High salinity growth conditions promote Tat-independent secretion of Tat substrates in *Bacillus subtilis*

René van der Ploeg[1#], Carmine G. Monteferrante[1#], Sjouke Piersma[1], James P. Barnett[2], Thijs R.H.M. Kouwen[1], Colin Robinson[2], and Jan Maarten van Dijl[1]

[1]*Department of Medical Microbiology, University of Groningen and University Medical Center Groningen and, Hanzeplein 1, P.O. box 30001, 9700 RB Groningen, the Netherlands*

[2]*School of Life Sciences, University of Warwick, Coventry, CV4 7AL, UK.*

[#]These authors contributed equally to this work

* To whom correspondence should be addressed: Department of Medical Microbiology, University Medical Center Groningen, Hanzeplein 1, P.O. box 30001, 9700 RB Groningen, the Netherlands, Telephone: +31-50-3615187, Fax: +31-50-3619105, E-mail: j.m.van.dijl01@umcg.nl

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Abstract (250 max)

The Gram-positive bacterium *Bacillus subtilis* contains two Tat translocases, which can facilitate transport of folded proteins across the plasma membrane. Previous research has shown that Tat-dependent protein secretion in *B. subtilis* is a highly selective process, and that heterologous proteins, such as the green fluorescent protein (GFP) are poor Tat substrates in this organism. Nevertheless, when expressed in *Escherichia coli*, both *B. subtilis* Tat translocases facilitated exclusively Tat-dependent export of folded GFP when the twin-arginine (RR) signal peptides of the *E. coli* AmiA, DmsA or MdoD proteins were attached. Therefore, the present studies were aimed at determining whether the same RR-signal peptide-GFP precursors would also be exported Tat-dependently in *B. subtilis*. In addition, we investigated the secretion of GFP fused to the full-length YwbN protein, a strict Tat substrate in *B. subtilis*. Several investigated GFP fusion proteins were indeed secreted in *B. subtilis*, but this secretion was shown to be completely Tat-independent. At high salinity growth conditions, the Tat-independent secretion of GFP as directed by the RR-signal peptides from the *E. coli* AmiA, DmsA or MdoD proteins was significantly enhanced, and this effect was strongest in strains lacking the TatAy-TatCy translocase. This implies that high environmental salinity has a negative influence on the avoidance of Tat-independent secretion of AmiA-GFP, DmsA-GFP and MdoD-GFP. We conclude that as yet unidentified control mechanisms reject the investigated GFP fusion proteins for translocation by the *B. subtilis* Tat machinery and, at the same time, set limits to their Tat-independent secretion presumably via the Sec pathway.

Introduction

Protein secretion is an important feature for the survival and competitive success of bacterial cells in their natural habitats. The ability to secrete proteins is particularly well developed in the Gram-positive bacterium *Bacillus subtilis*, which is of interest both from applied and fundamental scientific points of view [3, 47, 48, 51]. Combined genetic, proteomic and bioinformatic analyses have revealed that the vast majority of proteins secreted by *B. subtilis* leave the cytoplasm in an unfolded state via the general secretion (Sec) pathway [47]. Upon translocation these proteins fold into their active and protease-resistant conformation [19]. A limited number of proteins are secreted via the so-called twin-arginine (Tat) pathway which, in contrast to the Sec pathway, can facilitate the transport of fully folded proteins [16, 35, 37, 38, 42, 45, 53].

The proteins destined for export via the Sec or Tat pathways are synthesized with N-terminal signal peptides. These have a characteristic tripartite structure consisting of a positively charged N-terminal region, a hydrophobic H-region and a C-terminal region [37, 48]. The C-region contains a signal peptidase cleavage site for signal peptide removal during or shortly after membrane translocation of the attached protein [10, 52]. Although the signal peptides of Sec and Tat substrates are similar in structure, particular signal peptide features promote the specific targeting of proteins to the Tat pathway. These include a twin-arginine (RR) recognition motif in the N-region with the consensus sequence K/R-R-x-#-#, where # marks hydrophobic residues and x can be any residue [6, 12, 14, 33, 46]. This RR-motif is specifically recognized by the Tat translocase [1, 8, 13]. Additionally, RR-signal peptides are “unattractive” for the Sec machinery, because their H-region has a relatively low hydrophobicity, and because the C-region often (but not always) contains a positively charged residue that strongly promotes “Sec avoidance” [7, 14, 49]. Importantly, the Sec incompatibility of Tat substrates is not only achieved through RR-signal peptide

features, but also through their rapid or controlled folding in the cytoplasm prior to translocation [15, 39]. In fact, some Tat-dependently exported proteins are subject to dedicated chaperone-mediated proofreading in the cytoplasm in order to prevent the initiation of their transport before folding or co-factor assembly have been completed [30, 38, 40, 43].

B. subtilis contains two independently working Tat translocases named TatAyCy and TatAdCd, which are of the TatAC type that is commonly found in Gram-positive bacteria [21, 22, 23]. Unlike the TatABC type translocases that are present in Gram-negative bacteria, these “minimal” TatAC translocases lack a TatB subunit [4, 5, 24]. In *B. subtilis*, the TatAyCy and TatAdCd translocases have distinct specificities for the Dyp-type peroxidase YwbN and the phosphodiesterase PhoD respectively, at least when the cells are grown in a standard LB medium [21, 22, 23]. Also, a hybrid precursor of the subtilisin AprE fused to the YwbN signal peptide was secreted in a TatAyCy-specific manner, suggesting a preferential interaction between the YwbN signal peptide and the TatAyCy translocase [25]. Nevertheless, the specificities of TatAyCy and TatAdCd overlap at least to some extent as was recently shown by the heterologous expression of TatAdCd or TatAyCy in *Escherichia coli* strains lacking their own TatABC translocase [4, 5]. The latter studies revealed that both *B. subtilis* Tat translocases are able to translocate the green fluorescent protein (GFP) fused to the RR-signal peptides of the *E. coli* AmiA, DmsA or MdoD proteins (Fig. 1). A specificity difference was, however, observed as the TMAO reductase (TorA) and a TorA-GFP fusion were transported by TatAdCd but not by TatAyCy [4, 5].

An interesting conclusion from the heterologous Tat expression studies in *E. coli* was that both *B. subtilis* TatAC translocases were able to translocate active GFP when expressed in *E. coli*. By contrast, earlier experiments had indicated that this was not possible in *B. subtilis* [25, 32]. Therefore, the aim of the present studies was to assess whether the same RR-signal peptide-GFP hybrid precursors that were Tat-

dependently translocated in *E. coli* would also lead to Tat-dependent GFP secretion in *B. subtilis*. In addition we investigated whether a fusion of GFP to the full-size YwbN protein might facilitate GFP export. Briefly, the results show that none of the GFP fusion constructs were Tat-dependently secreted. Instead, Tat-independent GFP secretion was observed, which was most pronounced when the cells were grown in LB medium of high salinity. Taken together, our findings show that the GFP fusion proteins are rejected for translocation by the *B. subtilis* Tat machinery. Furthermore, the avoidance of Tat-independent secretion of all three hybrid GFP precursors, presumably via the Sec pathway, seems to be suppressed when cells are grown in medium with 6% salt.

Materials and Methods

Plasmids, bacterial strains, media and growth conditions

The plasmids and bacterial strains used in this study are listed in Table 1. Strains were grown with agitation at 37ºC in either Lysogeny Broth (LB), or Paris minimal (PM) medium. LB medium consisted of 1% tryptone and 0.5% yeast extract with or without NaCl (1% or 6%), pH 7.4. Notably, LB with 1% NaCl is the standard LB medium that has been used in all our previous studies. PM consisted of 10.7 mg ml^{-1} K_2HPO_4, 6 mg ml^{-1} KHPO, 1 mg ml^{-1} trisodium citrate, 0.02 mg ml^{-1} $MgSO_4$, 1% glucose, 0.1% casamino acids (Difco), 20 mg ml^{-1} L-tryptophan, 2.2 mg ml^{-1} ferric ammonium citrate and 20 mM potassium glutamate. To activate a phosphate starvation response and, accordingly, induce the expression of the TatAdCd translocase, the strains were grown overnight in HPDM (high phosphate defined medium), which is rich in phosphate. The next morning, cells were transferred to LPDM (low phosphate defined medium). Both media were prepared according to Müller *et al*. (1997) [34]. *Lactococcus lactis* was grown at 30°C in M17 broth supplemented with 0.5% glucose. When required, media for *E. coli* were

supplemented with erythromycin (Em; 100 μg ml^{-1}), kanamycin (Km; 20 μg ml^{-1}), chloramphenicol (Cm; 5 μg ml^{-1}), or spectinomycin (Sp; 100 μg ml^{-1}); media for *B. subtilis* were supplemented with Em (1 μg ml^{-1}), Km (20 μg ml^{-1}), Cm (5 μg ml^{-1}), Phleomycin (Phleo; 4 μg ml^{-1}) or Sp (100 μg ml^{-1}); media for *L. lactis* were supplemented with Em (2 μg ml^{-1}).

DNA techniques

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of competent *E. coli* cells were carried out as previously described [44]. *B. subtilis* was transformed as described by Kunst and Rapoport [28]. PCR was carried out with the Pwo DNA polymerase. PCR products were purified using the PCR purification kit from Roche. Restriction enzymes were obtained from New England Biolabs. Plasmid DNA from *E. coli* was isolated using the alkaline lysis method [44], or the Invisorb®Plasmid Isolation Kit (Invitek). All constructs were checked by sequencing (serviceXS, Leiden the Netherlands).

