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

3

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21

22    **Running title:** Salt-suppressed Sec avoidance in *Bacillus*

23    **Key words:** *Bacillus subtilis*, GFP, Tat, AmiA, DmsA, MdoD, YwbN

24

25 **Abstract** (250 max)

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

48

## 49 Introduction

50

51 Protein secretion is an important feature for the survival and competitive success of  
52 bacterial cells in their natural habitats. The ability to secrete proteins is particularly  
53 well developed in the Gram-positive bacterium *Bacillus subtilis*, which is of interest  
54 both from applied and fundamental scientific points of view [3, 47, 48, 51]. Combined  
55 genetic, proteomic and bioinformatic analyses have revealed that the vast majority of  
56 proteins secreted by *B. subtilis* leave the cytoplasm in an unfolded state via the  
57 general secretion (Sec) pathway [47]. Upon translocation these proteins fold into their  
58 active and protease-resistant conformation [19]. A limited number of proteins are  
59 secreted via the so-called twin-arginine (Tat) pathway which, in contrast to the Sec  
60 pathway, can facilitate the transport of fully folded proteins [16, 35, 37, 38, 42, 45,  
61 53].

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

77 features, but also through their rapid or controlled folding in the cytoplasm prior to  
78 translocation [15, 39]. In fact, some Tat-dependently exported proteins are subject to  
79 dedicated chaperone-mediated proofreading in the cytoplasm in order to prevent the  
80 initiation of their transport before folding or co-factor assembly have been completed  
81 [30, 38, 40, 43].

82 *B. subtilis* contains two independently working Tat translocases named  
83 TatAyCy and TatAdCd, which are of the TatAC type that is commonly found in Gram-  
84 positive bacteria [21, 22, 23]. Unlike the TatABC type translocases that are present in  
85 Gram-negative bacteria, these “minimal” TatAC translocases lack a TatB subunit [4,  
86 5, 24]. In *B. subtilis*, the TatAyCy and TatAdCd translocases have distinct  
87 specificities for the Dyp-type peroxidase YwbN and the phosphodiesterase PhoD  
88 respectively, at least when the cells are grown in a standard LB medium [21, 22, 23].  
89 Also, a hybrid precursor of the subtilisin AprE fused to the YwbN signal peptide was  
90 secreted in a TatAyCy-specific manner, suggesting a preferential interaction between  
91 the YwbN signal peptide and the TatAyCy translocase [25]. Nevertheless, the  
92 specificities of TatAyCy and TatAdCd overlap at least to some extent as was recently  
93 shown by the heterologous expression of TatAdCd or TatAyCy in *Escherichia coli*  
94 strains lacking their own TatABC translocase [4, 5]. The latter studies revealed that  
95 both *B. subtilis* Tat translocases are able to translocate the green fluorescent protein  
96 (GFP) fused to the RR-signal peptides of the *E. coli* AmiA, DmsA or MdoD proteins  
97 (Fig. 1). A specificity difference was, however, observed as the TMAO reductase  
98 (TorA) and a TorA-GFP fusion were transported by TatAdCd but not by TatAyCy [4,  
99 5].

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

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

115

## 116 **Materials and Methods**

117

### 118 *Plasmids, bacterial strains, media and growth conditions*

119 The plasmids and bacterial strains used in this study are listed in Table 1. Strains  
120 were grown with agitation at 37°C in either Lysogeny Broth (LB), or Paris minimal  
121 (PM) medium. LB medium consisted of 1% tryptone and 0.5% yeast extract with or  
122 without NaCl (1% or 6%), pH 7.4. Notably, LB with 1% NaCl is the standard LB  
123 medium that has been used in all our previous studies. PM consisted of 10.7 mg ml<sup>-1</sup>  
124 K<sub>2</sub>HPO<sub>4</sub>, 6 mg ml<sup>-1</sup> KHPO, 1 mg ml<sup>-1</sup> trisodium citrate, 0.02 mg ml<sup>-1</sup> MgSO<sub>4</sub>, 1%  
125 glucose, 0.1% casamino acids (Difco), 20 mg ml<sup>-1</sup> L-tryptophan, 2.2 mg ml<sup>-1</sup> ferric  
126 ammonium citrate and 20 mM potassium glutamate. To activate a phosphate  
127 starvation response and, accordingly, induce the expression of the TatAdCd  
128 translocase, the strains were grown overnight in HPDM (high phosphate defined  
129 medium), which is rich in phosphate. The next morning, cells were transferred to  
130 LPDM (low phosphate defined medium). Both media were prepared according to  
131 Müller *et al.* (1997) [34]. *Lactococcus lactis* was grown at 30°C in M17 broth  
132 supplemented with 0.5% glucose. When required, media for *E. coli* were

133 supplemented with erythromycin (Em; 100  $\mu\text{g ml}^{-1}$ ), kanamycin (Km; 20  $\mu\text{g ml}^{-1}$ ),  
134 chloramphenicol (Cm; 5  $\mu\text{g ml}^{-1}$ ), or spectinomycin (Sp; 100  $\mu\text{g ml}^{-1}$ ); media for *B.*  
135 *subtilis* were supplemented with Em (1  $\mu\text{g ml}^{-1}$ ), Km (20  $\mu\text{g ml}^{-1}$ ), Cm (5  $\mu\text{g ml}^{-1}$ ),  
136 Phleomycin (Phleo; 4  $\mu\text{g ml}^{-1}$ ) or Sp (100  $\mu\text{g ml}^{-1}$ ); media for *L. lactis* were  
137 supplemented with Em (2  $\mu\text{g ml}^{-1}$ ).

138

### 139 *DNA techniques*

140 Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and  
141 transformation of competent *E. coli* cells were carried out as previously described  
142 [44]. *B. subtilis* was transformed as described by Kunst and Rapoport [28]. PCR was  
143 carried out with the Pwo DNA polymerase. PCR products were purified using the  
144 PCR purification kit from Roche. Restriction enzymes were obtained from New  
145 England Biolabs. Plasmid DNA from *E. coli* was isolated using the alkaline lysis  
146 method [44], or the Invisorb®Plasmid Isolation Kit (Invitex). All constructs were  
147 checked by sequencing (serviceXS, Leiden the Netherlands).

