# The pupylation machinery is involved in iron homeostasis by targeting the iron storage protein ferritin

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**The balance of sufficient iron supply and avoidance of iron toxicity by iron homeostasis is a prerequisite for cellular metabolism and growth. Here we provide evidence that, in Actinobacteria, pupylation plays a crucial role in this process. Pupylation is a posttranslational modification in which the prokaryotic ubiquitin-like protein Pup is covalently attached to a lysine residue in target proteins, thus resembling ubiquitination in eukaryotes. Pupylated proteins are recognized and unfolded by a dedicated AAA+ ATPase (*Mycobacterium* proteasomal AAA+ ATPase; ATPase forming ring-shaped complexes). In Mycobacteria, degradation of pupylated proteins by the proteasome serves as a protection mechanism against several stress conditions. Other bacterial genera capable of pupylation such as *Corynebacterium* lack a proteasome, and the fate of pupylated proteins is unknown. We discovered that *Corynebacterium glutamicum* mutants lacking components of the pupylation machinery show a strong growth defect under iron limitation, which was caused by the absence of pupylation and unfolding of the iron storage protein ferritin. Genetic and biochemical data support a model in which the pupylation machinery is responsible for iron release from ferritin independent of degradation.**

iron limitation | prokaryotic ubiquitin-like protein | ATPase ARC | *Corynebacterium* | *Mycobacterium*

Pupylation is a posttranslational protein modification occurring in the phylum Actinobacteria and some other bacterial lineages, such as Nitrospirae (1, 2). It resembles eukaryotic ubiquitination and was first identified in *Mycobacterium tuberculosis* (3). Target proteins are covalently linked to the small prokaryotic ubiquitin-like protein (Pup), which, in mycobacteria, can serve as a tag for degradation via the proteasome (3–5). The proteasomal genes *prcA* and *prcB* are encoded within the *pup* gene cluster of mycobacteria (6). However, several Actinobacteria harbor genes of the pupylation machinery but lack the genes encoding the proteasome, raising the question of the fate of pupylated proteins in proteasome-free species (1, 6).

The process of Pup-mediated protein degradation in mycobacteria comprises several steps. First, the 64-aa residue-protein Pup is activated via deamidation of the C-terminal glutamine residue to glutamate, catalyzed by the deamidase of Pup (Dop) (3, 7, 8). Pup is then covalently attached to target proteins by the proteasome accessory factor A (PafA). PafA catalyzes the ATP-dependent formation of an isopeptide bond between the γ-carboxyl group of Pup and the ε-amino group of a lysine residue within the target protein (8–10). *Mycobacterium* proteasomal AAA+ ATPase (Mpa), termed ATPase forming ring-shaped complexes (ARCs) in nonmycobacterial species, recognizes pupylated proteins, unfolds them, and directs them into the proteasome for degradation (11, 12). Besides its function as a deamidase, Dop was also shown to catalyze the depupylation of substrates (13, 14). Some species, such as members of the genera *Corynebacterium* and *Streptomyces*, encode Pup variants with a carboxyl-terminal glutamate residue, which therefore do not require the deamidation step. In these bacteria, Dop may serve exclusively as depupylase.

Hitherto, proteome-wide searches revealed several pupylated proteins (pupylomes) in a range of proteasome-bearing Actinobacteria (15–19). The proteins making up the pupylomes covered a broad spectrum of functional categories, which might be explained by a general recycling function fulfilled by pupylation. In this view, protein degradation mediated by pupylation is assumed to recycle amino acids under several stress conditions in *Mycobacterium smegmatis* (20). Although pupylation was shown to target proteins to proteasome-mediated degradation, not all pupylated proteins are subject to this fate (15, 21). Furthermore, the activity of the mycobacterial ATPase Mpa itself was shown to be reversibly regulated by pupylation, which renders Mpa functionally inactive (22). In view of these results, the investigation of pupylation in proteasome-lacking Actinobacteria promises new insights into its physiological role(s) and the fate of pupylated proteins.

