Inactivation of the general transcription factor TnrA in *Bacillus subtilis* by proteolysis

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Under conditions of nitrogen limitation, the general transcription factor TnrA in *Bacillus subtilis* activates the expression of genes involved in assimilation of various nitrogen sources. Previously, TnrA activity has been shown to be controlled by protein–protein interaction with glutamine synthetase, the key enzyme of ammonia assimilation. Furthermore, depending on ATP and 2-oxoglutarate levels, TnrA can bind to the GlnK–AmtB complex. Here, we report that upon transfer of nitrate-grown cells to combined nitrogen-depleted medium, TnrA is rapidly eliminated from the cells by proteolysis. As long as TnrA is membrane-bound through GlnK–AmtB interaction it seems to be protected from degradation. Upon removal of nitrogen sources, the localization of TnrA becomes cytosolic and degradation occurs. The proteolytic activity against TnrA was detected in the cytosolic fraction but not in the membrane, and its presence does not depend on the nitrogen regime of cell growth. The proteolytic degradation of TnrA as a response to complete nitrogen starvation might represent a novel mechanism of TnrA control in *B. subtilis*.

INTRODUCTION

Nitrogen is a macronutrient for all known types of living organisms, since it is included in most biomolecules. In natural environments, the nitrogen sources may differ in both composition and concentration. To optimize their utilization, bacteria have developed regulatory systems that control gene expression in response to changes in the nitrogen availability (reviewed by Forchhammer, 2007).

TnrA is the major transcription factor in *Bacillus subtilis* that controls gene expression in response to nitrogen availability. Under nitrogen-limited growth, TnrA binds to a dyad symmetry element with the consensus sequence 5'-TGTAAN7TNACA-3' (Wray et al., 1997, 2000), and serves as either an activator or a repressor of genes. TnrA activates its own gene (Fisher, 1999), the *nasABCDE* genes (nitrate and nitrite utilization; Nakano et al., 1995, 1998), the *nrgAB* (*amtBglK*) operon (ammonium transport; Wray et al., 1998), the *ureABC* operon (urea utilization; Wray et al., 1997) and the genes for purine utilization, and interacts with some other target promoters. Under nitrogen-limited growth, TnrA is a negative regulator of *glnA* and *gltAB*, which encode the ammonium assimilatory enzymes glutamine synthetase (GS) and glutamate synthase, respectively (Wray et al., 1996; Fisher & Debarbouille, 2002; Belitsky et al., 2000). Interestingly, the induction of the *gltAB* operon depends on the pleiotropic regulator of carbon metabolism CcpA, and requires sugars that can be catabolized via glycolysis (Faires et al., 1999; Blencke et al., 2003; Wacker et al., 2003).

Several lines of evidence indicate that GS acts as a sensor of nitrogen availability in *B. subtilis* (Fisher, 1999). TnrA-activated genes are expressed constitutively in *glnA* mutants, implying that GS produces or transmits an inhibitory regulatory signal to TnrA during growth with excess nitrogen (Fisher et al., 2002). Indeed, the feedback-inhibited GS forms a complex with TnrA, preventing its binding to DNA (Wray et al., 2001). The most effective feedback inhibitors of GS biosynthetic activity are glutamine and AMP, while partial inhibition has been observed with alanine, glycine, serine and tryptophan (Deuel & Prusiner, 1974). Mutations in TnrA that result in constitutive expression of the TnrA-activated *amtB* promoter all lie within the carboxy-terminal region of TnrA and impair the interaction between GS and TnrA (Wray et al., 2001; Wray & Fisher, 2006). Thus, the feedback inhibitors of GS are the metabolic signals that cause inhibition of TnrA through its GS interaction.

Other regulators of TnrA activity have been found recently. When *B. subtilis* cells were grown with the poor nitrogen source nitrate, TnrA was found, in cell-free extracts, to be almost completely associated with the cell membrane via the ammonium-uptake proteins AmtB and GlnK, originally termed NrgA and NrgB, respectively (Heinrich et al., 2008).
et al. 2006). AmtB is a homotrimeric transmembrane ammonium transporter that is active under nitrogen-limited conditions (Wray et al., 1994; Khademi & Stroud, 2006). GlnK is a small regulatory protein that belongs to the PI family. As shown in various bacteria, GlnK homologues bind to AmtB and regulate their activity depending on the nitrogen availability (Javelle et al., 2004). The B. subtilis GlnK protein, although exhibiting unique features, has been shown to bind to the membrane in an AmtB-dependent manner (Detsch & Stülke, 2003; Heinrich et al., 2006). Depending on the GlnK effector molecules, B. subtilis GlnK can be soluble or membrane-bound: 4 mM ATP causes almost full solubilization of GlnK. TnrA has been shown to bind to the membrane-bound GlnK–AmtB complex alone, however, and not to soluble GlnK (Heinrich et al., 2006).