148 To construct the plasmids pHB-AmiA-GFP, pHB-DmsA-GFP and pHB-MdoD-  
149 GFP, the *amiA-gfp*, *dmsA-gfp* and *mdoD-gfp* hybrid genes were PCR-amplified from  
150 the respective pBAD24-based plasmids carrying these genes [5] (Table 1). The 5'  
151 primers used for PCR contained the *mntA* ribosome-binding site and start codon, as  
152 well as a *SpeI* restriction site, and the 3' primer contained a *BamHI* restriction site  
153 (Table 2). The resulting PCR products were cleaved with *SpeI* and *BamHI*, and  
154 ligated to *SpeI-BamHI*-cleaved pHB201. Ligation mixtures were used to transform *E.*  
155 *coli*, resulting in the identification of plasmids pHB-AmiA-GFP, pHB-DmsA-GFP and  
156 pHB-MdoD-GFP. Next, these plasmids were used to transform the *B. subtilis* strains  
157 168, *tatAyCy*, *tatAdCd* and total-*tat*<sub>2</sub>. To construct the plasmids pSURE-SpYwbN-  
158 GFP and pSURE-YwbN-GFP, the *ywbN* signal sequence and the full-length *ywbN*  
159 gene were PCR-amplified from chromosomal DNA of *B. subtilis* 168. The 5' primer

160 used for PCR contained a *KpnI* restriction site, and the 3' primer contained a *HindIII*  
161 restriction site (Table 2). The resulting PCR products were cleaved with *KpnI* and  
162 *HindIII*, and ligated to *KpnI-HindIII*-cleaved pSG1154 [29], which contains the  
163 *gfpmut1* gene. The fusion products Sp(YwbN)-GFP and YwbN-GFP were then  
164 amplified from these vectors using a 5' primer containing a *BspHI* restriction site and  
165 a 3' primer containing a *HindIII* restriction site, and they were cloned into the *NcoI*-  
166 *HindIII*-cleaved pNZ8910 plasmid. Ligation mixtures were used to transform *L. lactis*,  
167 resulting in the isolation of plasmids pSURE-SpYwbN-GFP and pSURE-YwbN-GFP.  
168 The plasmids were then used to transform the *B. subtilis ywbN*, *tatAyCy ywbN* or  
169 *tatAdCd ywbN* strains.

170

#### 171 *SDS-PAGE and Western blotting*

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

184

#### 185 *Fluorescence microscopy*

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



188 density at 600 nm ( $OD_{600}$ ) was measured. The strains containing pGFP, pSURE-  
189 SpYwbN-GFP or pSURE-YwbN-GFP were grown till an  $OD_{600}$  of 1.0, induced with  
190 1.0% (v/v) supernatant of *B. subtilis* ATCC 6633. In this respect it is noteworthy that  
191 the subtilin produced by *B. subtilis* ATCC6633 is secreted into its growth medium.  
192 Addition of this spent medium in a 100-fold dilution to *B. subtilis* cells containing  
193 pGFP, pSURE-SpYwbN-GFP or pSURE-YwbN-GFPI induces the *spaS* promoter on  
194 these plasmids thereby driving the high-level transcription of the downstream GFP  
195 genes. Upon growth for 2 additional hours, cells were spotted on M9 agarose slides  
196 containing the appropriate salt concentrations. These slides were prepared by  
197 transfer of M9 agarose medium into a 65  $\mu$ l Frame-Seal Slide Chamber (SLF-0601,  
198 Bio-Rad). Fluorescence microscopy was performed with a Leica DM5500 B  
199 microscope. Fluorescence images were recorded using a Leica EL6000 lamp with  
200 the intensity set to 55%. The exposure time was 256 ms. Quantification of GFP  
201 fluorescence was done using the ImageJ software package (<http://rsbweb.nih.gov/ij/>).  
202 Cellular fluorescence values were measured in grey scale values. Background  
203 fluorescence was calculated by averaging the grey scale values of the area outside  
204 the cells. Finally the background fluorescence was subtracted from the cellular  
205 fluorescence.

206

## 207 **Results**

208

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

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

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

244

245 *Rejection of the chimeric YwbN-GFP protein by Tat*

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

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

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

273

#### 274 *Phosphate starvation conditions result in Tat-independent GFP secretion*

275 Studies on the *B. subtilis* Tat translocases (following expression in both *B. subtilis*  
276 and *E. coli*) have shown that the TatAdCd translocase is the most permissive of the  
277 two translocases present in *B. subtilis* [4, 17]. However, production of the TatAdCd  
278 complex of *B. subtilis* is fully induced only under phosphate starvation conditions [23,  
279 36]. We thus investigated whether this translocase can facilitate the secretion of  
280 AmiA-GFP, DmsA-GFP or MdoD-GFP under conditions of phosphate starvation. As  
281 shown in Figure 5, all three precursors were produced by cells grown in LPDM  
282 medium with the cells also containing mature GFP in varying amounts. Furthermore,  
283 secretion of mature-sized GFP was observed in the AmiA-GFP- and DmsA-GFP-  
284 producing strains (Figure 5A, right panel). The secretion of GFP was however, mostly  
285 Tat-independent, since bands corresponding to mature-size GFP were detected in  
286 the medium of mutant strains lacking the *tatAyCy*, *tatAdCd*, or all *tat* genes. In  
287 contrast, no GFP secretion was observed for cells producing MdoD-GFP. In control  
288 experiments the secretion of PhoD was found to be dependent upon the production  
289 of the TatAdCd complex, as shown by the lack of PhoD secreted by the *tatAdCd* and  
290 total-*tat* mutant strains, in addition to the PhoD secretion observed in the strain  
291 lacking the *tatAyCy* genes. Furthermore, secretion of the Sec-dependent protein  
292 PhoB was not affected by any of the tested *tat* mutations. These findings show that  
293 induction of the TatAdCd translocase does not preclude the rejection of GFP by the  
294 *B. subtilis* Tat system.