To construct the plasmids pHB-AmiA-GFP, pHB-DmsA-GFP and pHB-MdoD-GFP, the *amiA-gfp*, *dmsA-gfp* and *mdoD-gfp* hybrid genes were PCR-amplified from the respective pBAD24-based plasmids carrying these genes [5] (Table 1). The 5' primers used for PCR contained the *mntA* ribosome-binding site and start codon, as well as a *Spe*I restriction site, and the 3' primer contained a *Bam*HI restriction site (Table 2). The resulting PCR products were cleaved with *Spe*I and *Bam*HI, and ligated to *Spe*I-*Bam*HI-cleaved pHB201. Ligation mixtures were used to transform *E. coli*, resulting in the identification of plasmids pHB-AmiA-GFP, pHB-DmsA-GFP and pHB-MdoD-GFP. Next, these plasmids were used to transform the *B. subtilis* strains 168, *tatAyCy*, *tatAdCd* and total-tat_2. To construct the plasmids pSURE-SpYwbN-GFP and pSURE-YwbN-GFP, the *ywbN* signal sequence and the full-length *ywbN* gene were PCR-amplified from chromosomal DNA of *B. subtilis* 168. The 5' primer

used for PCR contained a *Kpn*I restriction site, and the 3' primer contained a *Hin*dIII restriction site (Table 2). The resulting PCR products were cleaved with *Kpn*I and *Hin*dIII, and ligated to *Kpn*I-*Hin*dIII-cleaved pSG1154 [29], which contains the *gfpmut1* gene. The fusion products Sp(Ywbn)-GFP and YwbN-GFP where then amplified from these vectors using a 5' primer containing a *Bsp*HI restriction site and a 3' primer containing a *Hin*dIII restriction site, and they were cloned into the *Nco*I-*Hin*dIII-cleaved pNZ8910 plasmid. Ligation mixtures were used to transform *L. lactis*, resulting in the isolation of plasmids pSURE-SpYwbN-GFP and pSURE-YwbN-GFP. The plasmids were then used to transform the *B. subtilis ywbN*, *tatAyCy ywbN* or *tatAdCd ywbN* strains.

SDS-PAGE and Western blotting

Cellular or secreted proteins were separated by PAGE using pre-cast Bis-Tris NuPAGE gels (Invitrogen). The presence of GFP, YwbN or LipA in cellular or growth medium fractions was detected by Western blotting. For this purpose, proteins separated by PAGE were semi-dry blotted (75 min at 1 mA/cm^2) onto a nitrocellulose membrane (Protran®, Schleicher & Schuell). Subsequently, GFP was detected with monoclonal antibodies (Clontech), YwbN-Myc was detected with monoclonal antibodies against the Myc-tag attached to this protein (Gentaur), YwbN, LipA, TrxA, PhoD and PhoB were detected with specific polyclonal antibodies raised in rabbits. Visualisation of bound antibodies was performed with fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit or goat anti-mouse from LiCor Biosciences) in combination with the Odyssey Infrared Imaging System (LiCor Biosciences). Fluorescence was recorded at 800 nm.

Fluorescence microscopy

Cells carrying plasmids pHB-AmiA-GFP, pHB-DmsA-GFP and pHB-MdoD-GFP were grown in LB supplemented with 1 or 6% NaCl. After 7 hours of growth the optical

density at 600 nm (OD_{600}) was measured. The strains containing pGFP, pSURE-SpYwbN-GFP or pSURE-YwbN-GFP were grown till an OD_{600} of 1.0, induced with 1.0% (v/v) supernatant of *B. subtilis* ATCC 6633. In this respect it is noteworthy that the subtilin produced by *B. subtilis* ATCC6633 is secreted into its growth medium. Addition of this spent medium in a 100-fold dilution to *B. subtilis* cells containing pGFP, pSURE-SpYwbN-GFP or pSURE-YwbN-GFPl induces the *spaS* promoter on these plasmids thereby driving the high-level transcription of the downstream GFP genes. Upon growth for 2 additional hours, cells were spotted on M9 agarose slides containing the appropriate salt concentrations. These slides were prepared by transfer of M9 agarose medium into a 65 µl Frame-Seal Slide Chamber (SLF-0601, Bio-Rad). Fluorescence microscopy was performed with a Leica DM5500 B microscope. Fluorescence images were recorded using a Leica EL6000 lamp with the intensity set to 55%. The exposure time was 256 ms. Quantification of GFP fluorescence was done using the ImageJ software package (http://rsbweb.nih.gov/ij/). Cellular fluorescence values were measured in grey scale values. Background fluorescence was calculated by averaging the grey scale values of the area outside the cells. Finally the background fluorescence was subtracted from the cellular fluorescence.

Results

The AmiA and MdoD RR-signal peptides mediate Tat-independent GFP secretion in B. subtilis.

When heterologously expressed in *E. coli*, the TatAdCd and TatAyCy translocases can transport the AmiA-GFP, DmsA-GFP and MdoD-GFP precursors across the inner membrane, leading to an accumulation of active GFP in the periplasm [4, 5]. To assess whether the very same RR-signal peptide-GFP precursors would also be exported Tat-dependently in *B. subtilis*, we expressed them in *B. subtilis* 168 and

corresponding *tat* mutant strains. For this purpose, the respective hybrid genes were provided with the ribosome-binding site *plus* start codon of the *B. subtilis mntA* gene, that are well suited for heterologous protein expression in *B. subtilis* [26]. The resulting constructs were then constitutively expressed at relatively low levels from the *E. coli* - *B. subtilis* shuttle vector pHB201. Cells containing these constructs were subsequently grown in standard LB medium (1% NaCl). It should be noted that under these conditions, the cells produce mainly the TatAyCy translocase and the TatAdCd translocase is expressed at barely detectable levels [23, 36]. As shown in Figure 2A (left panels), all three precursors were synthesized in *B. subtilis* cells when grown overnight in this medium. However, only in the case of AmiA-GFP and MdoD-GFP was processing to the mature form and release of this mature form into the growth medium observed (Fig. 2A, left and right panels). The strains producing AmiA-GFP secreted relatively higher amounts of mature GFP into the medium than strains producing MdoD-GFP. Notably, the secretion of mature-sized GFP by strains producing AmiA-GFP was not influenced by the absence of *tatAyCy*, *tatAdCd* or even all *tat* genes, and the same was true for strains producing MdoD-GFP, although in this case the GFP was secreted at lower levels (Fig. 2A). No secretion of GFP was detectable for wild-type or *tat* mutant strains producing the DmsA-GFP precursor (Fig. 2A). Consistent with this observation, barely any mature-sized GFP was detectable in cells producing DmsA-GFP. This suggests that the DmsA-GFP precursor is neither an acceptable substrate for the two TatAC translocases nor the Sec translocase when produced in *B. subtilis* cells grown in standard LB medium (1% NaCl). By contrast, under these conditions the control protein YwbN-Myc was secreted in a strictly TatAyCy-dependent manner, as evidenced by the fact that it was secreted only by the parental strain 168 and the *tatAdCd* mutant, but not by the *tatAyCy* or total-*tat2* mutants (Fig. 2B). These findings show that under the tested conditions, the precursors of AmiA-GFP, DmsA-GFP and MdoD-GFP are rejected by the Tat system of *B. subtilis*.

Rejection of the chimeric YwbN-GFP protein by Tat

Our previous studies have shown that the RR-signal peptide of the Tat substrate YwbN can redirect the normally Sec-dependent protein AprE into the *B. subtilis* Tat pathway, leading to TatAyCy-dependent secretion of this protein [25]. We decided therefore to challenge the Tat system with a chimeric protein consisting of GFP fused to the C-terminus of full-length YwbN (YwbN-GFP). As controls we used strains producing GFP with or without the RR-signal peptide (denoted SpGFP and GFP respectively). Subsequently, the YwbN-GFP, SpGFP or GFP proteins were produced using the subtilin- induced SURE system [9]. The possible secretion of YwbN-GFP or GFP was assessed by Western blotting using specific antibodies for GFP and YwbN. As shown in Figure 3, neither GFP nor SpGFP was secreted into the growth medium. In contrast, small amounts of the YwbN-GFP fusion protein were secreted, but this was independent of the TatAyCy or TatAdCd translocases. These findings show that GFP produced in *B. subtilis* is rejected by the Tat system, irrespective of its fusion to a full-size Tat substrate or an RR-signal peptide only.

To test whether the GFP protein produced with the different signal peptide fusions was active, we analysed the producing cells by fluorescence microscopy. As can be observed in Figure 4, the production of the authentic GFP protein with the control plasmid pGFP resulted in a very bright fluorescent signal throughout the *B. subtilis* cells. Fusion of the YwbN signal peptide to GFP largely abolished the fluorescent signal and the remaining signal was most clearly detectable at the cell poles. Notably, production of the YwbN-GFP fusion protein resulted in a spotted pattern of GFP fluorescence that was not altered in the absence of the *tatAyCy* or *tatAdCd* genes. Together with the Western blotting data, these findings suggest that fusion of YwbN or the YwbN signal peptide to GFP may interfere with its folding into an active and stable conformation and/or to an altered sub-cellular localization,

possibly in an aggregated state. Alternatively, the GFP might correctly fold and then aggregate.