We have found that during transition from medium containing nitrate to conditions of complete nitrogen starvation, TnrA degrades in B. subtilis cells. The aim of this work was to characterize this process in detail and to gain insights into the protease activity involved.

**METHODS**

**Bacterial strains and growth conditions.** B. subtilis strains used in this study, strain 168 (wild-type), the AmtB-deficient strain B. subtilis GP 254 and the GlnK-deficient mutant B. subtilis GP 253, have been described previously (Detsch & Stülke, 2003). For the generation of the clpP and lonA mutants, B. subtilis 168 cells were transformed with chromosomal DNA from clpP and lonA mutants (Msadek et al., 1998; Riethdorf et al., 1994), as described in Anagnostopoulos & Spizizen (1961), and selected on LB agar plates containing 5 μg chloramphenicol ml⁻¹ for clpP mutants or 75 μg spectinomycin ml⁻¹ for lonA mutants. B. subtilis cells were grown in Spizizen minimal medium (SMM) (Anagnostopoulos & Spizizen, 1961) containing glucose (0.5 %, w/v) or tri-sodium citrate (0.6 %, w/v) as a carbon source. Sources of nitrogen were added as indicated. L-Tryptophan was added to a final concentration of 50 mg l⁻¹.

**Immunoblot analysis.** For immunoblot analysis, B. subtilis cell-free extracts containing 30 μg total cell protein per lane were separated on 15 % SDS–polyacrylamide gels. After electrophoresis, the proteins were transferred to a nitrocellulose membrane by semi-dry electroblotting. Antibodies were visualized by using anti-rabbit IgG-peroxidase-conjugated secondary antibodies (Sigma) and the LumiLight detection system (Roche Diagnostics). For control experiments, the presence of GS was assayed using polyclonal antibodies directed against B. subtilis GS.

**Preparation of membrane fractions.** Overnight cultures of the appropriate B. subtilis cells, which had been grown with 20 mM NaNO₃ as the nitrogen source, were diluted to OD₆₀₀ 0.1 with SMM (20 mM NaNO₃ final concentration). Cells were harvested at the late exponential phase of growth at OD₆₀₀ ~0.8 by centrifugation (14000 r.p.m., 10 min, 4 °C), resuspended in disruption buffer A (50 mM Tris, pH 7.5, 50 mM KCl, 2 mM MgCl₂) and broken in a RiboLyser (Hybaid). The cellular debris was removed by centrifugation (3500 g, 2 min, 4 °C), and the fractions of the cell-free extract were separated by ultracentrifugation (100000g, 1 h, 4 °C). The supernatant was divided into equal parts in an upper (S1) and a lower (S2) fraction. The sediment (P1) was resuspended in the initial volume of buffer A, and a part of P1 was centrifuged again as before.

**RESULTS**

**TnrA degrades following nitrate deprivation**

B. subtilis 168 cells were grown in SMM supplemented with 20 mM nitrate as sole nitrogen source, which had been found previously to be optimal for GlnK and TnrA accumulation in the cells (Heinrich et al., 2006). To investigate the response of these proteins to a transfer to complete nitrogen deprivation, cells were harvested at the late exponential growth phase (OD₆₀₀ 0.8), washed with SMM and shifted into SMM containing no combined nitrogen source. Surprisingly, the amount of TnrA rapidly diminished: already after 15 min it was strongly reduced, and after 30 min TnrA almost completely disappeared from the cells (Fig. 1a). To examine any influence of harvesting and washing of the cells on TnrA degradation, cells were treated as above but shifted into SMM with nitrate. No decrease of TnrA after 30 min was detected, and even after 60 min no degradation occurred (not shown), confirming that the observed disappearance of TnrA was a specific response to severe nitrogen deprivation (Fig. 1b). In contrast, no difference in GlnK abundance was observed during this shift experiment (Fig. 1g), indicating that rapid degradation was specific for TnrA. To examine the in vivo stability of TnrA, the nitrate-grown cells were washed and shifted to SMM containing 30 μg chloramphenicol l⁻¹ and nitrate. In spite of the fact that novel protein synthesis was arrested, no decrease in TnrA abundance was observed, indicating that TnrA is highly stable within the cells (Fig. 1d). However, when the cells were shifted to nitrate-free SMM containing chloramphenicol, TnrA was degraded to the same extent as in the absence of chloramphenicol (compare Fig. 1a and Fig. 1c). This indicates that the TnrA-degrading activity is already present in the cells and is not newly synthesized upon nitrate depletion. Furthermore, arrest of TnrA synthesis is not responsible for the decrease in TnrA abundance.
Since under nitrogen-poor (nitrate) conditions, TnrA is membrane-bound by complex formation with GlnK–AmtB (Heinrich et al., 2006), the degradation of TnrA was also analysed in GlnK- and AmtB-deficient mutants. There was no degradation of the TnrA protein in *B. subtilis* strains GP253 and GP254, which have deleted *glnK* and *amtB* genes, respectively (Fig. 1e, f). Thus, TnrA degradation seems to be a specific cellular response to nitrogen deprivation, which is impaired in *glnK* and *amtB* mutants.