*Corynebacterium glutamicum* is a member of the Actinobacteria harboring genes for the pupylation machinery (*pup*, *dop*, *pafA*, *arc*; Fig. 1*A*) but lacking the proteasomal genes *prcAB*. In a recent proteomics study, we identified 55 pupylated proteins in this species (23). *C. glutamicum* is a nonpathogenic Gram-positive soil bacterium, which has become a model organism for studying metabolism and regulation (24). As the physiological function of pupylation in this organism remained enigmatic, we screened for a phenotype of the Δ*pup* mutant during growth with various carbon sources and under different stress conditions. A severe growth defect was observed only under iron limitation. Detailed studies revealed that this phenotype is caused almost exclusively by the lack of pupylation of the iron-storage protein ferritin, and we provide evidence that this could result from a defect in iron release from nonpupylated ferritin. These results disclose a distinctive role

## Significance

**Pupylation is a posttranslational protein modification discovered in *Mycobacterium tuberculosis* in which it tags proteins for degradation via the proteasome. It thus resembles eukaryotic ubiquitination. In mycobacteria, pupylation plays a role under oxidative stress and under carbon and nitrogen starvation. Intriguingly, many bacteria containing the pupylation machinery lack a proteasome, such as corynebacteria, leaving the function of this protein modification open. We show that pupylation in *Corynebacterium glutamicum* plays a dedicated role in iron homeostasis by targeting the iron-storage protein ferritin. Evidence is provided that pupylation triggers the disassembly of 24-meric ferritin by ARC to support the release of the stored iron without using a protease. Thus, a physiological function of pupylation was discovered for a proteasome-free bacterial species.**

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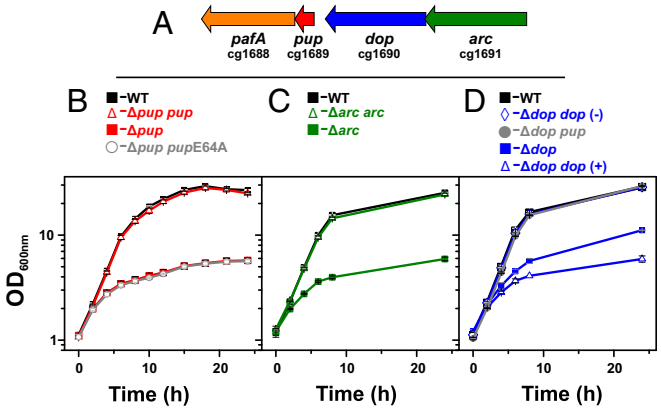


**Fig. 1.** Importance of the pupylation machinery for growth of *C. glutamicum* ATCC 13032 under iron limitation. (*A*) Genetic organization of pupylation-related genes. (*B–D*) Growth of the WT (black squares) and pupylation-deficient mutants cultivated in glucose minimal medium under iron limitation (1 μM $FeSO_4$). The strains are indicated at the top and represent: (*B*) Δ*pup* (red squares), Δ*pup*/pVWEx1-*pup* (red triangles), and Δ*pup*/pVWEx1-*pup*E64A (open circles); (*C*) Δ*arc*/pVWEx1 (green squares) and Δ*arc*/pVWEx1-*arc* (green triangles); and (*D*) Δ*dop*/pVWEx1 (blue squares) and Δ*dop*/pVWEx1-*dop* in the presence (blue triangles) and absence (blue diamonds) of 1 mM IPTG, and Δ*dop*/pVWEx1-*pup* (filled circles). Mean values and SDs were obtained from three independent biological replicates.

of pupylation in ferritin-mediated iron homeostasis and add another level of complexity to the control of iron homeostasis.

## Results

**Deletion of *pup* Results in a Growth Defect Under Iron Limitation.** We recently identified 55 pupylated proteins, including their pupylation sites in *C. glutamicum* cells cultivated in a rich medium. However, pupylation was dispensable under standard cultivation conditions as a mutant strain lacking the *pup* gene showed no growth defect (23). To get hints on the physiological function of this posttranslational modification in *C. glutamicum*, we compared the growth of the Δ*pup* mutant and WT in media with different carbon or nitrogen sources and under various stress conditions (Table S1). The two strains grew comparably under almost all tested conditions, including nitrite stress, which was shown to affect pupylation-defective mycobacteria (25). However, a severe growth defect was observed when the Δ*pup* mutant of *C. glutamicum* was cultivated under iron limitation using glucose minimal medium supplemented with 1 μM $FeSO_4$ instead of the standard 36 μM. The final biomass (measured as $OD_{600}$) of the WT was four to five times higher than that of the Δ*pup* mutant (26.6 ± 0.9 vs. 6.1 ± 0.3) in these cultures (Fig. 1*B*). The growth defect was observed at iron concentrations of ≤1 μM (Fig. S1) for cultures that had been precultivated under iron limitation (Fig. S2). This suggests that pupylation is particularly important for long-term adaptation to iron limitation. Based on these results, all following experiments were performed with glucose minimal medium supplemented with 1 μM $FeSO_4$ in the preculture and main culture if not stated otherwise. Successful complementation and suppression of the growth phenotype was obtained by transformation of the Δ*pup* mutant with the plasmid pVWEx1-*pup*, driving expression of native Pup using an isopropyl-β-D-thiogalactoside (IPTG)-inducible *tac* promoter. In contrast, transformation of the Δ*pup* mutant with the plasmid pVWEx1-*pup*-E64A, encoding a pupylation-incompetent Pup-E64A variant with a C-terminal alanine residue, did not allow reversal of the growth defect (Fig. 1*B*). This result confirmed that the growth phenotype was indeed a result of the lack of a functional Pup protein and not caused by any secondary mutations in the Δ*pup* mutant.