Phosphate starvation conditions result in Tat-independent GFP secretion

Studies on the *B. subtilis* Tat translocases (following expression in both *B. subtilis* and *E. coli*) have shown that the TatAdCd translocase is the most permissive of the two translocases present in *B. subtilis* [4, 17]. However, production of the TatAdCd complex of *B. subtilis* is fully induced only under phosphate starvation conditions [23, 36]. We thus investigated whether this translocase can facilitate the secretion of AmiA-GFP, DmsA-GFP or MdoD-GFP under conditions of phosphate starvation. As shown in Figure 5, all three precursors were produced by cells grown in LPDM medium with the cells also containing mature GFP in varying amounts. Furthermore, secretion of mature-sized GFP was observed in the AmiA-GFP- and DmsA-GFP-producing strains (Figure 5A, right panel). The secretion of GFP was however, mostly Tat-independent, since bands corresponding to mature-size GFP were detected in the medium of mutant strains lacking the *tatAyCy*¸ *tatAdCd*, or all *tat* genes. In contrast, no GFP secretion was observed for cells producing MdoD-GFP. In control experiments the secretion of PhoD was found to be dependent upon the production of the TatAdCd complex, as shown by the lack of PhoD secreted by the *tatAdCd* and total-*tat* mutant strains, in addition to the PhoD secretion observed in the strain lacking the *tatAyCy* genes. Furthermore, secretion of the Sec-dependent protein PhoB was not affected by any of the tested *tat* mutations. These findings show that induction of the TatAdCd translocase does not preclude the rejection of GFP by the *B. subtilis* Tat system.

High salinity growth conditions result in elevated levels of Tat-independent GFP secretion

298 We have previously shown that the specificity of Tat-dependent protein transport in
299 *B. subtilis* is influenced by the salinity of the growth medium (50). This was most
300 clearly evidenced by the finding that some YwbN was secreted completely Tat-
301 independently when LB medium was supplemented with 6% NaCl (instead of the
302 standard 1% NaCl). To investigate whether the secretion of AmiA-GFP, DmsA-GFP,
303 MdoD-GFP, SpYwbN-GFP or YwbN-GFP might be influenced by a growth medium
304 with high salinity, cells producing these hybrid precursors were grown in LB medium
305 with 6% NaCl. As shown by Western blotting of cellular and growth medium samples,
306 the increased salt concentration in the medium resulted in a drastically improved
307 secretion of DmsA-GFP, with mature-sized GFP now clearly detectable in both the
308 cellular and growth medium fractions (Fig. 6A). The highest levels of secreted GFP
309 were observed for the *tatAyCy* and total-*tat* mutant strains, suggesting that the
310 TatAyCy translocase interferes with the Tat-independent translocation of DmsA-GFP
311 during growth in LB medium with 6% salt. Consistent with these findings, the high
312 salinity growth conditions clearly had a stimulating effect on the secretion of mature
313 GFP by cells producing AmiA-GFP or MdoD-GFP. Again the highest levels of mature
314 GFP were secreted by the *tatAyCy* and total-*tat* mutant strains. The high salt
315 concentration had no effect on secretion of SpYwbN-GFP or YwbN-GFP (not shown).
316 Under the same conditions, Tat-independent secretion of YwbN was observed
317 (Figure 6B) as previously reported (50). These observations show that the Tat-
318 independent secretion of GFP and YwbN is strongly stimulated when cells are grown
319 in LB medium with 6% NaCl. As the Tat-independent secretion most likely takes
320 place via the Sec pathway [25, 50], these findings imply that the high salinity growth
321 conditions result (at least partially) in a suppressed “Sec avoidance” of the respective
322 precursor proteins. Since both Tat-dependent protein translocation and Sec
323 avoidance are not only determined by features of the signal peptide, but also by the
324 folding state of the respective precursor protein, we used fluorescence microscopy to
325 determine whether folded and active GFP is detectable in cells producing AmiA-GFP,

DmsA-GFP or MdoD-GFP. Indeed Figure 7 shows that at least some of the GFP within cells producing AmiA-GFP, DmsA-GFP or MdoD-GFP is active when cells were grown in LB with 6% NaCl. Nevertheless, little if any GFP seems to be secreted by the Tat translocases of the respective cells. It should be noted here that the cellular GFP expression levels and fluorescence were not substantially different when cells were grown in LB with 1% or with 6% NaCl, suggesting that salt does not directly affect the folding state of cytoplasmic GFP (data not shown). This view is supported by the finding that cells producing the authentic GFP (without signal peptide) did not show significant differences in fluorescence upon growth in LB with 1% or 6% NaCl (Figure 8).

Discussion

The present studies were aimed at investigating the possible Tat-dependent secretion in *B. subtilis* of hybrid GFP precursor proteins that contain the RR-signal peptides of the *E. coli* AmiA, DmsA or MdoD proteins. While these precursors were previously shown to be transported to the periplasm of *E. coli* by the heterologously expressed TatAdCd or TatAyCy translocases of *B. subtilis* [4, 5], we now show that these precursors are not accepted by the *B. subtilis* TatAC translocases when expressed in *B. subtilis*. Instead, Tat-independent secretion of GFP was observed in strains producing the AmiA-GFP or MdoD-GFP precursors under standard growth conditions (*i.e.* LB medium with 1% NaCl), and this Tat-independent secretion was significantly enhanced when the strains were grown in LB medium with 6% NaCl. While cells expressing the DmsA-GFP precursor under standard growth conditions did not secrete GFP, these cells did secrete GFP Tat-independently when grown in LB with 6% NaCl. Under these high salinity growth conditions, we also observed Tat-independent secretion of the known *B. subtilis* Tat substrate YwbN. These findings

imply that the Sec avoidance of *B. subtilis* RR-precursor proteins under standard growth conditions is suppressed under high salinity growth conditions.

To investigate whether a full-size Tat-dependent protein might serve as a carrier for Tat-dependent translocation of GFP in *B. subtilis*, the possible secretion of a YwbN-GFP fusion protein was investigated. However, the results showed unambiguously that this fusion protein was not exported Tat-dependently, as was the case when only the YwbN signal peptide was fused to GFP. While YwbN-GFP was effectively produced, degradation within the *B. subtilis* cells was observed, and small amounts were found to be secreted Tat-independently. The finding that the YwbN signal peptide can direct Tat-independent secretion is in agreement with previous studies indicating that this RR-signal peptide is able to direct either Tat- or Sec-dependent secretion of particular proteins to which it was fused [25]. This was even true for the authentic *E. coli* Tat substrate SufI, which was secreted Tat-independently in *B. subtilis* when fused to the YwbN signal peptide [25]. In contrast to the AmiA-GFP, DmsA-GFP or MdoD-GFP, no difference in GFP secretion was observed when the strains producing YwbN-GFP or SpYwbN-GFP were grown in LB with 6% NaCl (data not shown). This suggests that the altered behaviour of AmiA-GFP, DmsA-GFP or MdoD-GFP under high salinity growth conditions may relate to specific properties of the respective signal peptides.

Previous studies have indicated that the Tat pathway in *B. subtilis* is able to facilitate the secretion of GFP, albeit in an inactive state [32]. It is therefore not clear why the *B. subtilis* TatAC translocases do not facilitate the secretion of mature GFP when the AmiA-GFP, DmsA-GFP, MdoD-GFP, SpYwbN-GFP or YwbN-GF precursors are produced in *B. subtilis*. At least three possible reasons for this finding are conceivable. Firstly, the respective RR-signal peptides may not be presented to the TatAC translocases in the right way. This would then expose these signal peptides to the Sec machinery of *B. subtilis*, resulting in Tat-independent GFP secretion via the Sec pathway. Consistent with this idea, the RR-motifs in the AmiA,

DmsA and MdoD signal peptides do not show a perfect match with the consensus RR-motif S/T-R-R-x-F-L-K (Fig. 1). Nevertheless, at least under high salinity growth conditions, the RR-signal peptides of AmiA, DmsA and MdoD seem to be recognized somehow by TatAyCy as was evidenced by the observation that Tat-independent GFP secretion was enhanced in *B. subtilis* strains lacking *tatAyCy*. Secondly, the GFP attached to the AmiA, DmsA or MdoD signal peptides may not fold rapidly enough in *B. subtilis* to allow Tat-dependent translocation of the fusion proteins. This seems to be the case for the SpYwbN-GFP fusion, the production of which resulted in substantially lower levels of cell fluorescence than the production of GFP without an attached signal peptide. This was despite the protein production levels of GFP with or without the YwbN signal peptide being very similar (Figure 3). Furthermore, foci of fluorescence were observed in cells producing SpYwbN-GFP or YwbN-GFP suggesting that aggregation of GFP might occur thereby precluding its efficient export via Tat. On the other hand, the identification of GFP foci at the cell poles is in agreement with previous reports, which showed a polar and septal localization of Tat-machinery components in *B. subtilis* [31, 41]. However, mutations in the *tatAyCy* or *tatAdCd* genes did not seem to influence the appearance of GFP foci suggesting that this phenomenon is not directly related to interactions with the Tat machinery. Thirdly, *B. subtilis* may be missing some chaperones that are needed to coordinate the export of the investigated GFP fusion proteins. This might apply to the fusions containing *E. coli* RR-signal peptides, like the DmsA signal peptide, which is known to be recognized by the DmsD chaperone [38, 43]. On the other hand, if the absence of an appropriate chaperone were the main problem, we would expect that fusing GFP to a native Tat substrate of *B. subtilis*, such as YwbN, would result in productive Tat-dependent GFP export provided that the fused GFP is folded.