295

#### 296 *High salinity growth conditions result in elevated levels of Tat-independent GFP* 297 *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,

326 DmsA-GFP or MdoD-GFP. Indeed Figure 7 shows that at least some of the GFP  
327 within cells producing AmiA-GFP, DmsA-GFP or MdoD-GFP is active when cells  
328 were grown in LB with 6% NaCl. Nevertheless, little if any GFP seems to be secreted  
329 by the Tat translocases of the respective cells. It should be noted here that the  
330 cellular GFP expression levels and fluorescence were not substantially different  
331 when cells were grown in LB with 1% or with 6% NaCl, suggesting that salt does not  
332 directly affect the folding state of cytoplasmic GFP (data not shown). This view is  
333 supported by the finding that cells producing the authentic GFP (without signal  
334 peptide) did not show significant differences in fluorescence upon growth in LB with  
335 1% or 6% NaCl (Figure 8).

336

### 337 **Discussion**

338

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

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

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

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

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

406         Analyses of cells producing AmiA-GFP, DmsA-GFP or MdoD-GFP by  
407 fluorescence microscopy showed that these cells contained little or no active GFP.  
408 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

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

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

459

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471 **Competing interests**

472 The authors declare that they have no competing interests

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475 **References**

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695 **Figure Legends**

696

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

702

703 **Fig. 2. Secretion of AmiA-GFP, DmsA-GFP or MdoD-GFP by cells grown in**  
704 **standard LB medium with 1% NaCl. A.** Cell and growth medium fractions of *B.*  
705 *subtilis* strains producing AmiA-GFP, DmsA-GFP or MdoD-GFP were separated by  
706 centrifugation and used for SDS-PAGE and Western blotting with specific antibodies.  
707 For this purpose, the cells of *tatAyCy*, *tatAdCd* or total-*tat* mutant strains or the  
708 parental strain 168 were grown for 7 hours in LB medium, supplemented with 1%  
709 NaCl. Protein loading was corrected for OD<sub>600</sub>. “pG”, cells harbouring pHB-AmiA-  
710 GFP, pHB-DmsA-GFP or pHB-MdoD-GFP; “ev”, cells harbouring the empty vector  
711 pHB201. **B.** Cell and growth medium fractions of *B. subtilis* strains producing YwbN-  
712 Myc were prepared for SDS-PAGE and Western blotting with specific antibodies as  
713 indicated for panel A. For this purpose, the cells of *tatAyCy*, *tatAdCd* or total-*tat*  
714 mutant strains or the parental strain 168 contained the *XywbN* cassette in *amyE*.  
715 “Xy”, cells containing the *XywbN* cassette.

716

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

718 Cell and growth medium fractions of *B. subtilis* strains producing GFP, GFP fused to  
719 the signal peptide of YwbN (SpGFP) or the fusion protein YwbN-GFP were separated  
720 by centrifugation and used for SDS-PAGE and Western blotting with specific  
721 monoclonal antibodies directed against GFP and polyclonal antibodies against  
722 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<sub>600</sub>. 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<sub>600</sub> 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<sub>600</sub>.  
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<sub>600</sub>. 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<sub>600</sub> 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	<i>B. subtilis</i> - <i>E. coli</i> expression vector; ori-pBR322; ori-pTA1060; <i>cat86::lacZa</i> ; Cm <sup>R</sup> ; Em <sup>R</sup>	[10]
pHB-AmiA-GFP	pHB201 vector carrying the <i>amiA-gfp</i> hybrid gene; Cm <sup>R</sup> ; Em <sup>R</sup>	This study
pHB-DmsA-GFP	pHB201 vector carrying the <i>dmsA-gfp</i> hybrid gene; Cm <sup>R</sup> ; Em <sup>R</sup>	This study
pHB-MdoD-GFP	pHB201 vector carrying the <i>mdoD-gfp</i> hybrid gene; Cm <sup>R</sup> ; Em <sup>R</sup>	This study
pSG1554	<i>bla amyE3'</i> spc P <sub>xyl</sub> - <i>gfpmut1 amyE5'</i>	[29]
pNZ8910	SURE expression vector, P <sub>spaS</sub> , Em <sup>R</sup>	[9]
pSG1554-SpYwbN	pSG1154 vector carrying the signal sequence of <i>ywbN</i> fused to <i>gfpmut1</i> ; Ap <sup>R</sup> ; Sp <sup>R</sup>	This study
pSG1554-YwbN	pSG1154 vector carrying <i>ywbN</i> fused to <i>gfpmut1</i> ; Ap <sup>R</sup> ; Sp <sup>R</sup>	This study
pSURE-SpYwbN-GFP	pNZ8910 vector carrying the <i>ywbN</i> signal sequence- <i>gfp</i> gene fusion; Em <sup>R</sup>	This study
pSURE-YwbN-GFP	pNZ8910 vector carrying the <i>ywbN-gfp</i> gene fusion; Em <sup>R</sup>	This study
pGFP	Originally known as pNZ8907; P <sub>spaS</sub> translationally fused to <i>gfp</i> ; only the full-size GFP is produced; Em <sup>R</sup>	[9]
<b>Strains</b>		
<b><i>E. coli</i></b>		
DH5α	<i>supE44; hsdR17; recA1; gyrA96; thi-1; relA1</i>	[44]
<b><i>L. Lactis</i></b>		
MG1363	Plasmid-free derivative of NCDO 712	[18]
<b><i>B. subtilis</i></b>		
168	<i>trpC2</i>	[2]
ATCC6633	Subtilin producer	[9]
tatAyCy	<i>trpC2; tatAy-tatCy::Sp; Sp<sup>R</sup></i>	[21]
tatAdCd	<i>trpC2; tatAd-tatCd::Km; Km<sup>R</sup></i>	[22]
tatAdCd	<i>trpC2; tatAd-tatCd::Cm; Cm<sup>R</sup></i>	[21]
total-tat <sub>2</sub>	<i>trpC2; tatAd-tatCd::Km; Km<sup>R</sup>; tatAy-tatCy::Sp; Sp<sup>R</sup>; tatAc::Em; Em<sup>R</sup></i>	[22]
ywbN	<i>trpC2; ywbN::Phleo; Phleo<sup>R</sup></i>	This study
ywbN spaRK	<i>trpC2; ywbN:: Phleo; Phleo<sup>R</sup>; amyE:: spaRK, Km<sup>R</sup></i>	This study
tatAyCy ywbN spaRK	<i>trpC2; ywbN:: Phleo; Phleo<sup>R</sup>; amyE:: spaRK, Km<sup>R</sup>; tatAy-tatCy::Sp; Sp<sup>R</sup></i>	This study
tatAdCd ywbN spaRK	<i>trpC2; ywbN:: Phleo; Phleo<sup>R</sup>; amyE:: spaRK, Km<sup>R</sup>; tatAd-tatCd::Cm; Cm<sup>R</sup></i>	This study
ywbN pGFP	<i>trpC2; ywbN:: Phleo; Phleo<sup>R</sup>; amyE:: spaRK, Km<sup>R</sup>; pNZ8907</i>	This study
ywbN pSURE-SpYwbN-GFP	<i>trpC2; ywbN:: Phleo; Phleo<sup>R</sup>; amyE:: spaRK, Km<sup>R</sup>; pSURE-SpYwbN-GFP Em<sup>R</sup></i>	This study
ywbN pSURE-YwbN-GFP	<i>trpC2; ywbN:: Phleo; Phleo<sup>R</sup>; amyE:: spaRK, Km<sup>R</sup>; pSURE-YwbN-GFP Em<sup>R</sup></i>	This study
AyCy ywbN	<i>trpC2; ywbN:: Phleo; Phleo<sup>R</sup>; amyE:: spaRK, Km<sup>R</sup>; pSURE-SpYwbN-GFP Em<sup>R</sup>; tatAy-tatCy::Sp; Sp<sup>R</sup></i>	This study
pSURE-YwbN-GFP		
AdCd ywbN	<i>trpC2; ywbN:: Phleo; Phleo<sup>R</sup>; amyE:: spaRK, Km<sup>R</sup>; pSURE-SpYwbN-GFP Em<sup>R</sup>; tatAd-tatCd::Cm; Cm<sup>R</sup></i>	This study
pSURE-YwbN-GFP		
168 XywbN	<i>trpC2; amyE::xylA-ywbN-myc; Cm<sup>R</sup></i>	[22]
tatAyCy XywbN	<i>trpC2; tatAy-tatCy::Sp; Sp<sup>R</sup>; amyE::xylA-ywbN-myc; Cm<sup>R</sup></i>	[22]
tatAdCd XywbN	<i>trpC2; tatAd-tatCd::Km; Km<sup>R</sup>; amyE::xylA-ywbN-myc; Cm<sup>R</sup></i>	[22]
total-tat <sub>2</sub> XywbN	<i>trpC2; tatAd-tatCd::Km; Km<sup>R</sup>; tatAy-tatCy::Sp; Sp<sup>R</sup>; tatAc::Em; Em<sup>R</sup>; amyE::xylA-ywbN-myc; Cm<sup>R</sup></i>	[22]
168 pHB201	<i>trpC2; pHB201; Em<sup>R</sup>; Cm<sup>R</sup></i>	This study
168	<i>trpC2; pHB-AmiA-GFP; Em<sup>R</sup>; Cm<sup>R</sup></i>	This study