**ARC and Dop Are Also Involved in the Adaptation to Iron Limitation.** In *M. tuberculosis*, pupylated proteins are recognized by the AAA+ ATPase Mpa, which unfolds Pup targets for subsequent proteasomal degradation. We therefore tested whether ARC, the homolog of Mpa in *C. glutamicum*, is also required for optimal growth under iron limitation. A mutant with an in-frame deletion of *arc* was constructed and exhibited a growth defect under iron limitation similar to the Δ*pup* mutant, which could be reversed by plasmid-based expression of *arc* using plasmid pVWEx1-*arc* (Fig. 1*C*).

Besides Pup and ARC, the deamidase/depupylase Dop plays an important role during pupylation. An in-frame *dop* deletion mutant of *C. glutamicum* also showed a growth defect under iron limitation, which was, however, not as severe as the one observed for the Δ*pup* and Δ*arc* mutants. The Δ*dop* mutant reached approximately 50% of the final biomass of the WT (Fig. 1*D*), indicating that Dop is required for optimal adaptation to iron limitation, but not as important as Pup or ARC. In contrast to the successful complementation of the Δ*pup* and Δ*arc* mutants, IPTG-induced overexpression of the *dop* gene in the Δ*dop* strain with plasmid pVWEx1-*dop* resulted in an even more pronounced growth defect under iron limitation rather than in its suppression (Fig. 1*D*). In contrast, no growth defect caused by *dop* overexpression was observed under iron-replete conditions. Presumably, overexpression of *dop* led to an unphysiologically high depupylase activity, which antagonized pupylation and caused a growth defect similar to the one observed for the Δ*pup* and the Δ*arc* mutants (Fig. 1 *B* and *C*). On the contrary, when the Δ*dop* strain carrying pVWEx-*dop* was cultivated in the absence of IPTG, the growth defect could be reversed, presumably because the basal *tac* promoter activity in the absence of IPTG allowed the synthesis of physiological Dop levels (Fig. 1*D*).

In summary, the results suggest that Dop is necessary for ensuring a critical level of free or recycled Pup in the cell. If this assumption holds true, artificially increased Pup levels should also result in a suppression of the growth defect of the Δ*dop* mutant. Indeed, overexpression of *pup* in the Δ*dop* strain using plasmid pVWEx1-*pup* led to WT-like growth under iron limitation (Fig. 1*D*).

**The Δ*pup* Mutant Senses a Stronger Iron Limitation than WT.** Neither the Δ*pup* mutant (23) nor the Δ*arc* and Δ*dop* mutants (Fig. S3*A*) showed a growth defect under iron sufficiency (36 μM $FeSO_4$), pointing to a specific role of pupylation for adaptation to iron limitation. To understand why pupylation is critical for this process, genome-wide mRNA levels of the Δ*pup* mutant and the WT strain were compared under iron-limiting growth conditions by using DNA microarrays. The samples for RNA isolation were taken in the early exponential phase ($OD_{600}$ of 3), when the growth curves of the two strains just started to diverge (Fig. 1*B*). Overall, 121 genes showed at least a twofold change in transcript levels in the Δ*pup* mutant, including 33 genes (~50%) of the DtxR regulon and the entire RipA regulon (Fig. 2*A* and Table S2). DtxR is the master regulator of iron homeostasis, serving as a sensor for chelatable cytosolic $Fe^{2+}$, which binds to its target promoters only when complexed with $Fe^{2+}$ (26). In *C. glutamicum,* DtxR represses 59 genes, the majority being involved in iron acquisition, and activates five genes, including *ftn* and *dps* encoding the iron-storage proteins ferritin (cg2782) and DNA-binding protein from starved cells (Dps; cg3327) (27, 28). One of the repressed genes encodes the AraC-type regulator of iron proteins, RipA, which itself represses a number of prominent iron-containing proteins under iron limitation, such as aconitase or succinate dehydrogenase, serving an analogous function as the regulatory small RNA RyhB in *Escherichia coli* (29). To independently confirm the DNA microarray data, we followed *ripA* expression in the WT and the Δ*pup* mutant by using a plasmid-based transcriptional fusion with the autofluorescent reporter protein E2-Crimson. The cell-specific median fluorescence of