**Localization of TnrA upon nitrate depletion**

In exponentially growing cells, GlnK protein can be soluble or membrane-bound, and its location determines the binding of TnrA to the membrane (Heinrich et al., 2006). The next step was therefore to reveal the localization of TnrA in cells before and immediately after the shift to conditions of nitrogen deprivation. Crude extracts of shifted and non-shifted *B. subtilis* wild-type cells were fractionated into cytosolic and a membrane fractions as described in Methods, and analysed by immunoblotting. The quality of the fractionation was verified by Western blot analysis using antibodies against the strictly cytoplasmic enzyme GS. GS was detected in the cytosolic but not in the membrane fractions, confirming that the membrane preparations were essentially free of cytoplasmic proteins (not shown). In agreement with data published previously, TnrA was found to be fully membrane-bound in the nitrate-grown *B. subtilis* wild-type cells (Fig. 2a, lane 1). Already immediately upon the shift, the amount of membrane-bound TnrA was drastically reduced, whereas TnrA protein was now detectable in the cytosolic fraction (Fig. 2a, lane 2). After 7 min, no more membrane-bound TnrA could be detected, whereas a small amount of soluble TnrA was still detectable (Fig. 2a, lane 3). From this experiment, it appears that TnrA is localized to the cytoplasm prior to degradation. However, in the GlnK- and AmtB-deficient strains GP 254 and GP 253, TnrA was soluble, irrespective of the conditions and was not degraded (Fig. 2b, c).

When, in SMM medium, the carbon source glucose was replaced by sodium citrate, the amount of TnrA did not decrease upon a shift from nitrate-supplemented medium to medium containing no nitrogen source (Fig. 3a). Furthermore, TnrA did not change its cellular localization following nitrate withdrawal and remained membrane-bound (Fig. 3b).

**Fig. 1.** TnrA degrades in *B. subtilis* wild-type cells following nitrate depletion. *B. subtilis* cells were grown in SMM supplemented with nitrate as sole nitrogen source. At late exponential growth phase, an aliquot was removed (lane 1), and the remaining cells were washed and shifted into medium without a combined nitrogen source (a, c, e, f, g) following incubation at 37 °C with shaking. Samples were taken immediately after the shift, and after 7, 15 and 30 min of incubation (lanes 2, 3, 4 and 5, respectively). The crude cell extracts were prepared and analysed by immunoblotting using anti-TnrA (a–f) or anti-GlnK antibodies (g). As a control, wild-type cells were shifted into the previous medium containing 20 mM nitrate (b, d). To examine the intracellular stability of TnrA under shift conditions, the cells were shifted to SMM containing 30 μg chloramphenicol l⁻¹ (c, d). (a, b, c, d, g) Fractions prepared from the wild-type strain; (e) fractions from *B. subtilis* GP254; (f) fractions from *B. subtilis* GP253.

Since under nitrogen-poor (nitrate) conditions, TnrA is membrane-bound by complex formation with GlnK–AmtB (Heinrich et al., 2006), the degradation of TnrA was also analysed in GlnK- and AmtB-deficient mutants. There was no degradation of the TnrA protein in *B. subtilis* strains GP253 and GP254, which have deleted *glnK* and *amtB* genes, respectively (Fig. 1e, f). Thus, TnrA degradation seems to be a specific cellular response to nitrogen deprivation, which is impaired in *glnK* and *amtB* mutants.