Analyses of cells producing AmiA-GFP, DmsA-GFP or MdoD-GFP by fluorescence microscopy showed that these cells contained little or no active GFP. Furthermore, Western blotting revealed that some of the produced GFP is secreted

409 Tat-independently, possibly via the Sec pathway. Such secretion via Sec would
410 suggest slow folding of GFP since the Sec pathway is known to translocate only
411 proteins in an unfolded state. Notably, Tullman-Ercek *et al*. [49] reported that the
412 signal peptides of AmiA, DmsA and MdoD can direct attached proteins, such as
413 GFP, the alkaline phosphatase PhoA and the maltose-binding protein MBP to both
414 the Sec and Tat pathways of *E. coli*. The Tat-specificity of the AmiA and MdoD signal
415 peptides was found to be especially low when fused to the alkaline phosphatase
416 PhoA, which is a regular Sec substrate [49]. However, the Tat-independent export of
417 GFP fused to the AmiA and MdoD signal peptides was also substantial (about 25-
418 30%), which is consistent with our present finding that these hybrid precursors are
419 Tat-independently exported in *B. subtilis*. Furthermore, the export of DmsA-GFP in *E.*
420 *coli*, as reported by Tullman-Ercek *et al*. was only to less than 10% Tat-independent,
421 which is in line with our present observations that the synthesis of this precursor does
422 not lead to detectable levels of Tat-independent secretion of GFP. The observed
423 strong Sec avoidance of DmsA-GFP is consistent with the presence of two positively
424 charged residues in the C-region of the DmsA signal peptide (*i.e.* Arg and His; Fig.
425 1). Such positively charged residues with a possible role in Sec avoidance are absent
426 from the AmiA and MdoD signal peptides.
427 Interestingly, an increased salinity of the growth medium seems to result in a
428 suppression of Sec avoidance, not only by the AmiA-GFP, DmsA-GFP and MdoD-
429 GFP precursors, but also by authentic Tat-dependently secreted proteins such as
430 YwbN. It is at present not clear why this happens, but the finding suggests that
431 electrostatic interactions and/or a salt-sensitive factor are involved in Sec avoidance.
432 A possible involvement of electrostatic interactions in Sec avoidance would be in line
433 with the finding that positively charged residues in the C-region of the signal peptide
434 facilitate Sec avoidance. However, high salinity of the growth medium might also
435 slow down the folding of precursor proteins, for example through changes in the
436 cytoplasmic concentrations of compatible solutes, which would then make these

proteins more attractive for the Sec translocase [11, 20, 50],. One additional Sec-avoidance determinant seems to be the TatAyCy translocase itself, since the absence of this translocase resulted in increased levels of GFP secretion under high salinity growth conditions. It thus seems that TatAyCy can be directly involved in Sec avoidance, possibly by targeting unfolded GFP precursors for degradation, or by redirecting them into the cytoplasm where they fold into a Sec incompatible state. Notably, in *B. subtilis* an increased TatAdCd-dependent secretion in the absence of TatAyCy has previously been shown for the phosphodiestase PhoD [23]. This supports the view that interactions of certain precursor proteins with TatAyCy may lead to the rejection of these precursors for translocation via Tat in *B. subtilis*.

In conclusion, the present results indicate that as yet unidentified control mechanisms reject the AmiA-GFP, DmsA-GFP and MdoD-GFP fusion proteins for translocation by the *B. subtilis* Tat machinery and, at the same time, set limits to their Sec-dependent secretion. At least the Sec avoidance of all three hybrid GFP precursors seems to be overruled when cells are grown in LB medium with 6% NaCl. Further studies to characterize this phenomenon should involve the systematic mutagenesis of the C-regions of the AmiA, DmsA MdoD and YwbN signal peptides. In addition, at least under these high salinity growth conditions, the TatAyCy translocase seems to be a determinant in Sec avoidance, probably due to preferential signal peptide recognition. Most likely, the identification and subsequent elimination or modulation of the control systems that limit GFP secretion will be key to unlocking the *B. subtilis* Tat pathway for the production of heterologous proteins.

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Competing interests

The authors declare that they have no competing interests

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Figure Legends

Fig. 1. Signal peptide sequences. The amino acid sequences of the RR-signal peptides of AmiA, DmsA and MdoD of *E. coli*, and YwbN and PhoD of *B. subtilis* are shown. Twin-arginine motifs are underlined, hydrophobic H-regions are printed in italics, and the C-regions are marked in bold with residues flanking the signal peptidase cleavage sites underlined.

Fig. 2. Secretion of AmiA-GFP, DmsA-GFP or MdoD-GFP by cells grown in standard LB medium with 1% NaCl. A. Cell and growth medium fractions of *B. subtilis* strains producing AmiA-GFP, DmsA-GFP or MdoD-GFP were separated by centrifugation and used for SDS-PAGE and Western blotting with specific antibodies. For this purpose, the cells of *tatAyCy*, *tatAdCd* or total-*tat* mutant strains or the parental strain 168 were grown for 7 hours in LB medium, supplemented with 1% NaCl. Protein loading was corrected for OD_{600}. “pG”, cells harbouring pHB-AmiA-GFP, pHB-DmsA-GFP or pHB-MdoD-GFP; “ev”, cells harbouring the empty vector pHB201. **B.** Cell and growth medium fractions of *B. subtilis* strains producing YwbN-Myc were prepared for SDS-PAGE and Western blotting with specific antibodies as indicated for panel A. For this purpose, the cells of *tatAyCy*, *tatAdCd* or total-*tat* mutant strains or the parental strain 168 contained the X*ywbN* cassette in *amyE*. “Xy”, cells containing the X*ywbN* cassette.

Fig. 3. Secretion of a chimeric YwbN-GFP fusion protein

Cell and growth medium fractions of *B. subtilis* strains producing GFP, GFP fused to the signal peptide of YwbN (SpGFP) or the fusion protein YwbN-GFP were separated by centrifugation and used for SDS-PAGE and Western blotting with specific monoclonal antibodies directed against GFP and polyclonal antibodies against YwbN. Notably, the full-size YwbN-GFP fusion protein was only efficiently detected

723 with antibodies against YwbN. Specifically, the cells of parental strain 168, as well as
724 the mutant strains ywbN (mutant lacking *ywbN* gene), ywbN pGFP (producing
725 'unfused' GFP), ywbN pSpGFP (producing SpGFP), ywbN pYwbNGFP (producing
726 YwbN-GFP), ywbN AyCy pYwbNGFP (lacking TatAyCy and producing YwbN-GFP)
727 or ywbN AdCd pYwbNGFP (lacking TatAdCd and producing YwbN-GFP) were grown
728 for 7 hours in LB medium, supplemented with 1% NaCl . Protein loading was
729 corrected for OD_{600}. The positions of GFP, SpGFP, YwbNGFP, the secreted control
730 protein LipA, and the cytoplasmic lysis marker TrxA are indicated with arrows.
731 Positions of Mw markers are indicated on the left.
732
733 **Fig. 4. Fluorescence microscopic analysis of GFP, SpGFP and YwbNGFP**
734 **production.** Cells of *B. subtilis* 168 producing GFP, GFP fused to the signal peptide
735 of YwbN (SpGFP) or the YwbN-GFP fusion protein were grown in LB medium with
736 1% NaCl till an OD_{600} of 1.0. The strains were then induced with subtilin by the
737 addition of spent medium from *B. subtilis* ATCC6633 (1% v/v) and grown for 2
738 additional hours. After this time period cells were spotted onto M9 agarose slides with
739 1% NaCl and analyzed by phase contrast and fluorescence microscopy.
740
741 **Fig. 5. Secretion of AmiA-GFP, DmsA-GFP or MdoD-GFP by cells grown in**
742 **Phosphate starvation conditions.**
743 Cell and growth medium fractions of *B. subtilis* strains producing AmiA-GFP, DmsA-
744 GFP or MdoD-GFP (**A**), PhoD (**B**), or PhoB (**C**) were separated by centrifugation and
745 used for SDS-PAGE and Western blotting with specific antibodies. For this purpose,
746 the cells of *tatAyCy*, *tatAdCd* or total-*tat* mutant strains or the parental strain 168
747 were grown for 7 hours in LPDM medium. Protein loading was corrected for OD_{600}.
748 Lanes are labelled as in Figure 2, and the positions of precursor and mature forms of
749 PhoD and PhoB are marked with arrows. Positions of Mw markers are indicated on

750 the left. Note that PhoD and PhoB are produced through expression of the authentic
751 genes from their own promoters.
752
753 **Fig. 6. Secretion of AmiA-GFP, DmsA-GFP or MdoD-GFP by cells grown in LB**
754 **medium with 6% NaCl.** Cell and growth medium fractions of *B. subtilis* strains
755 producing AmiA-GFP, DmsA-GFP or MdoD-GFP (**A**), or YwbN-Myc (**B**) were
756 separated by centrifugation and used for SDS-PAGE and Western blotting with
757 specific antibodies. For this purpose, the cells of *tatAyCy*, *tatAdCd* or total-*tat* mutant
758 strains or the parental strain 168 were grown for 7 hours in LB medium,
759 supplemented with 6% NaCl. Protein loading was corrected for OD_{600}. Lanes are
760 labelled as in Figure 2, and the positions of precursor and mature forms of GFP and
761 YwbN-Myc are marked with arrows. Positions of Mw markers are indicated on the
762 left.
763
764 **Fig. 7. Fluorescence microscopic analysis of AmiA-GFP, DmsA-GFP or MdoD-**
765 **GFP production by cells grown in LB medium with 6% NaCl.** Cells of *B. subtilis*
766 168 producing AmiA-GFP (AmiA), DmsA-GFP (DmsA), MdoD-GFP (MdoD) or no
767 GFP (strain containing the empty vector pHB201) were grown in LB medium with 6%
768 NaCl for 7 h. Cells were spotted onto M9 agarose slides with 6% NaCl and analyzed
769 by phase contrast and fluorescence microscopy. The cellular fluorescence values
770 indicated in the fluorescence panels were determined as arbitrary grey scale units of
771 the cells and have been corrected for average background fluorescence. Please note
772 that the production levels of AmiA-GFP, DmsA-GFP, and MdoD-GFP are much lower
773 than the production levels of the subtilin-induced GFP constructs shown in Figure 4.
774
775 **Fig. 8. Fluorescence microscopic analysis of GFP production by cells grown in**
776 **LB medium with 1% or 6% NaCl.** Cells of *B. subtilis* 168 (pGFP) producing
777 'unfused' GFP were grown in LB medium with 1% or 6% NaCl till an OD_{600} of 1.0.