pHB-AmiA-GFP tatAyCy	<i>trpC2; tatAy-tatCy::Sp; pHB-AmiA-GFP; Sp<sup>R</sup>, Em<sup>R</sup>, Cm<sup>R</sup></i>	This study
pHB-AmiA-GFP tatAdCd	<i>trpC2; tatAd-tatCd::Km; pHB-AmiA-GFP; Km<sup>R</sup>, Em<sup>R</sup>, Cm<sup>R</sup></i>	This study
pHB-AmiA-GFP total-tat <sub>2</sub>	<i>trpC2; tatAd-tatCd::Km; tatAy-tatCy::Sp; tatAc::Em; pHB-AmiA-GFP Km<sup>R</sup>, Sp<sup>R</sup>, Em<sup>R</sup>, Cm<sup>R</sup></i>	This study
pHB-AmiA-GFP 168	<i>trpC2; pHB-DmsA-GFP; Em<sup>R</sup>, Cm<sup>R</sup></i>	This study
pHB-DmsA-GFP tatAyCy	<i>trpC2; tatAy-tatCy::Sp; pHB-DmsA-GFP; Sp<sup>R</sup>, Em<sup>R</sup>, Cm<sup>R</sup></i>	This study
pHB-DmsA-GFP tatAdCd	<i>trpC2; tatAd-tatCd::Km; pHB-DmsA-GFP; Km<sup>R</sup>, Em<sup>R</sup>, Cm<sup>R</sup></i>	This study
pHB-DmsA-GFP total-tat <sub>2</sub>	<i>trpC2; tatAd-tatCd::Km; tatAy-tatCy::Sp; tatAc::Em; pHB-DmsA-GFP Km<sup>R</sup>, Sp<sup>R</sup>, Em<sup>R</sup>, Cm<sup>R</sup></i>	This study
pHB-DmsA-GFP 168	<i>trpC2; pHB-MdoD-GFP; Em<sup>R</sup>, Cm<sup>R</sup></i>	This study
pHB-MdoD-GFP tatAyCy	<i>trpC2; tatAy-tatCy::Sp; pHB-MdoD-GFP; Sp<sup>R</sup>, Em<sup>R</sup>, Cm<sup>R</sup></i>	This study
pHB-MdoD-GFP tatAdCd	<i>trpC2; tatAd-tatCd::Km; pHB-MdoD-GFP; Km<sup>R</sup>, Em<sup>R</sup>, Cm<sup>R</sup></i>	This study
pHB-MdoD-GFP total-tat <sub>2</sub>	<i>trpC2; tatAd-tatCd::Km; tatAy-tatCy::Sp; tatAc::Em; pHB-MdoD-GFP Km<sup>R</sup>, Sp<sup>R</sup>, Em<sup>R</sup>, Cm<sup>R</sup></i>	This study

