of the polyadenylation in MicA RNA decay in stationary phase. (A) The MicA RNA stability in the wild type and in the absence of PAP I-dependent polyadenylation ($\Delta pcnB$ mutation) was verified by Northern blot analysis. MicA RNA half-lives are shown in minutes (min). (*) Nonspecific band that was used as loading control. (B) Relative amount of the full-length MicA RNA remaining at each timepoint (as determined by PhosphorImager analysis) plotted as function of time.

degradation (Carpousis 2002). To determine the importance of this association in MicA RNA degradation, we performed RNA decay experiments with the *rne131* mutant (that is impaired in degradosome assembly) (Fig. 6C). In the absence of the degradosome, MicA RNA presented a more than twofold stabilization. However, the *pnp* single mutant presents a higher stabilization, revealing that the role of PNPase exceeds the role of the degradosome activity. In fact, the double mutant *rne131 pnp* showed an increased stability when compared with the *rne131* mutant (Fig. 6C,D). These results confirmed that PNPase can act in this sRNA in a degradosome-independent manner.

The noncoding RybB RNA is under the control of PNPase

The results described above raised the question if the effect of PNPase was particular to MicA or whether it could be extended to other sRNAs. We have evaluated the stability of the small RybB in both the wild-type and *pnp* mutant strains. RybB acts as an antisense RNA and regulates the expression of both membrane proteins OmpC and OmpW in stationary phase (Guillier et al. 2006; Johansen et al. 2006). We have shown that RybB is accumulated in the absence of PNPase, and that this is due to an increasing

stability found in the *pnp* mutant (Fig. 7). Moreover, PNPase is also shown to be important for the degradation of breakdown products. As observed, also smaller species of this sRNA are strongly accumulated in the absence of PNPase. These results emphasize the role of PNPase in the control of sRNAs levels and outer membrane protein expression in stationary phase.

DISCUSSION

We have shown here that 3' exonucleolytic degradation is more important in the regulation of sRNA levels than was previously recognized. Transcription was shown to be important in the control of MicA RNA levels that are increased in stationary phase (Figueroa-Bossi et al. 2006; Johansen et al. 2006; Papenfort et al. 2006; Udekwi and Wagner 2007). Here, we have shown for the first time that the growth phase regulation of MicA RNA is highly dependent on post-transcriptional control. Most interestingly, from the major exonucleases involved in mRNA decay, only PNPase was shown to be important in the control of MicA. During stationary phase, the level of MicA RNA was significantly higher in cells containing a mutant allele of the *pnp* gene, which encodes the 3' exonuclease polynucleotide phosphorylase. Moreover, this increase in the level of MicA was associated with a decrease in the cellular level of the target *ompA* mRNA and the OmpA protein. Assay of the half-life of MicA showed that this RNA was more stable (about threefold) in the *pnp* mutant. This result provided an explanation for the increase in MicA level and indicated that this RNA is a substrate for PNPase.

Previous studies in which MicA was overproduced from a plasmid have demonstrated that an increase in the level of this RNA correlates with the down-regulation of *ompA* mRNA (Rasmussen et al. 2005; Udekwi et al. 2005). We also confirmed that the reduction in the *ompA* mRNA level in the *pnp* mutant was dependent to a large extent on the presence of MicA. Given the above findings, it seems likely that the inactivation of PNPase will impact the levels of other MicA targets such as the mRNA of LamB, recently reported in *Salmonella* (Bossi and Figueroa-Bossi 2007). While the level of *ompA* mRNA increased in a *pnp* mutant background when MicA was removed, it was not restored to the level in wild-type cells. This indicates that there is a second, MicA-independent pathway by which PNPase can influence the levels of *ompA* mRNA. RseX, one other sRNA implicated in *ompA* expression, is thus a good candidate to be also regulated by PNPase (Douchin et al. 2006).

Interestingly, PNPase affected MicA when cells were in the stationary phase, but it did not change MicA stability in exponential phase cultures. This indicates that the PNPase-mediated decay of MicA RNA is growth-phase dependent. It has also been reported that PNPase is the only 3' exonuclease involved in the degradation of certain mRNAs in stationary phase in *Streptococcus* (Barnett et al. 2007).