778 The strains were then induced with subtilin by the addition of spent medium from *B.*
779 *subtilis* ATCC6633 (1% v/v) and grown for 2 additional hours. After this time period
780 cells were spotted onto M9 agarose slides with 1% or 6% NaCl and analyzed by
781 fluorescence microscopy.
782

Tables

Table 1. Strains and Plasmids used in this study.

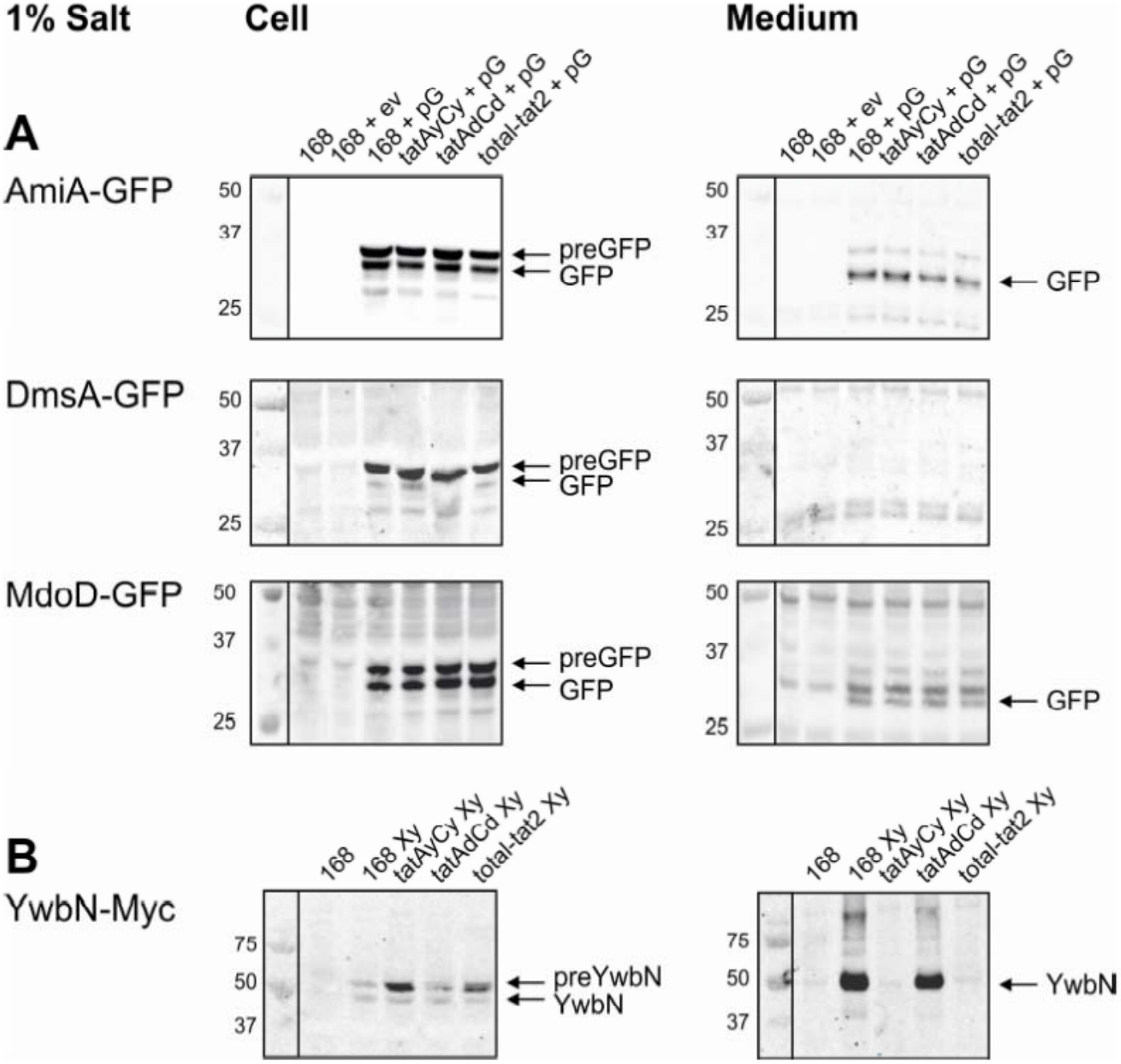
Plasmids	Relevant properties	Reference
pHB201	*B. subtilis-E.coli* expression vector; ori-pBR322; ori-pTA1060; *cat86::lacZa*; Cm^R; Em^R	[10]
pHB-AmiA-GFP	pHB201 vector carrying the *amiA-gfp* hybrid *gene*; Cm^R; Em^R	This study
pHB-DmsA-GFP	pHB201 vector carrying the *dmsA-gfp* hybrid *gene*; Cm^R; Em^R	This study
pHB-MdoD-GFP	pHB201 vector carrying the *mdoD-gfp* hybrid gene; Cm^R; Em^R	This study
pSG1554	bla *amyE3′* spc Pxyl–′gfpmut1 *amyE5′*	[29]
pNZ8910	SURE expression vector, PspaS, Em^R	[9]
pSG1554-SpYwbN	pSG1154 vector carrying the signal sequence of *ywbN* fused to gfpmut1; Ap^R; Sp^R	This study
pSG1554-YwbN	pSG1154 vector carrying *ywbN* fused to gfpmut1; Ap^R; Sp^R	This study
pSURE-SpYwbN-GFP	pNZ8910 vector carrying the *ywbN* signal sequence-*gfp* gene fusion; Em^R	This study
pSURE-YwbN-GFP	pNZ8910 vector carrying the *ywbN-gfp* gene fusion; Em^R	This study
pGFP	Originally known as pNZ8907; P_{spaS} translationally fused to *gfp*; only the full-size GFP is produced; Em^R	[9]
Strains		
E. coli		
DH5α	*supE44*; *hsdR17*; *recA1*; *gyrA96*; *thi-1*; *relA1*	[44]
L.Lactis		
MG1363	Plasmid-free derivative of NCDO 712	[18]
B. subtilis		
168	*trpC2*	[2]
ATCC6633	Subtilin producer	[9]
tatAyCy	*trpC2*; *tatAy-tatCy*::Sp; Sp^R	[21]
tatAdCd	*trpC2*; *tatAd-tatCd*::Km; Km^R;	[22]
tatAdCd	*trpC2*; *tatAd-tatCd*::Cm; Cm^R;	[21]
total-tat_2	*trpC2*; *tatAd-tatCd*::Km; Km^R; *tatAy-tatCy*::Sp; Sp^R; tatAc::Em; Em^R	[22]
ywbN	*trpC2*; *ywbN*::Phleo; $Phleo^R$	This study
ywbN spaRK	*trpC2*; *ywbN*:: Phleo; $Phleo^R$;*amyE*:: *spaRK*, Km^R	This study
tatAyCy ywbN spaRK	*trpC2*; *ywbN*:: Phleo; $Phleo^R$;*amyE*:: *spaRK*, Km^R; *tatAy-tatCy*::Sp; Sp^R	This study
tatAdCd ywbN spaRK	*trpC2*; *ywbN*:: Phleo; $Phleo^R$;*amyE*:: *spaRK*, Km^R; *tatAd-tatCd*::Cm; Cm^R	This study
ywbN pGFP	*trpC2*; *ywbN*:: Phleo; $Phleo^R$;*amyE*:: *spaRK*, Km^R; pNZ8907	This study
ywbN pSURE-SpYwbN-GFP	*trpC2*; *ywbN*:: Phleo; $Phleo^R$;*amyE*:: *spaRK*, Km^R; pSURE-SpYwbN-GFP Em^R	This study
ywbN pSURE-YwbN-GFP	*trpC2*; *ywbN*:: Phleo; $Phleo^R$;*amyE*:: *spaRK*, Km^R; pSURE-YwbN-GFP Em^R	This study
AyCy ywbN pSURE-YwbN-GFP	*trpC2*; *ywbN*:: Phleo; $Phleo^R$;*amyE*:: *spaRK*, Km^R; pSURE-SpYwbN-GFP Em^R; *tatAy-tatCy*::Sp; Sp^R;	This study
AdCd ywbN pSURE-YwbN-GFP	*trpC2*; *ywbN*:: Phleo; $Phleo^R$;*amyE*:: *spaRK*, Km^R; pSURE-SpYwbN-GFP Em^R; *tatAd-tatCd*::Cm; Cm^R;	This study
168 *XywbN*	*trpC2; amyE*::*xylA-ywbN-myc*; Cm^R	[22]
tatAyCy *XywbN*	*trpC2*; *tatAy-tatCy*::Sp; Sp^R; *amyE*::*xylA-ywbN-myc*; Cm^R	[22]
tatAdCd *XywbN*	*trpC2*; *tatAd-tatCd*::Km; Km^R; *amyE*::*xylA-ywbN-myc*; Cm^R	[22]
total-tat_2 *XywbN*	*trpC2*; *tatAd-tatCd*::Km; Km^R; *tatAy-tatCy*::Sp; Sp^R; tatAc::Em; Em^R; *amyE*::*xylA-ywbN-myc*; Cm^R	[22]
168 pHB201	*trpC2;* pHB201; Em^R; Cm^R	This study
168	*trpC2;* pHB-AmiA-GFP; Em^R; Cm^R	This study