**Table 2 Primers used in this study**

Primer	Sequence	Remarks
RBS-MntA-AmiA-F	GGGGGACTAGTAAGAGGAGGAGAAAT ATGAGCACTTTTAAACCACTA	<i>SpeI</i> , RBS <i>mntA</i> start <i>amiA</i>
RBS-MntA-DmsA-F	GGGGGACTAGTAAGAGGAGGAGAAAT ATGAAAACGAAAAATCCCTGAT	<i>SpeI</i> , RBS <i>mntA</i> start <i>dmsA</i>
<i>SpeI</i> -MntA-MdoD-F	GGGGGACTAGTAAGAGGAGGAGAAAT ATGGATCGTAGACGATTTATT	<i>SpeI</i> , RBS <i>mntA</i> start <i>mdoD</i>
GFP-Rev-BamHI	CCCCCGGATCCTTATTTGTATAGTTCATCCATGC	<i>BamHI</i> , end <i>gfp</i>
YwbN_LW-F	GGCGGTACCATGAGCGATGAACAGAAAAAGCCA GAACAA	<i>KpnI</i>
SPywbN_LW-R	GGGGAATTCAACAAGCGGAGCGAGACCGCC	<i>EcoRI</i>
YwbN_LW-R	GGGGGAATTCTGATTCCAGCAAACGCTG	<i>EcoRI</i>
F-YwbN-SURE	GGGGGTCATGAGCGATGAACAGAAAAAGCCAGA ACAAATTC	<i>RcaI</i>
GFP-Rev-HindIII	GCCCAAGCTTATTATTTGTAGAGCTCATCCATGCC ATGTG	<i>HindIII</i> , end <i>gfpmut1</i>

AmiA MSTFKPLKTLTSRRQVLKAGLAALTLSGMS**QAI**AKDELLKTSNGHS

DmsA MKTKIPDAVLAAEVSRRGLVKTTAIGGLAMASSALTLPFS**RIA**HAV

MdoD MDRRRFIKGSMAMAAVCGTSGIASLFS**QA**AFAA DSDIADGQTQRFD

YwbN MSDEQKKPEQIHRRDILKWGAMAGAAVAIGASGLGGLAP**LVQ**TAAK

PhoD MAYDSRFDEWVQKLKEESFQNNTFDRRKFIQGAGKIAGLSLGLTIAQ**SV**GAFE

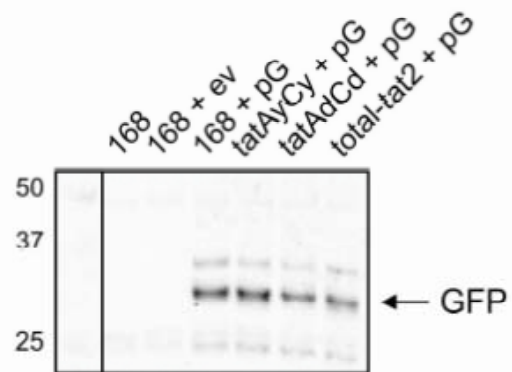
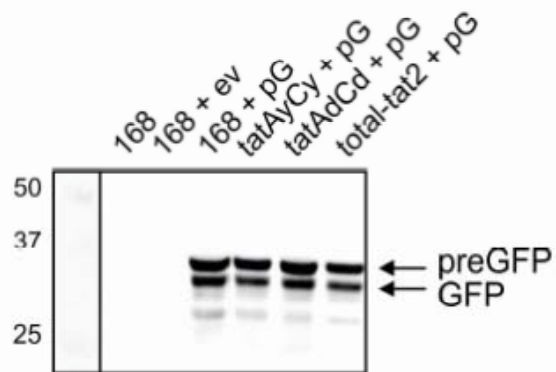
1% Salt

Cell

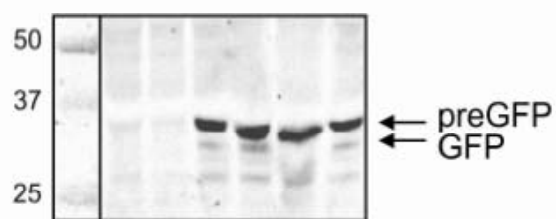
Medium

**A**

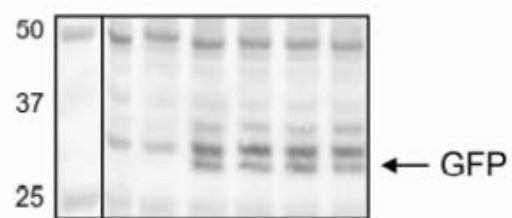
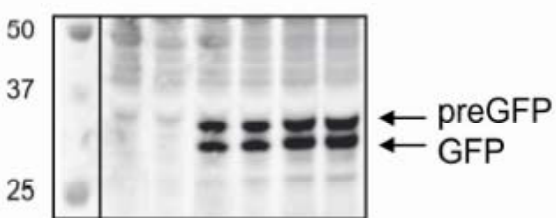
AmiA-GFP



DmsA-GFP

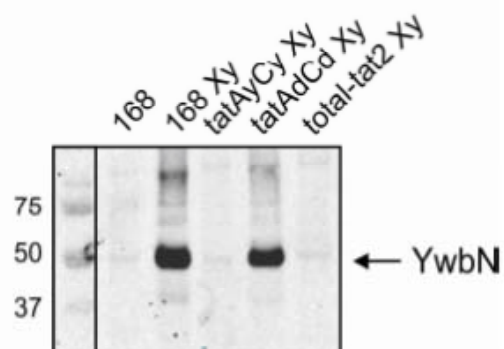
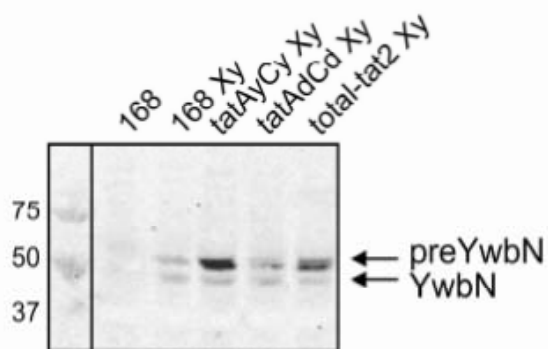