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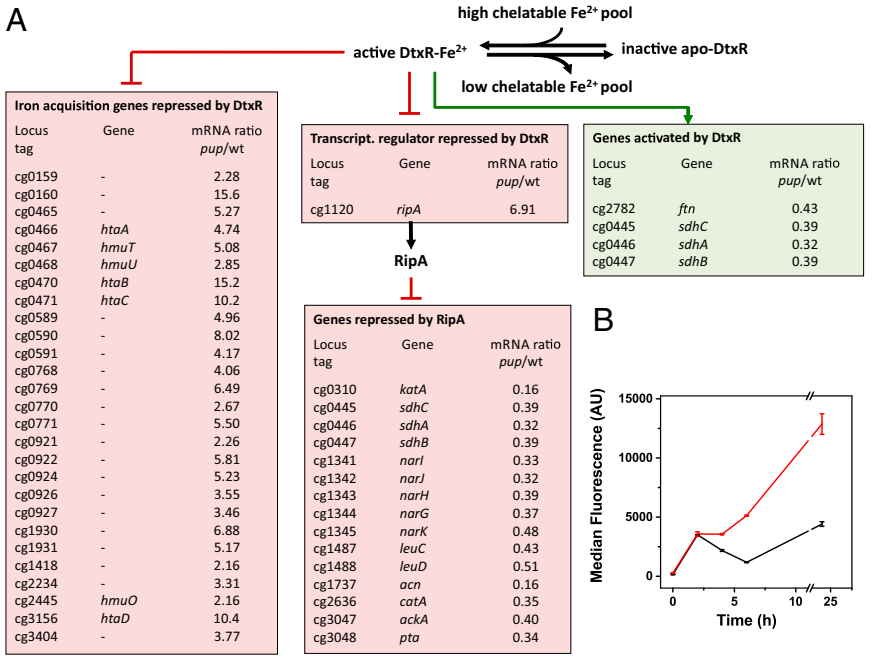


**Fig. 2.** (*A*) Influence of a *pup* deletion on global gene expression under iron-limitation conditions. The transcriptional network controlling iron homeostasis in *C. glutamicum* involves the $Fe^{2+}$-sensing master regulator DtxR and the AraC-type regulator RipA. Under iron-replete conditions, DtxR is complexed with $Fe^{2+}$, and only then does it bind to its target promoters. The boxes show those DtxR and RipA target genes, whose mRNA ratio was changed at least twofold in a transcriptome comparison of the Δ*pup* mutant and the WT under iron-limitation conditions. A list of all genes showing differential expression in the Δ*pup* mutant is given in Table S2. (*B*) Comparison of *ripA* promoter activity in *C. glutamicum* WT (black) and the Δ*pup* mutant (red) using the reporter plasmid pJC1-P*ripA*-crimson. The two strains were cultivated under iron limitation, and samples were taken after 0, 2, 4, 6, and 24 h. A total of 100,000 cells of each sample were analyzed by flow cytometry for Crimson fluorescence. Two biological replicates were performed, and error bars indicate the deviation between the samples. Scatter plots of the samples are shown in Fig. S9.

100,000 cells each was determined by flow cytometry at different points during cultivation under iron limitation. As shown in Fig. 2*B*, the *ripA* promoter was much more strongly activated in the Δ*pup* mutant than in the WT, in agreement with the transcriptome data. These results suggest that the Δ*pup* mutant has a lower chelatable cytosolic $Fe^{2+}$ concentration than the WT, causing a shift of DtxR to the DNA binding-incompetent apo-form.

Determination of the total cellular iron content of WT and Δ*pup* mutant by using a ferrozine-based assay revealed no significant differences between the two strains (Fig. S4). The total iron content is considered to be composed of the three pools, namely chelatable cytosolic iron, iron present in iron-dependent proteins, and iron stored in Ftn and Dps. As the prominent iron-dependent proteins repressed by RipA showed decreased mRNA levels in the Δ*pup* strain (Fig. 2*A*), the low chelatable cytosolic iron concentration of this mutant revealed by the transcriptome data should be the result of increased iron content of the iron storage proteins Ftn and/or Dps.

**Ferritin Is the Key Pupylation Target for Adaptation to Iron Limitation.** The list of 55 proteins recently shown to be pupylated in *C. glutamicum* includes Ftn and Dps (23). Thus, the stronger iron starvation response of the Δ*pup* strain and its growth defect under iron limitation might be related to a defective pupylation of the iron storage proteins. Because the 24-meric Ftn has a much higher iron storage capacity than the 12-mer Dps (4,500 vs. 500 iron atoms) (30), it was chosen as prime target for subsequent studies.