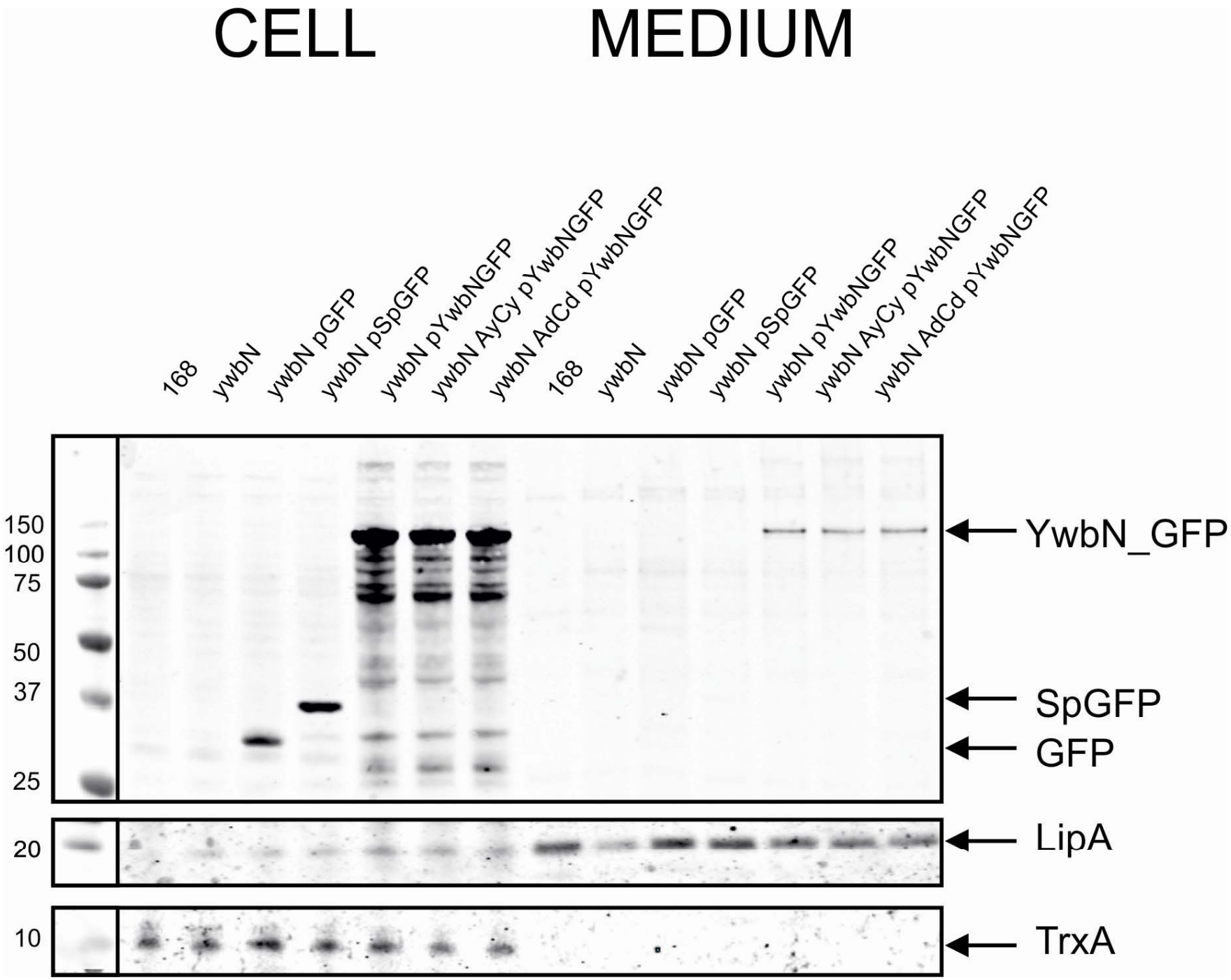
pHB-AmiA-GFP tatAyCy	*trpC2*; *tatAy-tatCy*::Sp; pHB-AmiA-GFP; Sp^R; Em^R; Cm^R	This study
pHB-AmiA-GFP tatAdCd	*trpC2*; *tatAd-tatCd*::Km; pHB-AmiA-GFP; Km^R; Em^R; Cm^R	This study
pHB-AmiA-GFP total-tat_2	*trpC2*; *tatAd-tatCd*::Km; *tatAy-tatCy*::Sp; tatAc::Em; pHB-AmiA-GFP Km^R; Sp^R; Em^R; Cm^R	This study
pHB-AmiA-GFP 168	*trpC2;* pHB-DmsA-GFP; Em^R; Cm^R	This study
pHB-DmsA-GFP tatAyCy	*trpC2*; *tatAy-tatCy*::Sp; pHB-DmsA-GFP; Sp^R; Em^R; Cm^R	This study
pHB-DmsA-GFP tatAdCd	*trpC2*; *tatAd-tatCd*::Km; pHB-DmsA-GFP; Km^R; Em^R; Cm^R	This study
pHB-DmsA-GFP total-tat_2	*trpC2*; *tatAd-tatCd*::Km; *tatAy-tatCy*::Sp; tatAc::Em; pHB-DmsA-GFP Km^R; Sp^R; Em^R; Cm^R	This study
pHB-DmsA-GFP 168	*trpC2;* pHB-MdoD-GFP; Em^R; Cm^R	This study
pHB-MdoD-GFP tatAyCy	*trpC2*; *tatAy-tatCy*::Sp; pHB-MdoD-GFP; Sp^R; Em^R; Cm^R	This study
pHB-MdoD-GFP tatAdCd	*trpC2*; *tatAd-tatCd*::Km; pHB-MdoD-GFP; Km^R; Em^R; Cm^R	This study
pHB-MdoD-GFP total-tat_2	*trpC2*; *tatAd-tatCd*::Km; *tatAy-tatCy*::Sp; tatAc::Em; pHB-MdoD-GFP Km^R; Sp^R; Em^R; Cm^R	This study