**Fig. 2.** TnrA localization following nitrate downshift. Nitrate-grown *B. subtilis* cells at late exponential growth phase (lane 1) were washed and shifted into combined nitrogen-free medium. Samples were taken immediately after the shift, and after 7, 15 and 30 min of incubation (lanes 2, 3, 4 and 5, respectively). Whole-cell extracts were fractionated by centrifugation into an upper (S) and lower soluble fraction and a particulate (P) fraction (see Methods). P was washed and centrifuged again to yield a washed particulate (Pw) fraction. The fractions were subjected to SDS-PAGE followed by immunoblotting using TnrA-specific antibodies. (a) Fractions prepared from *B. subtilis* 168. (b) Fractions prepared from *B. subtilis* GP254. (c) Fractions prepared from *B. subtilis* GP253.
The data above suggested that the rapid disappearance of TnrA in vivo is accomplished by specific proteolysis. To further examine the proteolytic activity, in vitro reconstitution of TnrA degradation by B. subtilis cell extracts was performed (see Methods for details). B. subtilis wild-type cells were harvested after the shift into nitrogen-depleted medium and cell-free extracts were prepared. Almost complete degradation of purified TnrA by crude extract of shifted cells was observed within 30 min of incubation (Fig. 4), whereas the crude extract of non-shifted cells degraded TnrA much more slowly.

To elucidate the cellular localization of the activity responsible for in vitro TnrA proteolysis, B. subtilis 168 cell extracts were separated into membrane and cytosolic fractions, as described above, and cytoplasmic (S1) and washed membrane (Pw) fractions were used for TnrA in vitro degradation assays. TnrA degradation was observed in the cytoplasmic fraction of extracts from both shifted and non-shifted cells, whereas no degradation activity was found in membrane fractions (Fig. 5a), indicating a cytosolic localization of the TnrA-degrading activity. In contrast to crude extracts, TnrA-degrading activity in the cytosolic fraction was present in extracts of cells harvested before and after the shift to nitrogen deprivation, suggesting constitutive expression of the relevant protease. To check this suggestion, the in vitro TnrA degradation assay was performed with extracts prepared from ammonium-grown cells, which do not express TnrA, as well as from citrate-grown cells, in which TnrA does not degrade. As shown in Fig. 5, TnrA-degrading activity is present in the cytosolic fractions of the cells independently of their nitrogen or carbon growth regime (Fig. 5b, c). Surprisingly, no in vitro proteolysis whatsoever of TnrA was observed for extracts of B. subtilis GP 254 cells, and similarly for extracts of B. subtilis GP 253 cells. TnrA degradation was strongly impaired compared with extracts from wild-type cells.
The intracellular enzymes GS from several purified proteins were subjected to proteolysis specific for the TnrA protein or degraded many proteins, activity recovered from the Superdex 200 column was mass of ~480 kDa. To reveal whether the proteolytic activity against natural TnrA that is complexed with GlnK–AmtB, membrane fractions containing the TnrA/GlnK–AmtB complex were prepared from non-shifted, nitrate-grown B. subtilis wild-type cells and mixed with extracts that were active in degrading recombinant TnrA. Clearly, no endogenous TnrA proteolysis was observed under those assay conditions with cytosolic fractions of shifted and non-shifted cells, whereas recombinant TnrA was degraded by these extracts (Fig. 6). Thus, TnrA bound to the GlnK–AmtB complex seems to be protected from degradation.

Properties of TnrA-degrading activity

In order to characterize the type of protease responsible for TnrA degradation, the influence of protease inhibitors was studied. Two millimolar PMSF was found to completely suppress proteolytic activity, indicating that this protease is a serine protease. In contrast, EDTA and benzamidine did not affect TnrA proteolysis (Fig. 7). The addition of ATP to the reaction mixture up to a final concentration of 5 mM did not affect the activity (not shown), indicating that TnrA proteolysis is energy-independent. The optimal pH of this activity was in the range of 7.0–7.4 (not shown). To reveal the approximate molecular mass of the TnrA-degrading protease, the cytoplasmic fraction of B. subtilis wild-type cells was separated by FPLC gel filtration on a Superdex 200 column, yielding an apparent molecular mass of ~480 kDa. To reveal whether the proteolytic activity recovered from the Superdex 200 column was specific for the TnrA protein or degraded many proteins, several purified proteins were subjected to proteolysis assays. The intracellular enzymes GS from B. subtilis and N-acetyl-L-glutamate kinase (NAG-kinase) from Synechococcus elongatus were not subject to proteolysis under conditions that led to TnrA degradation (Fig. 8), whereas recombinant GlnK protein seemed to be partially degraded. The fact that TnrA was degraded by a protease-active fraction recovered after size-exclusion chromatography is further evidence for the ATP-independence of this proteolytic activity.