To confirm the previously detected in vivo pupylation of Ftn on K78 (23) and test for additional pupylation sites, we performed an in vitro pupylation assay using purified Ftn, Pup, and PafA (Fig. S5*A*). Peptide mass fingerprint analysis of trypsinized pupylated Ftn revealed five peptides (48% coverage; Fig. S5*B*). The one including K78 was confirmed to be pupylated using MALDI-TOF MS/MS (Fig. S5*C*). Ftn pupylation was additionally confirmed by immunoblot analyses using anti-Pup antiserum (Fig. S5*A*). As a negative control, we used DtxR, which was not found to be pupylated in our previous in vivo study (23). Also in vitro, no pupylation of DtxR was observed (Fig. S5*A*). To test whether K78 is the only pupylation site of Ftn in vivo, we purified His-tagged variants of Ftn and Ftn-K78A from a *C. glutamicum* Δ*pup*Δ*ftn* mutant harboring plasmid pVWEx1-*pup-ftn* or pVWEx1-*pup-ftn*-K78A, respectively. Pupylation of WT Ftn was confirmed by its apparent mass of 29 kDa, the presence of a pupylated peptide (*m*/*z*, 3,697.8) in the trypsinized protein, and immunoblotting with anti-Pup antiserum (Fig. 3*A*). In contrast, purified Ftn-K78A showed an apparent mass of 19 kDa, contained no detectable pupylated peptide, and was not detected by the anti-Pup antiserum (Fig. 3*A*).

A strain carrying a chromosomally encoded Ftn-K78A variant was constructed to test the relevance of Ftn pupylation for growth of *C. glutamicum* under iron limitation. Surprisingly, this strain carrying a single point mutation showed the same growth defect as the Δ*pup* mutant under iron limitation (Fig. 3*B*), whereas it grew like the WT under iron-replete conditions (Fig. S3*B*). A Ftn-K78R strain behaved like the Ftn-K78A strain, showing that the functionality of K78 could not be replaced by R78 (Fig. S3*C*). In contrast to the Ftn-K78 exchange mutants, a Δ*ftn* deletion mutant grew like the WT under iron-limited and iron-replete conditions. These results strongly indicate that defective pupylation of Ftn is responsible for the growth defect of the Δ*pup* mutant under iron limitation. To further validate this result, we cultivated the Δ*pup*Δ*ftn* mutant under iron limitation. In fact, this mutant grew much better than the Δ*pup* mutant, although not as good as the WT (Fig. 3*C*). Similarly, the growth defect of the Δ*arc* mutant could be largely abolished by deletion of *ftn* (Fig. 3*C*).

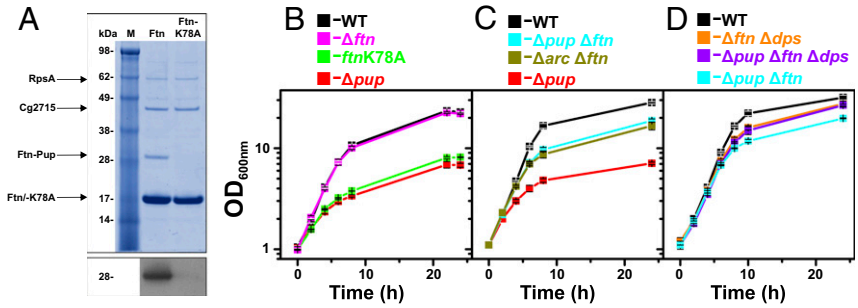


**Fig. 3.** (*A*) Requirement of K78 for in vivo pupylation of Ftn. *C. glutamicum* Δ*pup*Δ*ftn* carrying pVWEx1-*pup-ftn* or pVWEx1-*pup-ftn*-K78A was cultivated in BHI complex medium and harvested in the exponential growth phase. Crude cell extracts were subjected to $Ni^{2+}$-NTA affinity chromatography, and 3.3 μg protein of each eluate was analyzed by SDS/PAGE and Coomassie staining. Pupylation was confirmed by immunoblot analysis (*Lower*) using polyclonal anti-Pup antiserum. Protein bands visible in the Coomassie-stained gel were excised, trypsinized, and subjected to MS analysis to determine protein identity or the pupylated lysine residue. (*B–D*) Growth of *C. glutamicum* WT and the indicated mutant strains in glucose minimal medium under iron limitation (1 μM $FeSO_4$). The chromosomal *ftn*-K78A mutation prevents pupylation of Ftn. Mean values and SDs calculated from three independent biological replicates are shown.