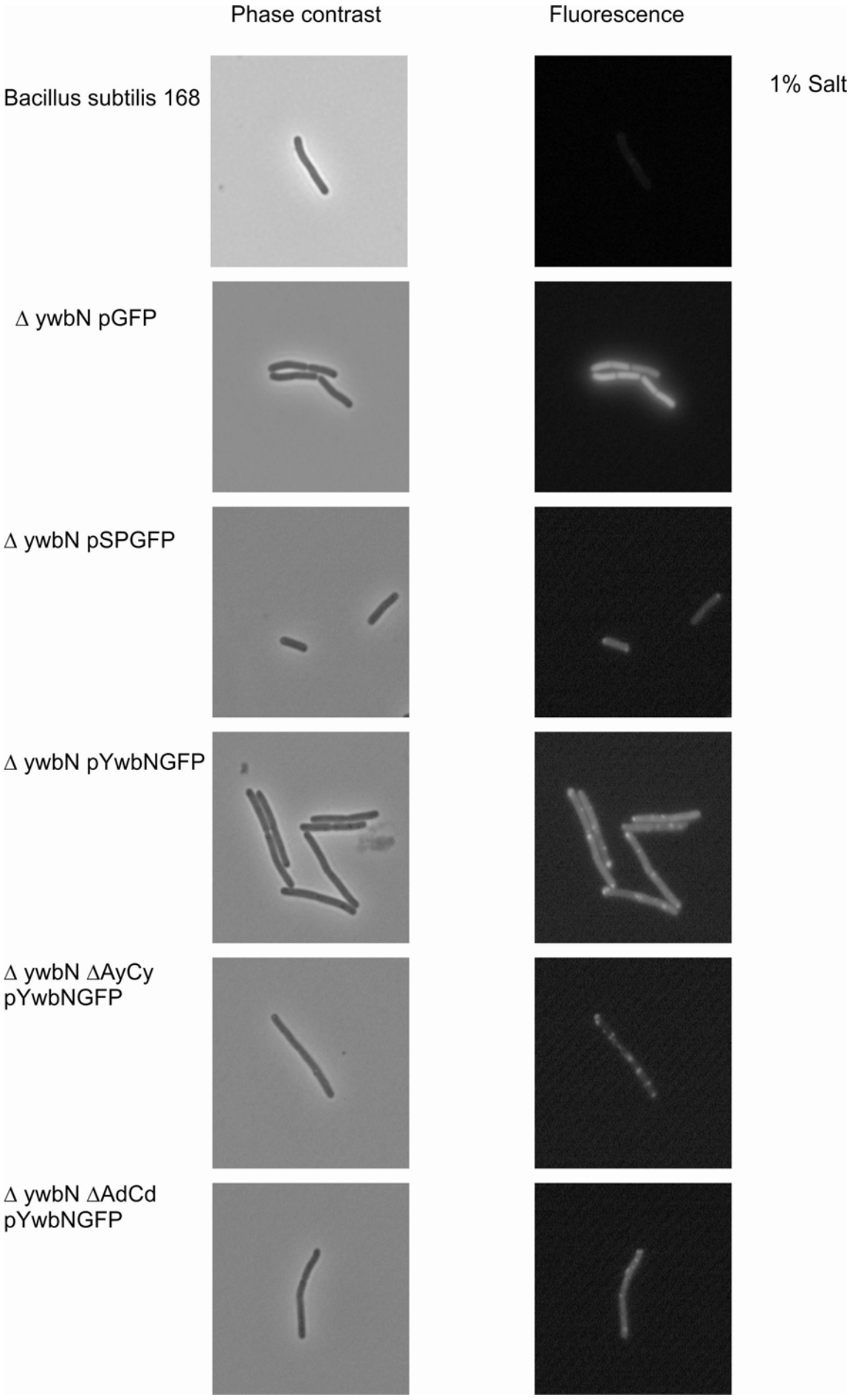
Table 2 Primers used in this study

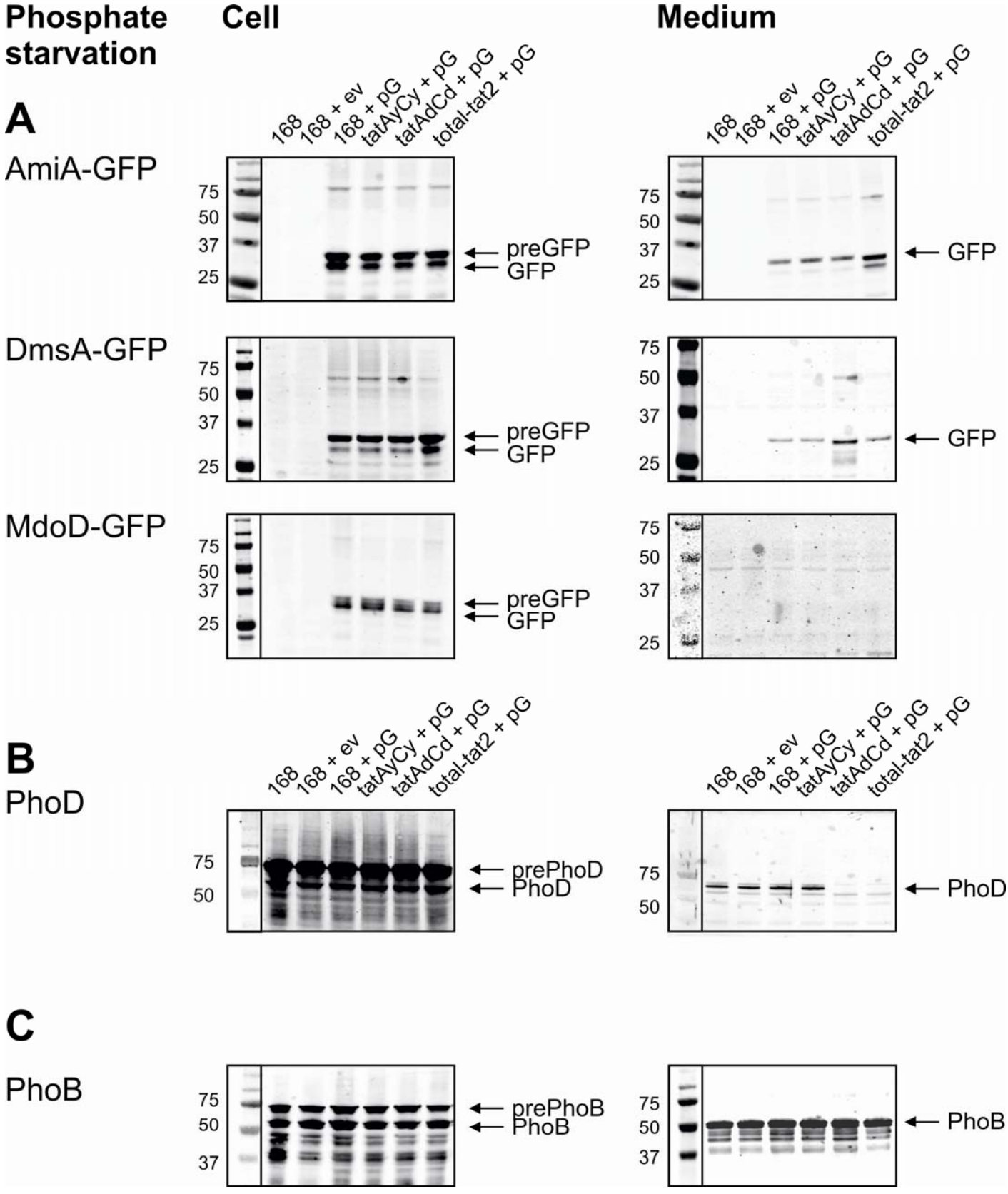
Primer	Sequence	Remarks
RBS-MntA-AmiA-F	GGGGGACTAGTAAGAGGAGGAGAAAT *ATGAGCACTTTTAAACCACTA*	*Spe*I, RBS *mntA* start *amiA*
RBS-MntA-DmsA-F	GGGGGACTAGTAAGAGGAGGAGAAAT *ATGAAAACGAAAATCCCTGAT*	*Spe*I, RBS *mntA* start *dmsA*
SpeI-MntA-MdoD-F	GGGGGACTAGTAAGAGGAGGAGAAAT *ATGGATCGTAGACGATTTATT*	*Spe*I, RBS *mntA* start *mdoD*
GFP-Rev-BamHI	CCCCCGGATCCTTATTTGTATAGTTCATCCATGC	*Bam*HI, end *gfp*
YwbN_LW-F	GGCGGTACCATGAGCGATGAACAGAAAAAGCCAGAACAA	*KpnI*
SPywbN_LW-R	GGGGAATTCAACAAGCGGAGCGAGACCGCC	*Eco*RI
YwbN_LW-R	GGGGGAATTCTGATTCCAGCAAACGCTG	*Eco*RI
F-YwbN-SURE	GGGGGTCATGAGCGATGAACAGAAAAAGCCAGAACAAATTC	*RcaI*
GFP-Rev-HindIII	GCCCAAGCTTATTATTTGTAGAGCTCATCCATGCCATGTG	*HindIII*, end *gfpmut1*