FIGURE 6. RNase E is involved in the control of the MicA RNA stability. (A) Cultures were grown at 30°C to an $OD_{600} \approx 2.0$ and then shifted to the nonpermissive temperature of 44°C. After 5 min, transcription was blocked with the addition of rifampicin, and samples were withdrawn at the times indicated. Total RNA was extracted as described in the Materials and Methods. MicA RNA stability was analyzed using an antisense RNA probe. (C) RNase E mutant cells impaired in degradosome assembly (*rne131* mutation) were analyzed with wild-type and *pnp* mutant strains. MicA RNA stability is shown in minutes (min). (*) Nonspecific band. (B,D) Full-length MicA RNA was quantified with a PhosphorImager and plotted versus time of extraction (min).

The 3' exonucleases RNase II and RNase R appear to be not required for the normal rate of decay of MicA RNA. Hfq, a pleiotrophic regulator with very important roles on RNA (Valentin-Hansen et al. 2004), also participates in the stability of MicA (J.M. Andrade and C.M. Arraiano, unpubl.). Hfq levels are described to decrease upon entrance to stationary phase (Ali Azam et al. 1999). Thereby, it is possible that down-regulation in Hfq levels results in a more unprotected MicA RNA, allowing PNPase to effectively degrade it.

The decay of MicA appears also to be independent of *ompA* mRNA in backgrounds that were wild-type or mutant with regard to PNPase. Thus, while binding between the sRNA and its target mRNA has been reported to be important for the decay of sRNA (Massé et al. 2003; Afonyushkin et al. 2005), this is not the case for PNPase-mediated decay of MicA. Interestingly, we have also shown that PNPase-mediated decay of MicA RNA during stationary phase is not dependent on Poly(A) Polymerase I. This may be related to the finding that PNPase can also work as a polymerase when inorganic phosphate is limiting, as can happen in stationary phase (Mohanty and Kushner 2000).

PNPase and RNase E can physically interact in the degradosome, a multiprotein complex involved in RNA turnover (Carpousis 2002), and these enzymes have been shown to interact functionally in the decay of some sRNAs such as RyhB, SgrS, and CrsB (Massé et al. 2003; Morita

et al. 2005; Suzuki et al. 2006). We found that MicA was stabilized in RNase E mutants that were temperature-sensitive or were unable to form the degradosome. These results revealed the importance of RNase E and clearly established that degradosome in the degradation of the MicA RNA. Moreover, when both of the corresponding *rne* mutations were combined with a *pnp* mutation, the level of stabilization was even higher, supporting the idea that RNase E and PNPase can act independently. Accordingly, the effect of PNPase on MicA is not exclusively due to the activity of PNPase in the degradosome.

PNPase activity on sRNAs appears to be not restricted to MicA; we found also that the small RybB is stabilized in the *pnp* mutant. Together with MicA, the RybB RNA is also a regulator of outer membrane protein expression (Guillier et al. 2006; Johansen et al. 2006). The notion that PNPase is a key player controlling OMP expression, namely through regulation of riboregulators stability, highlights the importance of this enzyme in RNA metabolism and unveils an important role in the adaptation to stationary phase (Fig. 8). The outer membrane provides the first cellular contact with the exterior, and the accurate control of outer membrane composition is essential for survival. A diversity of sRNAs was shown to play a major role on this regulation (Guillier et al. 2006). Earlier reports associate PNPase activity with cell membrane-associated processes such as

FIGURE 7. PNPase controls the small RybB RNA. (A) Northern blot analysis of the stability of the RybB RNA in the wt and *pnp* mutant strains in stationary phase. Full-length RybB and breakdown products of degradation are observable. (Lower panel) The same membrane was stripped and rehybridized with a 5S RNA riboprobe as a loading control. (B) The signal corresponding to the full-length RybB RNA was quantified with a PhosphorImager and plotted versus time of extraction (min).

antibiotic uptake (McMurphy and Levy 1987; Nicholson 1999). We now show that PNPase-mediated degradation of small RNAs controlling outer membrane protein expression can change the profile of OMP in the cell. This may promote membrane alterations with an impact in cellular functions such as cellular morphogenesis, permeability, or virulence.