**Pupylation of Dps Plays a Minor Role in Adaptation to Iron Limitation.** Because deletion of *ftn* in the Δ*pup* mutant did not fully reverse the growth defect under iron-limitation conditions (Fig. 3*C*), we speculated that pupylation of Dps might be responsible for the remaining difference. Thus, we constructed the mutant strains Δ*dps*, Δ*ftn*Δ*dps*, Δ*pup*Δ*dps*, and Δ*pup*Δ*ftn*Δ*dps*. Under iron-replete conditions, all these strains grew like the WT (Fig. S3 *D* and *E*). Under iron limitation, the strains Δ*dps* (Fig. S3*F*) and Δ*ftn* (Fig. 3*B*) grew like the WT, whereas the double-deletion mutant Δ*ftn*Δ*dps* showed a slight growth defect (Fig. 3*D*). Importantly, the Δ*pup*Δ*ftn*Δ*dps* strain grew as the Δ*ftn*Δ*dps* strain, suggesting that pupylation is no longer relevant under iron limitation when Ftn and Dps are absent. In contrast to the Δ*pup*Δ*ftn* mutant, the Δ*pup*Δ*dps* mutant grew as poorly as the Δ*pup* mutant (Fig. S3*G*), showing that Ftn pupylation rather than Dps pupylation is of prime importance for adaptation to iron limitation.

**Evidence Against Proteolysis of Pupylated Ferritin.** In mycobacteria, pupylated proteins are degraded by the proteasome (3, 4). *C. glutamicum* does not possess a proteasome but does possess the ATP-dependent proteases $ClpP_1P_2$ (31) and FtsH (32). To test whether pupylated Ftn is subject to proteolysis, we analyzed the Ftn protein levels in the WT and the Δ*pup* mutant under iron limitation. No significant differences in the Ftn levels were detected in the two strains, indicating that pupylated Ftn is not subject to proteolysis under the conditions tested (Fig. S6).

## Discussion

In mycobacteria and streptomycetes, pupylation was shown to target proteins to proteasomal degradation and to play an important role in the response to several stress conditions, such as nitrosative stress (25), oxidative stress (19), or nitrogen and carbon starvation (20), as well as in the development of cell morphology (21). However, members of other actinobacterial genera such as *Corynebacterium* do not harbor a *prcAB*-encoded proteasome; nevertheless, they possess the genes for the pupylation machinery including Pup, Dop, PafA, and Mpa/ARC. In the present study, we found that pupylation is specifically required for adaptation to iron limitation in *C. glutamicum*. Interestingly, our results indicate that this process is independent from protein degradation and involves the iron-storage protein ferritin as primary target. The crucial role of ferritin pupylation for adaptation to iron limitation was demonstrated by the finding that a single chromosomal point mutation exchanging the pupylated lysine residue K78 of Ftn to alanine caused the same growth defect as the deletion of *pup* or *arc* in *C. glutamicum*. Furthermore, the growth defect of the Δ*pup* and Δ*arc* mutants could be largely abolished by additional deletion of *ftn*. The ARC dependency indicates that adaptation to iron limitation requires ARC-catalyzed unfolding of ferritin. However, we did not find evidence that pupylated and unfolded ferritin is subsequently degraded.

In general, ferritins, but also bacterioferritins and Dps proteins, store iron in their cavities as a ferric ($Fe^{3+}$) mineral core (30). To release ferric iron for incorporation into cellular proteins, it has to be reduced to $Fe^{2+}$. The process of iron release from iron-storage proteins is still under investigation. Several different mechanisms have been reported for iron release from ferritin, including spontaneous dissolution of $Fe^{3+}$, lysosomal or proteasomal degradation of ferritin, and direct reduction of the ferric mineral in ferritin followed by $Fe^{2+}$ release through the ferritin pores (33). Our results for *C. glutamicum* suggest that iron stored in ferritin cannot be easily mobilized out of the intact 24-mer in vivo without pupylation, as the transcriptome data and the results of the reporter gene fusion disclosed that the Δ*pup* mutant shows a much stronger iron starvation response than the WT under iron-limiting conditions (Fig. 2).