MdoD-GFP



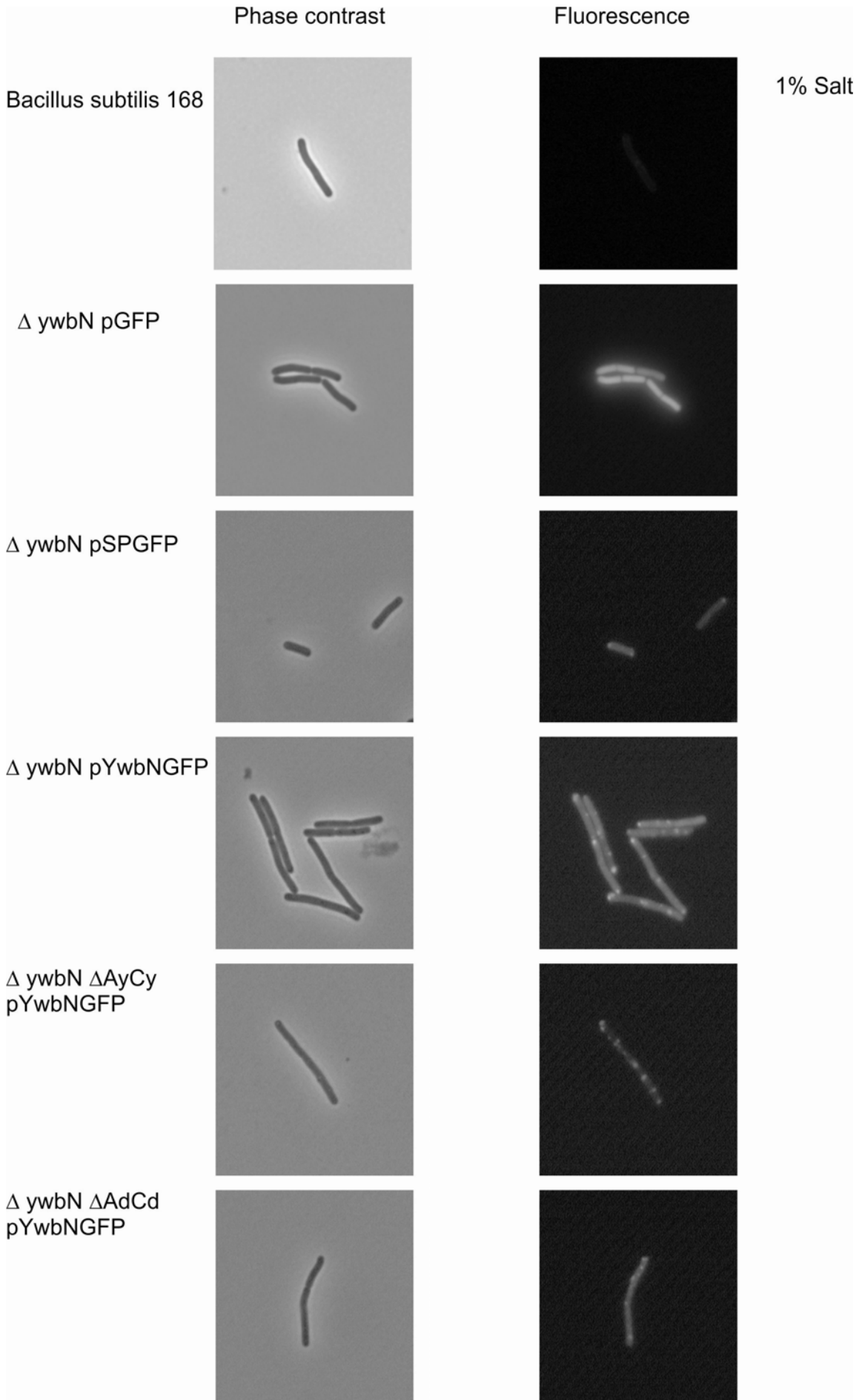
**B**

YwbN-Myc





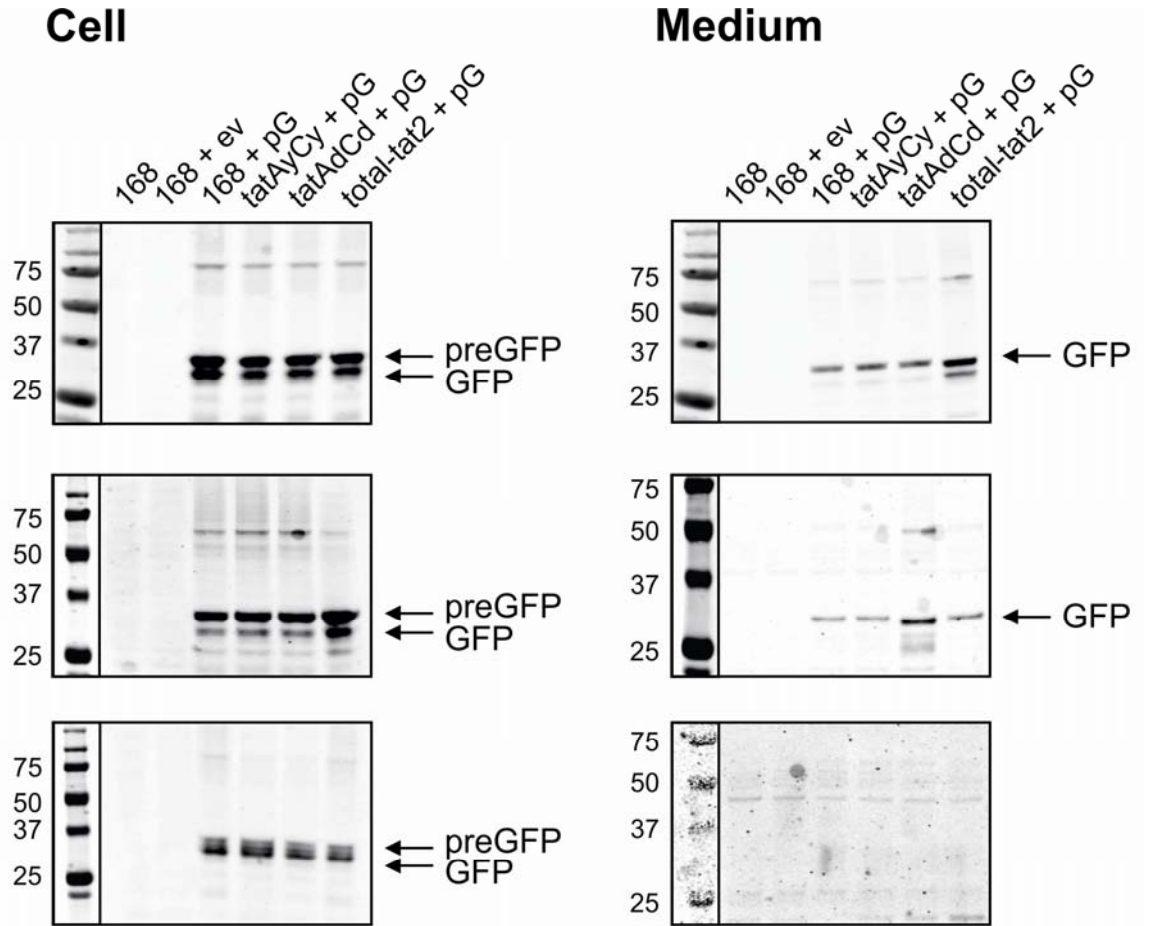




**Phosphate starvation**

**A**

AmiA-GFP

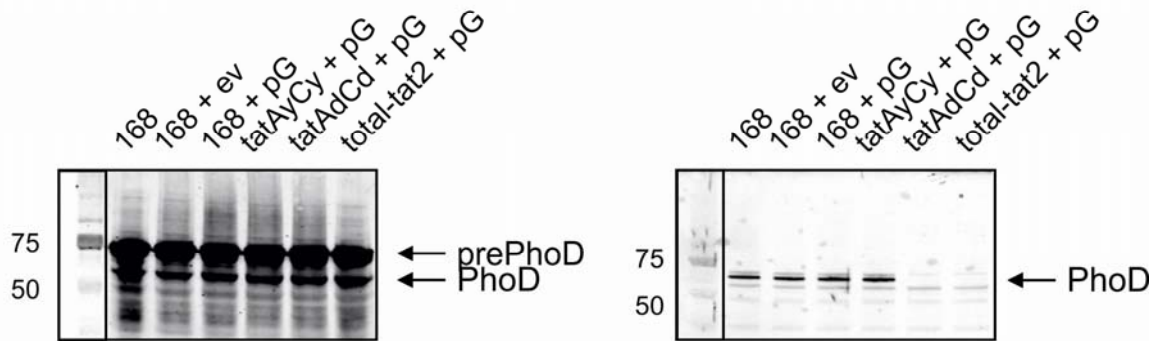


DmsA-GFP

MdoD-GFP

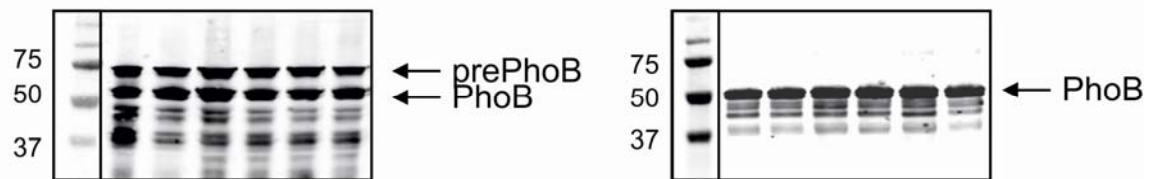
**B**

PhoD



**C**

PhoB