In eukaryotes, the exosome (a complex of exonucleases reminiscent of PNPase) was shown to be essential in the maturation of noncoding RNAs (Houseley et al. 2006). We envisage that PNPase may be involved in the degradation of other small RNAs, making PNPase-mediated decay a more general feature of stable RNA metabolism.

MATERIALS AND METHODS

Bacterial strains and plasmids

The strains used in this work are indicated in Table 1. All experiments were performed in the *E. coli* MG1693 (wild type) and its derivative isogenic strains. Strain $\Delta ompA::cam$ was constructed using a one-step inactivation system of chromosomal genes (Datsenko and Wanner 2000). Deletion of the *ompA* gene completely eliminates the binding site for the antisense MicA RNA. After transformation with the PCR fragment containing the *ompA*

FIGURE 8. PNPase-mediated degradation is a major regulatory event controlling the levels of sRNAs (namely MicA and RybB) that are required for the accurate expression of outer membrane proteins. These sRNAs act as antisense RNAs and bind to the 5' UTR of their target outer membrane mRNAs in an Hfq-dependent mechanism. This process inhibits translation and can help promoting the decay of the target mRNAs. Balanced outer membrane protein composition is essential for survival and affects many cellular processes, such as morphology, permeability, and virulence. PNPase emerges as an important enzyme in the growth phase adaptation to stationary phase.

deletion into the MG1693 strain, chloramphenicol-resistant colonies were isolated. Transformants were reisolated on selective plates, and mutation was reintroduced into MG1693. The mutations were transferred by P1vir transduction as described (Cairrão et al. 2003). Plasmid pKAK7 was described elsewhere (Donovan and Kushner 1986). Primers used in this work are listed in Table 2.

Growth conditions

Bacteria were grown at 37°C unless stated otherwise, in Luria-Bertani (LB) medium supplemented with thymine (50 µg/mL). Growth was performed as described (Andrade et al. 2006). When required, antibiotics were present at the following concentrations: chloramphenicol, 50 µg/mL; kanamycin, 50 µg/mL; tetracycline, 20 µg/mL; ampicillin, 150 µg/mL.

TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Reference
G897	MC4100 <i>relA</i> ⁺ <i>micA</i>	Udekwi et al. (2005)
HM104	<i>thyA715 rnr</i>	Cairrão et al. (2003)
MG1693	<i>thyA715</i>	Arraiano et al. (1988)
SK5665	<i>thyA715 rne1</i>	Arraiano et al. (1988)
SK5671	<i>thyA715 rne1 pnp7</i>	Arraiano et al. (1988)
SK5691	<i>thyA715 pnp7</i>	Arraiano et al. (1988)
SK7988	<i>thyA715 ΔpcnB</i>	O'Hara et al. (1995)
CMA201	<i>thyA715 Δrnb</i>	Cairrão et al. (2003)
CMA300	<i>thyA715 pnp7 ΔpcnB</i>	This study
CMA413	<i>thyA715 micA</i>	This study
CMA414	<i>thyA715 pnp7 micA</i>	This study
CMA418	<i>thyA715 pnp7 rne131</i>	This study
CMA440	<i>thyA715 pnp7 / pKAK7</i>	This study
CMA445	<i>thyA715 ΔompA</i>	This study
CMA446	<i>thyA715 pnp7 ΔompA</i>	This study
CMA501	<i>thyA715 rne131</i>	Viegas et al. (2005)