Therefore, our data support a model in which pupylation acts as a trigger for iron mobilization out of iron storage proteins, as illustrated for ferritin in Fig. 4. Pupylation of ferritin triggers ARC-catalyzed unfolding, which in turn causes partial or complete deoligomerization of the 24-meric ferritin shell. As a consequence, the ferric mineral stored in ferritin becomes accessible for reduction and dissolution, providing chelatable iron for incorporation into iron-dependent proteins. Unfolded pupylated ferritin can be depupylated by Dop and might be available for reassembling 24-meric ferritin after renaturation. In mutants lacking Pup or ARC, iron stored in ferritin is hardly accessible or is accessible at a rate too low to meet cellular demands. Under iron-sufficient conditions, such a defect has no impact on growth, as there is still enough iron available in the medium that can be taken up and used to synthesize iron-dependent proteins, such as proteins containing iron-sulfur clusters or heme. Under iron-limiting conditions, however, the absence of Pup or ARC has severe consequences, as, in this situation, the iron stored in ferritin is urgently required for essential iron-dependent proteins. Furthermore, our results suggest that the depupylase Dop is required to recycle Pup. However, Dop levels have to be carefully balanced to avoid a futile cycle of pupylation and depupylation (Fig. 1*D*). Growth stops when iron release from ferritin is blocked, but can be resumed when pupylation and ARC-catalyzed unfolding of ferritin become possible again. This was demonstrated by an experiment in which a delayed induction of a plasmid-borne *pup* gene (6 h after starting cultivation under iron-limitation conditions) could still rescue the growth defect of the Δ*pup* mutant (Fig. S3*H*). Deletion of *pup* in the Δ*ftn*Δ*dps* background had no further influence on growth, indicating that

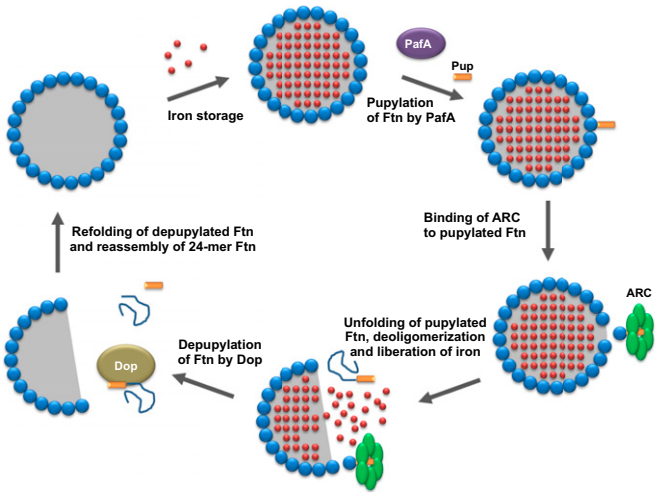


**Fig. 4.** Model illustrating the proposed role of pupylation for iron release from ferritin. A monomer of an iron-loaded 24-meric ferritin is pupylated at K78 by the Pup ligase PafA. The pupylated ferritin monomer is recognized by the AAA+ ATPase ARC, which catalyzes the unfolding of this monomer. As a consequence, the ferritin shell is partially or completely deoligomerized, making the ferric mineral inside accessible for reduction and solubilization. The unfolded pupylated ferritin monomer is depupylated by Dop, and, after renaturation, may reassemble to form intact 24-meric ferritin shells.

pupylation is not important under iron limitation when ferritin and Dps are absent.

In a first attempt to demonstrate the ARC-dependent deoligomerization of pupylated 24-meric Ftn, we tried to establish an in vitro assay based on native PAGE. For this purpose, iron-loaded 24-meric Ftn was pupylated in vitro with PafA and ATP, as demonstrated by a shift of the Ftn band via Coomassie and Prussian Blue staining (Fig. S7). When incubated with purified ARC and ATP, the band of pupylated 24-meric Ftn disappeared and a broad protein smear became visible (Fig. S7). This effect was strictly dependent on pupylated Ftn and did not occur when nonpupylated 24-meric Ftn was incubated with ARC and ATP. A more detailed kinetic characterization of ARC-dependent deoligomerization and iron release from pupylated Ftn requires further studies.

Iron homeostasis is closely interlinked with oxidative stress responses, as $Fe^{2+}$ triggers the Fenton reaction leading to the formation of the extremely damaging hydroxyl radical from hydrogen peroxide (34). Iron-storage proteins, in particular Dps (35, 36), contribute to the avoidance of toxic levels of free $Fe^{2+}$ within cells and are thus typical members of oxidative stress stimulons. In many bacteria, the response to oxidative stress is controlled by the $H_2O_2$-sensing transcriptional regulator OxyR (37). In contrast to *E. coli*, in which OxyR is an activator, *C. glutamicum* OxyR acts as a repressor that is inactivated by $H_2O_2$ stress (38, 39). Deletion of *oxyR* in *C. glutamicum* resulted in 3- and 12-fold up-regulation of *ftn* and *dps* expression, respectively, whereas *pup* and *pafA* were the most strongly down-regulated genes (10-fold) (38). Thus, $H_2O_2$ stress increases the levels of the iron storage proteins and, at the same time, decreases the levels of the pupylation machinery. This behavior fits to a protective role of Ftn and Dps by reduction of the free $Fe^{2+}$ levels, which is enhanced by the inhibition of pupylation-triggered iron release.