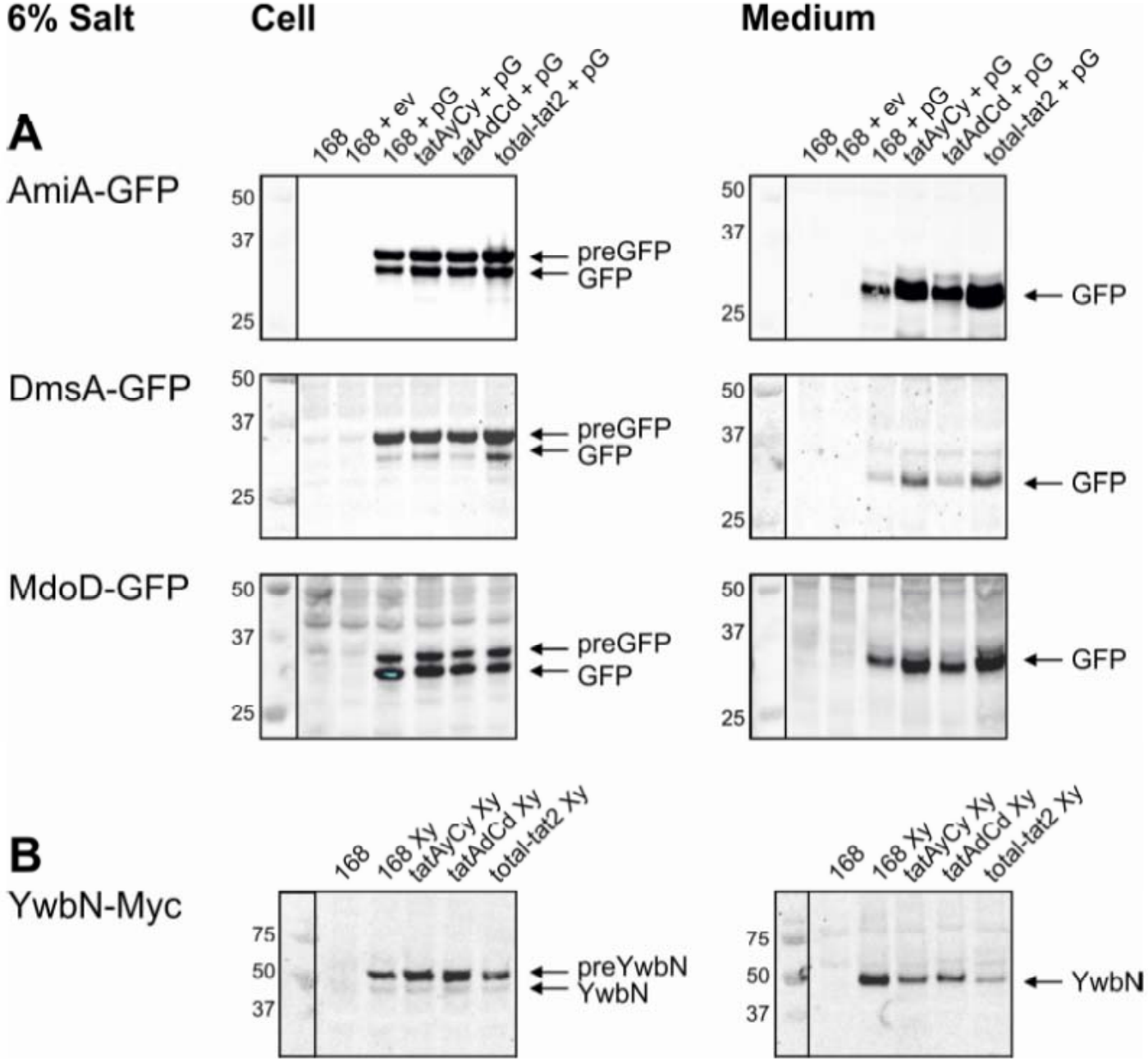

AmiA MSTFKPLKTLTSRRQVLKAGLAALTLSGMSQAIAKDELLKTSNGHS
DmsA MKTKIPDAVLAAEVSRRGLVKTTAIGGLAMASSALTLPFSRIAHAV
MdoD MDRRRFIKGSMAMAAVCGTSGIASLFSQAAFAADSDIADGQTQRFD
YwbN MSDEQKKPEQIHRRDILKWGAMAGAAVAIGASGLGGLAPLVQTAAK
PhoD MAYDSRFDEWVQKLKEESFQNNTFDRRKFIQGAGKIAGLSLGLTIAQSVGAFE


1% Salt
Cell
Medium
A
AmiA-GFP
DmsA-GFP
MdoD-GFP
168
168 + ev
168 + pG
tatAyCy + pG
tatAdCd + pG
total-tat2 + pG
50
37
25
preGFP
GFP
B
YwbN-Myc
168 Xy
tatAyCy Xy
tatAdCd Xy
total-tat2 Xy
75
preYwbN
YwbN


CELL
MEDIUM
168
ywbN
ywbN pGFP
ywbN pSpGFP
ywbN pYwbNGFP
ywbN AyCy pYwbNGFP
ywbN AdCd pYwbNGFP
168
ywbN
ywbN pGFP
ywbN pSpGFP
ywbN pYwbNGFP
ywbN AyCy pYwbNGFP
ywbN AdCd pYwbNGFP
150
100
75
50
37
25
20
10
YwbN_GFP
SpGFP
GFP
LipA
TrxA

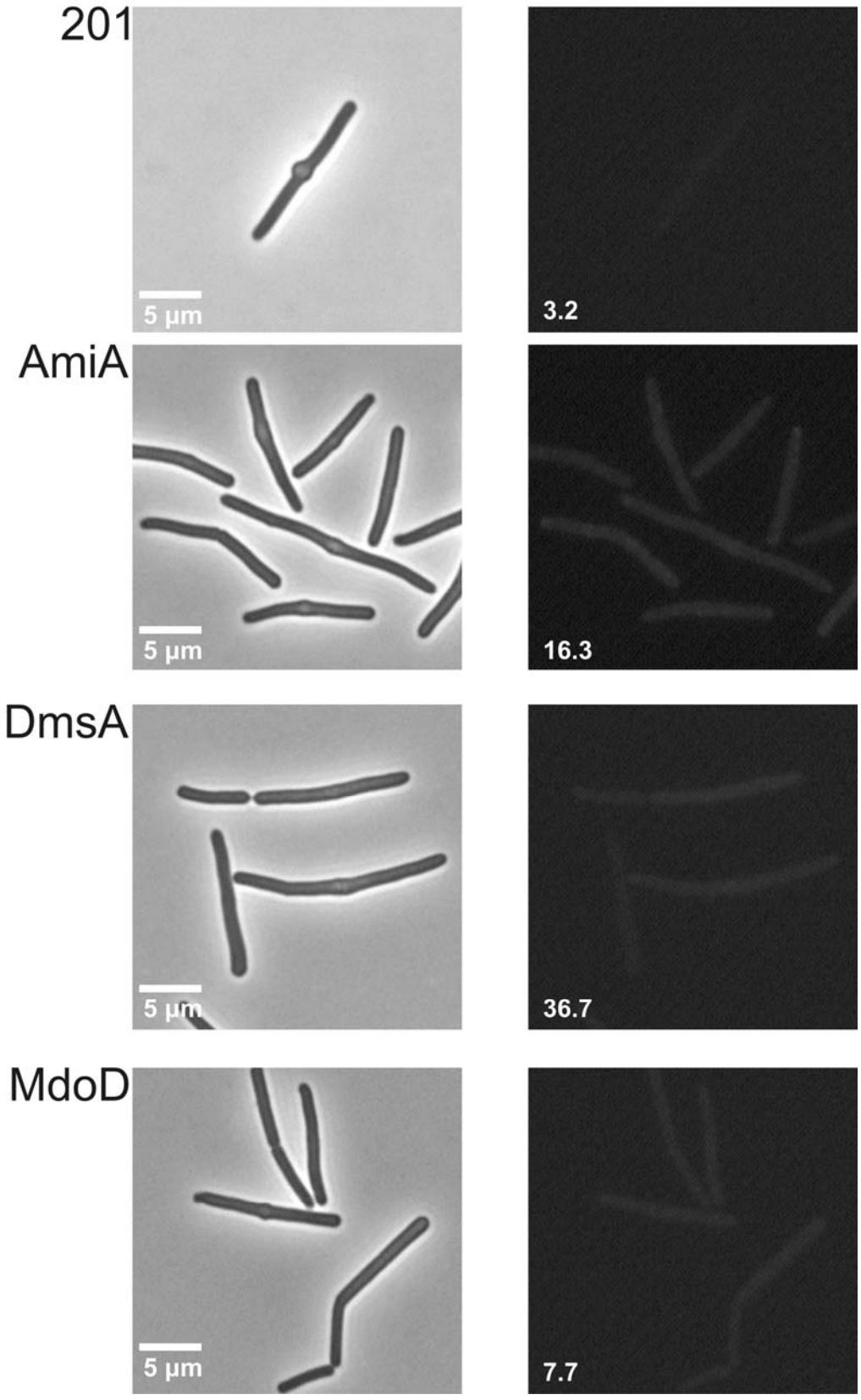

Phase contrast
Fluorescence
Bacillus subtilis 168
1% Salt
Δ ywbN pGFP
Δ ywbN pSPGFP
Δ ywbN pYwbNGFP
Δ ywbN ΔAyCy pYwbNGFP
Δ ywbN ΔAdCd pYwbNGFP


Phosphate starvation
Cell
Medium
A
AmiA-GFP
DmsA-GFP
MdoD-GFP
168
168 + ev
168 + pG
tatAyCy + pG
tatAdCd + pG
total-tat2 + pG
75
50
37
25
preGFP
GFP
B
PhoD
prePhoD
PhoD
C
PhoB
prePhoB
PhoB

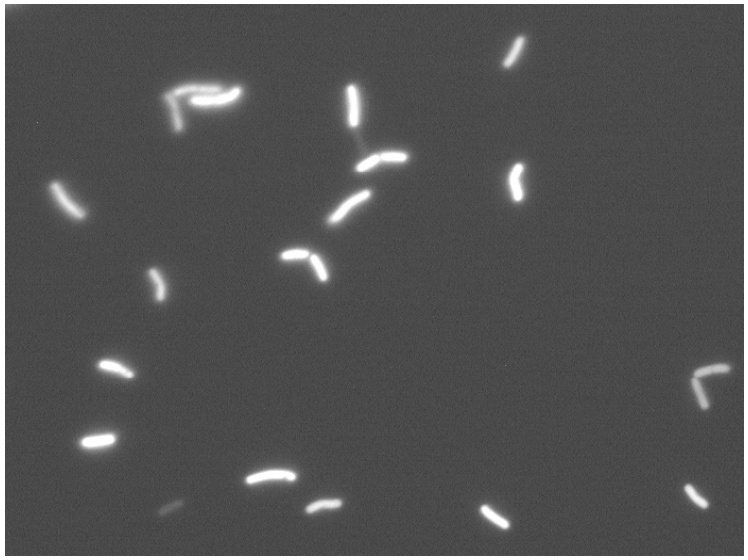

6% Salt
Cell
Medium
A
AmiA-GFP
DmsA-GFP
MdoD-GFP
168
168 + ev
168 + pG
tatAyCy + pG
tatAdCd + pG
total-tat2 + pG
50
37
25
preGFP
GFP
B
YwbN-Myc
168 Xy
tatAyCy Xy
tatAdCd Xy
total-tat2 Xy
75
preYwbN
YwbN

168

6% Salt


Phase contrast
Fluorescence
201
AmiA
DmsA
MdoD
5 µm
5 µm
5 µm
5 µm
3.2
16.3
36.7
7.7

1% NaCL



6% NaCL