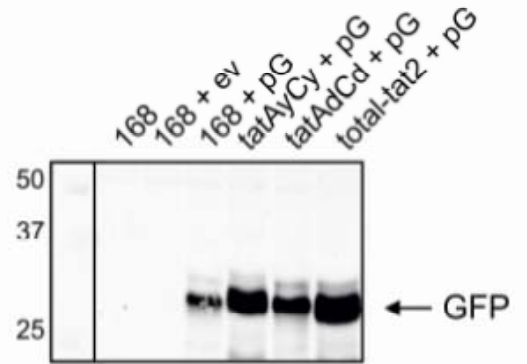
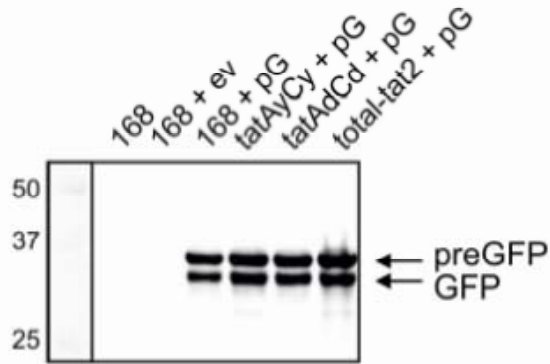
6% Salt

Cell

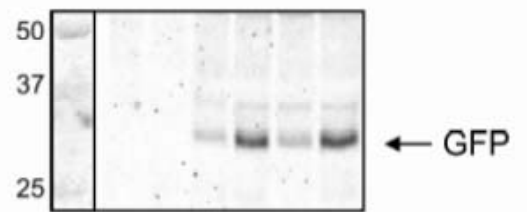
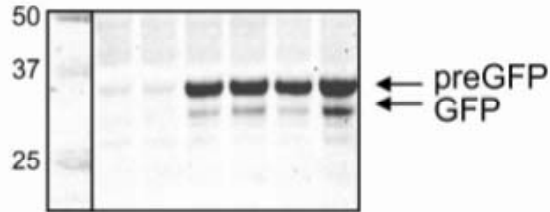
Medium

**A**

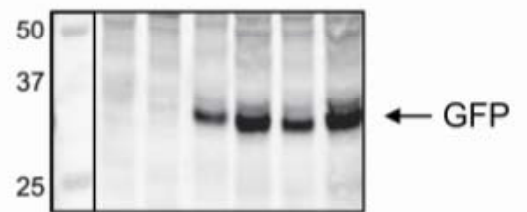
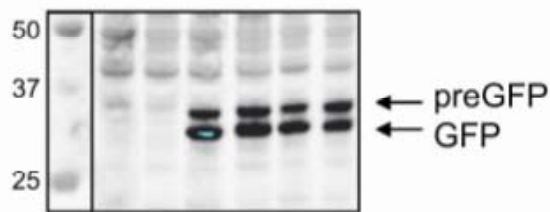
AmiA-GFP