The concept that the iron release from Ftn and Dps is triggered by pupylation is likely to occur not only in corynebacteria, but also in other bacteria harboring the pupylation machinery. The ferritin homolog BfrB of *M. smegmatis* has been shown to be pupylated (17), and BfrB of *M. tuberculosis* was enriched in a pupylome study, but pupylation was not detected yet (15). Analysis of the positions of lysine residues in ferritin and Dps proteins of known crystal structure revealed that the pupylation sites currently known, namely K78 and K14 in *C. glutamicum* Ftn and Dps, respectively, and K10 in *M. smegmatis* BfrB are located on the outer surface and are thus accessible for pupylation (Fig. S8). Moreover, all analyzed actinobacterial ferritin and Dps homologs appear to possess at least one surface-exposed lysine residue that could be a target for pupylation. Further support for a role of pupylation in mycobacterial iron homeostasis comes from the observation that *mpa* and *pafA* mutants of *M. tuberculosis* show an increased resistance to $H_2O_2$ (25). As outlined earlier, this might be a result of an inhibition of iron release from BfrB.

Our finding that pupylation of ferritin is required for adaptation of *C. glutamicum* to iron limitation adds another level of complexity to the control of iron homeostasis in bacteria. Whereas regulation by transcriptional regulators such as Fur (40) or by regulatory sRNAs like RyhB (41) has been well studied, regulation at the posttranslational level has not yet been described in bacteria to the best of our knowledge. Our results raise a number of questions that demand further investigation. How many subunits of the 24-meric ferritin need to be pupylated and unfolded to make the iron from the mineral core accessible? How is iron solubilized from the mineral core in vivo? Is pupylation in *C. glutamicum* generally independent from degradation or are there pupylation targets that are degraded, for example, by the protease $ClpP_1P_2$? How important is pupylation-triggered iron release from ferritin for the pathogenicity of *M. tuberculosis* in view of the limited iron availability in the host? Obviously, the newly discovered link between pupylation and ferritin opens up new avenues for understanding the physiological role of pupylation and the mechanism of iron release from ferritin.

## Materials and Methods

**Bacterial Strains and Cultivation Conditions.** Bacterial strains and plasmids used in this study are listed in Table S3. *C. glutamicum* American Type Culture Collection (ATCC) 13032 and derivatives were cultivated at 30 °C by using brain heart infusion (BHI) medium or CGXII minimal medium containing 4% (wt/vol) glucose and 30 mg/L protocatechuate (42). To obtain iron limitation, the trace salt solution used for CGXII was prepared without iron, which was added freshly dissolved in 10 mM HCl. Detailed cultivation procedures are described in *SI Materials and Methods*.

**Strain and Plasmid Constructions.** All oligonucleotides used in this study are listed in Table S4. Enzymes used for cloning were obtained from Thermo Fisher Scientific. Kits for plasmid DNA isolation (GeneJET Plasmid Miniprep Kit) and DNA purification (QIAquick PCR Purification Kit) were obtained from Thermo Fisher Scientific and Qiagen, respectively. Standard molecular cloning methods like DNA restriction and ligation were performed according to the manufacturers' instructions or standard protocols (43). Competent *C. glutamicum* cells were obtained as described previously (44). In-frame deletion mutants of *C. glutamicum* and codon exchanges in chromosomal genes were created by using a two-step homologous recombination protocol (45). Detailed information on the cloning steps and plasmid constructions are provided in *SI Materials and Methods*.

**DNA Microarray Analyses.** DNA microarray analyses were performed to compare the mRNA levels of the *C. glutamicum* Δ*pup* mutant and its parent WT. The two strains precultivated under iron limitation were inoculated into fresh medium to an $OD_{600}$ of 1.5, cultured for 2 h, and harvested on ice by centrifugation. RNA preparation, cDNA synthesis, hybridization, and data analysis were performed as described previously (46). The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database and are accessible in GEO through accession number GSE64866.

**Flow Cytometry.** Analyses of each 100,000 cells of *C. glutamicum* WT and Δ*pup* carrying pJC1-$P_{ripA}$-crimson reporter constructs was performed as described previously (47).

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