TABLE 2. Primers used in this study

Primer	Sequence	Description
micA-T7	5'- <u>TAATACGACTCACTATAG</u> GAAGGCCACTCGTGAGTGGCCAA-3'	MicA RNA antisense probe
micA-F	5'-GAAAGACGCGCATTTGTATC-3'	
micA1	5'-TTTTCGCCACCCGAAGTGCAGGC-3'	Confirmation of <i>micA</i> deletion
micA2	5'-AATGGAAGCTTCTGATACCGAACCG-3'	
ompA-T7	5'- <u>TAATACGACTCACTATAG</u> AAAAAAAAACCCGCGAGCAGC-3'	<i>ompA</i> RNA antisense probe
ompA-F	5'-TTGTAGACTTTACATCGCCAGGG-3'	
ompAdelF	5'-CAGACGAGAACTTAAGCCTGCGGCTGAGTTACAACGTCTTTGATAC CTTTAACGTGTAGGCTGGAGCTGCTTC-3'	<i>ompA</i> deletion
ompAdelR	5'-TTGTGCGTGACAAAAAAGATTAAACATACCTTATACAAGACTTTTTT TCGGTCCATATGAATATCCTCCTTAG-3'	
ompA1	5'-CCCAGCCAATGCTTCAGTCGTGAC-3'	Confirmation of <i>ompA</i> deletion
ompA2	5'-ACGCGCAAAAAATCGATCGATCTGG-3'	
rybB-T7	5'- <u>TAATACGACTCACTATAG</u> GAACAAAAACCCATCAACCTTGAACCG-3'	RybB RNA antisense probe
rybB-F	5'-ACTGCTTTTCTTTGATGTC-3'	
5S-RNA-F	5'-AAACAGAAATTTGCCTGGCGGCAGTAG-3'	5S-RNA probe
5S-RNA-R	5'- <u>TAATACGACTCACTATAG</u> GATGCCTGGCAGTTCCTACTCTCGC-3'	
23S-RNA-F	5'-TGTGAGGTTAAGCGACTAAGC-3'	23S-RNA probe
23S-RNA-R	5'-TTCGGGGAGAACCGACTATC-3'	

The T7 promoter sequence in the oligos is underlined. The sequence homology with the pDK3 is shown in bold.

RNA extraction and Northern blot analysis

Total RNA was extracted as previously described (Andrade et al. 2006). Ten micrograms of total RNA from each strain was used to analyze *ompA* mRNA on a 1.2% agarose MOPS/formaldehyde gel. RNA was transferred to Hybond-N⁺ membrane (Amersham Biosciences) by capillarity using 20× SSC as a transfer buffer. Thirty micrograms of total RNA was used to analyze MicA RNA on 10% polyacrylamide gels. RNA was electrotransferred onto Hybond-N⁺ membrane using 1× TAE. RNA was UV cross-linked to the membrane. A DNA template from PCR obtained with Taq DNA Polymerase (Fermentas) was used to generate the riboprobes by in vitro transcription with T7 RNA Polymerase (Promega) in the presence of [α -³²P]UTP (GE Healthcare). All riboprobes were purified on G50 Microspin columns (GE Healthcare). For loading control, the membranes used to detect *ompA* were stripped and rehybridized with a 23S-RNA probe. Hybridizations were carried out at 68°C with the PerfectHyb Plus Hybridization Buffer (Sigma).

RNA half-life determination

Quantification of full-length RNA was done after PhosphorImager scanning by the IMAGEQUANT program (Molecular Dynamics). The half-lives of RNA were determined by linear regression using the logarithm of the percentage of RNA remaining versus time, considering the amount of RNA at 0 min as 100%. The data presented are the average of different Northern blots (two to 10) from at least two independent RNA extractions.

Protein analysis

The protocol for the two-dimensional gel electrophoresis experiments was done as described with modifications (Andrade et al. 2006). Two hundred micrograms of total protein extracts (after precipitation with TCA 10%/acetone) was soluble in 250 μ L of rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% [v/v] IPG buffer [GE Healthcare], 50 mM DTT).

First-dimension electrophoresis was carried on linear immobilized pH 3–10 gradient (IPG) strips (13 cm, Immobiline DryStrip, GE Healthcare). The rehydration step and electrophoresis were performed in an IPGphor Isoelectric Focusing Unit (Pharmacia Biotech). Following a rehydration step at 30 V for 12 h, isoelectric focusing (IEF) was carried out for 32,000 Vhr, at a maximum of 8000 V. After IEF, the IPGs were either separated immediately for second dimension or kept at –80°C until use. The equilibrated IPGs were separated on equal 12% SDS–PAGEs using a Laemmli system. Gels were stained with Coomassie Brilliant Blue G-250. OmpA protein was identified based on the protein pattern (Andrade et al. 2006). Image acquisition from the gels was done using an ImageScanner (Amersham Biosciences) and the LabScan 5.0 software. The membrane protein fraction, from stationary phase cultures, was extracted as described (Matsuyama et al. 1984). Equal amounts of protein were run on a 12% SDS-polyacrylamide gel with 8 M urea. Gels were stained overnight with Coomassie R-250.

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