Two abundant proteases in B. subtilis are ClpP and LonA. To examine whether they could be involved in TnrA degradation, clpP and lonA mutants were assayed for TnrA degradation following nitrogen depletion. Neither the deletion of clpP nor that of lonA impaired TnrA degradation significantly (Fig. 9). The slightly delayed TnrA degradation in the lonA mutant could indicate a minor role of LonA in TnrA degradation.

DISCUSSION

TnrA is one of the major regulators of nitrogen metabolism in B. subtilis cells (Fisher, 1999). It has been shown that its activity is regulated by complex formation by a feedback-inhibited form of GS under conditions of nitrogen excess (Wray et al., 2001). Recently, binding of TnrA to the membrane-bound GlnK–AmtB complex has been identified, and this could have additional regulatory consequences for the subcellular relocalization of TnrA. Here, we present yet another option for TnrA activity regulation. When cells are acclimated to growth with a poor nitrogen source, such as nitrate, they express a maximal amount of TnrA (Heinrich et al., 2006). Withdrawal of this nitrogen source leads to rapid elimination of TnrA from the cells by proteolysis (Fig. 1). The regulation of transcription factor activity by proteolysis has been found previously for sigma factors, such as the heat-shock regulator σ32 (sigma H) from Escherichia coli (Herman et al., 1995a, b). The σ32 factor has been shown to be degraded under non-stressed conditions and to be stabilized following heat-shock
treatment (Yura & Nakahigashi, 1999). Another example is the stationary phase-specific sigma factor $\sigma^5$ from E. coli. Its proteolytic degradation is directed by the RssB protein, which acts as a recognition factor, and RssB affinity for $\sigma^5$ is modulated by phosphorylation (Becker et al., 2000; Hengge-Aronis, 2000; Zhou et al., 2001).

In exponentially growing cells, utilizing nitrate as the nitrogen source, TnrA was shown to bind to the membrane via the AmtB–GlnK complex, the ammonium channel together with its cognate regulator (Detsch & Stülke, 2003; Heinrich et al., 2006). Knockout of either $amtB$ or $glnK$ leads to solubilization of TnrA but does not disrupt its function, since these cells are able to grow on poor nitrogen sources. GlnK and AmtB seem to play an ambivalent role in regulating TnrA accumulation. On the one hand, GlnK and AmtB seem to be required for TnrA degradation, since in the respective mutants, no degradation of TnrA was observed, either in vivo or in vitro assays (see Figs 1e, f and 5d, e). On the other hand, TnrA in complex with GlnK and AmtB seems to be protected from proteolysis (Fig. 6). From the lack of TnrA degradation in AmtB- as well as in GlnK-deficient cells, it can be concluded either that AmtB and GlnK are involved together in regulation of the protease activity or that the expression of a component of the protease is coupled to the $glnK$–$amtB$ genes and consequently that mutation in $amtB$ or $glnK$ would impair protease activity. Another explanation may be that in both $amtB$ and $glnK$ mutants, TnrA has an abnormal cytosolic localization (see Fig. 2b, c), which may indirectly affect the interactions of externally added recombinant TnrA with DNA or GS, which could then modulate its accessibility to the protease. From kinetic analysis, it appears that cytosolic relocation of TnrA following nitrate downshift precedes its subsequent degradation (Fig. 2). In agreement with a cytoplasmic proteolytic degradation of TnrA, in vitro TnrA-degrading activity was found in the cytosolic fraction alone (Fig. 5). The slower TnrA degradation in crude extracts of non-shifted cells as opposed to the constitutive degradation in cytosolic fractions could arise from interference of membrane-bound GlnK–AmtB with the purified TnrA protein, which could protect it from degradation. It must be considered that TnrA was added at physiological concentrations in these assays. This interaction would not occur in extracts from nitrogen down-shifted cells, since TnrA apparently does not bind to the membrane under those conditions (Fig. 2). The resolution of TnrA from membrane-bound GlnK–AmtB complex makes it susceptible to proteolysis in wild-type cells. When glucose was replaced by citrate, the withdrawal of nitrate did not cause cellular relocation of TnrA, and consequently no degradation of TnrA occurred (Fig. 3). Possibly, the cytosolic relocation of TnrA in glucose-grown cells following nitrate withdrawal is the result of a modification of the GlnK–AmtB complex that disables TnrA binding and exposes it to proteolytic processing. In the presence of citrate, the signal that leads to TnrA solubilization is not generated. Since citrate is not the preferred carbon source of B. subtilis, the ATP and 2-oxoglutarate concentrations within the cell that are the effectors for GlnK localization could play a role (Heinrich et al., 2006). In citrate-grown cells, GlnK could remain in