DmsA-GFP

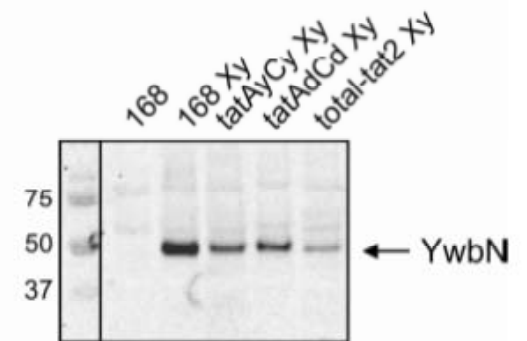
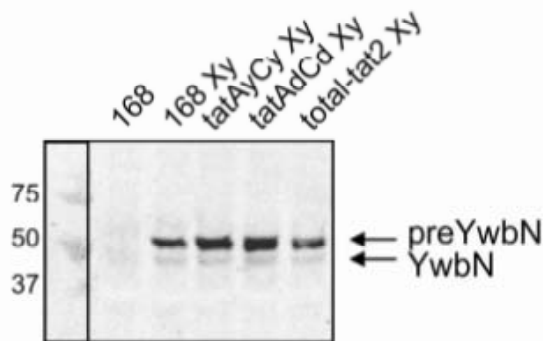


MdoD-GFP



**B**

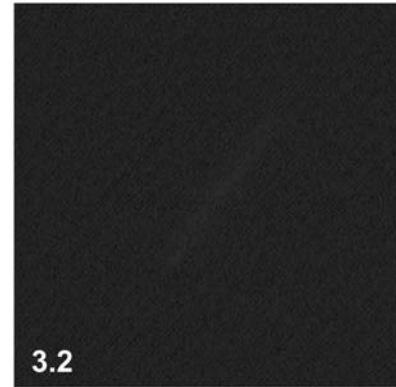
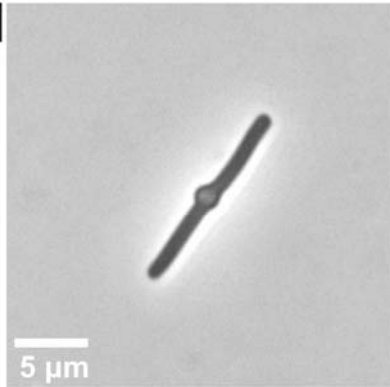
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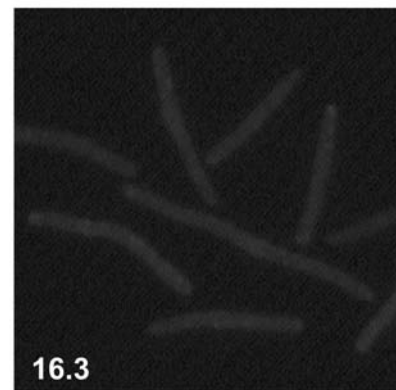
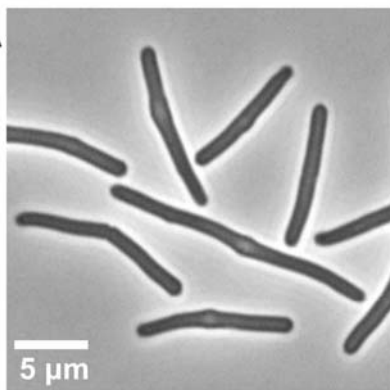
Phase contrast

Fluorescence

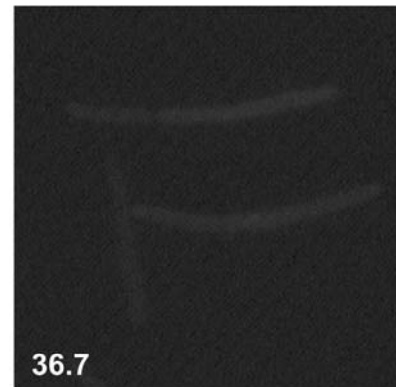
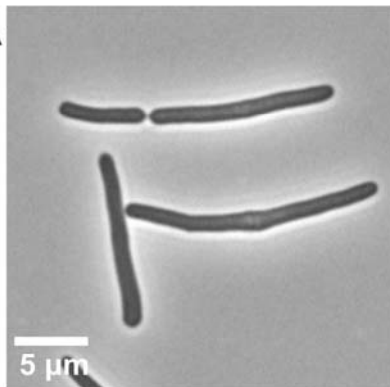
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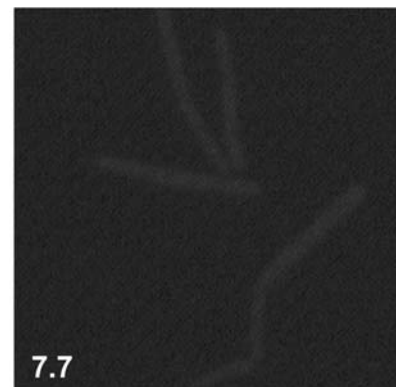
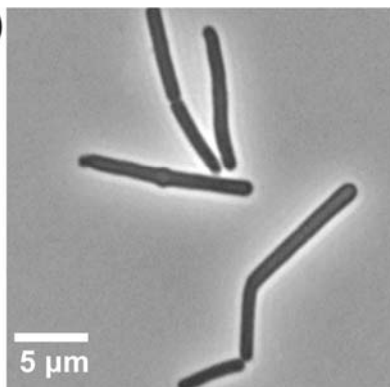
AmiA



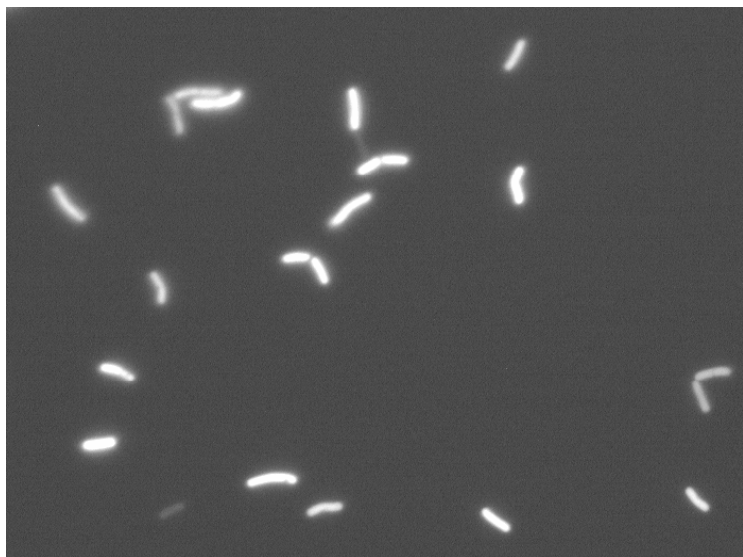
DmsA



MdoD



1% NaCl



6% NaCl