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**Fig. 7.** The influence of protease inhibitors on in vitro TnrA degradation. The cytosolic fractions of shifted (I) and non-shifted (II) B. subtilis 168 cells were used as a source of proteolytic activity. TnrA-degradation assays were performed as described in Methods. In lanes (I), the samples were preincubated with 2 mM PMSF, 5 mM benzamidine or 5 mM EDTA, as indicated. In lanes (s), the samples were preincubated with the solvents of the inhibitors alone (100 % ethanol for PMSF, and pure water for EDTA and benzamidine). As a further control, TnrA was incubated in buffer (lanes c).

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**Fig. 8.** Specificity of proteolytic activity. In vitro degradation assays were performed as described in Methods using the proteolytic activity recovered after size-exclusion chromatography from cytoplasmic fraction (S1) of B. subtilis 168. Several purified proteins were used as substrate, as indicated: B. subtilis TnrA, GS and GlnK, and NAG-kinase (from S. elongatus); the corresponding proteins incubated in reaction buffer served as controls (lanes c).
complex with the AmtB protein even under nitrogen depletion and sequester TnrA to the membrane, preventing its degradation.

TnrA in vitro degradation assays have revealed the presence of a TnrA-degrading activity in cytosolic extracts from wild-type cells independent of the nitrogen and carbon source used to grow the cells. Most likely, this protease belongs to the housekeeping proteases. Since the proteolytic activity is completely inhibited by PMSF, it probably belongs to serine proteases. It should be noted that the proteolysis of transcription factors described earlier was in most cases conducted by ATP-dependent proteases such as ClpP or FtsH, as well as Lon protease (Herman et al., 1995b; Zhou et al., 2001; Riethdorf et al., 1994; Reeves et al., 2007). In agreement with the ATP-independence of in vitro TnrA degradation, neither a lonA- nor a clpP-deficient mutant was impaired in TnrA degradation, although a minor role for LonA is possible (Fig. 9). The molecular mass of the protease activity was determined to be ~480 kDa, probably representing an oligomeric proteolytic complex including chaperone proteins and providing high specificity to target proteins, since other proteins such as GS and NAG-kinase were not subjected to proteolysis (Fig. 8). Our further research will be devoted to the identification and characterization of the protease that degrades TnrA.

The physiological benefit of degrading TnrA under conditions of nitrate deprivation remains speculative. An excess amount of TnrA that is released from the GlnK–AmtB complex could exceed the physiological need for TnrA. Under these nitrogen-starvation conditions, GS will be present almost completely in the active form that does not sequester TnrA. The excess active TnrA could bind to DNA at non-specific sites and might interfere with the fine-tuning of gene regulation through an overdosage effect. Under those conditions, degradation of TnrA by proteolysis might represent an efficient mechanism to re-establish the optimal cellular level of this central transcription factor. Another explanation could be that nitrogen starvation turns on a sporulation program that results in the replacement of vegetative sigma factors with sporulation sigma factors (Tam et al., 2007). Consequently, all vegetative transcription factors would be annihilated to stop any biosynthetic processes not essential for sporulation.

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Fig. 9. TnrA degradation in B. subtilis wild-type (WT), clpP and lonA mutant strains. B. subtilis 168 and lonA− cells were grown in nitrate-supplemented SMM to exponential growth phase (lane 1). Due to poor growth of the clpP− mutant, the cells were first grown in nitrate-rich medium to the end of exponential growth and were then transferred to SMM nitrate medium for 10 h (lane 1). Then, the cells were washed and shifted to medium lacking combined nitrogen and incubated at 37 °C with shaking. Samples were taken immediately after the shift, and after 7, 15 and 30 min of incubation (lanes 2, 3, 4 and 5, respectively), and analysed with anti-TnrA antibodies.

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