JB Accepts, published online ahead of print on 7 July 2014
J. Bacteriol. doi:10.1128/JB.01843-14
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1 Folding LacZ in the periplasm of *Escherichia coli*

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11 Running Head: Folding LacZ in the periplasm of *Escherichia coli*

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15 ABSTRACT

16 Targeted, translational LacZ fusions provided the initial support for the signal
17 sequence hypothesis in prokaryotes and allowed for selection of the mutations that
18 identified the Sec translocon. Many of these selections relied on the fact that expression
19 of targeted, translational *lacZ* fusions like *malE-lacZ* and *lamB-lacZ42-1* causes lethal
20 toxicity as folded LacZ jams the translocation pore. But there is another class of targeted
21 LacZ fusions that do not jam the translocon. These targeted, non-jamming fusions also
22 show toxic phenotypes that may be useful for selecting mutations in genes involved in
23 post-translocational protein folding and targeting; however, they have not been
24 investigated to the same extent as their jamming counterparts. In fact it is still unclear
25 whether LacZ can be fully translocated in these fusions. It may be that they simply
26 partition into the inner membrane where they can no longer participate in folding or
27 assembly. In the present study we systematically characterize the non-jamming fusions
28 and determine their ultimate localization. We report that LacZ can be fully translocated
29 into the periplasm where it is toxic. We show that this toxicity is likely due to LacZ
30 misfolding and that in the absence of the periplasmic disulfide bond catalyst DsbA, LacZ
31 folds in the periplasm. Using the novel phenotype of periplasmic β-galactosidase
32 activity, we show that the periplasmic chaperone FkpA contributes to LacZ folding in this
33 non-native compartment. We propose that targeted, non-jamming LacZ fusions may be
34 used to further study folding and targeting in the periplasm of *Escherichia coli*.
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36 INTRODUCTION

37 Translational LacZ fusions have been used to study protein targeting in
38 *Escherichia coli* for over thirty-five years. The hybrid proteins produced by such fusions
39 comprise an N-terminal targeting sequence fused to a C-terminal, often functional
40 fragment of LacZ. The targeting sequence may consist of an outer membrane protein
41 (OMP) like LamB, a soluble periplasmic protein, or an integral inner membrane (IM)
42 protein like MalF. Each of these hybrid proteins has in common an N-terminal signal
43 sequence, which targets it to the Sec translocon at the inner membrane (IM).

44 The first *lamB-lacZ* fusions were described in 1977, and a subset of these were
45 found to confer sensitivity to the inducer maltose (1). Induction of targeted hybrid
46 proteins like LamB-LacZ*42-1* causes massive accumulation of the precursor forms of
47 envelope proteins in the cytoplasm (2). It seems the hybrid proteins jam the secretion
48 machinery, causing a global reduction in translocation efficiency. Jamming in turn leads
49 to proteolytic degradation of the essential components of the Sec translocase and
50 eventually to cell death (3). Screens for mutations that relieve lethal jamming without
51 preventing synthesis of the LamB-LacZ*42-1* hybrid protein ($Mal^R$ $Lac^+$) return mutations
52 in *lamB* that prevent fusion targeting by altering the signal sequence (4). The Δ*60lamB-*
53 *lacZ* fusion, which contains a *lamB* signal sequence deletion, was identified in this way
54 (5). Such mutants provided important support for the signal sequence hypothesis in
55 bacteria. Together, findings with the *lamB-* and *malE-lacZ* (2) fusions made possible the
56 selections and screens that identified the *sec* and *prl* alleles, which defined the
57 components of the Sec translocon during the 1980s (6–9).

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58 The role of LacZ in hybrid jamming was determined in studies with two
59 derivatives of *lamB-lacZ42-1*, which show that envelope protein precursors do not
60 accumulate if LacZ folding is prevented prior to translocation. The first fusion, *lamB-*
61 *lacZX90*, contains an *ochre* mutation that prevents folding by truncating the last ten
62 amino acids of LacZ (10, 11). The second fusion, *H*lamB-lacZ*, prevents folding by
63 altering the targeting pathway of the hybrid protein. *H** denotes a mutant *lamB* signal
64 sequence with increased hydrophobicity, which reroutes the fusion from the usual post-
65 translational, SecB-dependent targeting pathway to the co-translational, signal
66 recognition particle (SRP) pathway (12). Because translation and translocation are
67 coupled in the SRP pathway, LacZ cannot fold prior to translocation (12). Both the
68 *lacZX90* and *H*lamB* mutations relieve the lethal jamming caused by LamB-LacZ*42-1*,
69 and, because neither fusion jams the secretion machinery, they must allow LacZ
70 sequences to exit the Sec translocon (11, 12).

71 Despite the fact that the *X90* and *H** mutations relieve jamming, induction of
72 either fusion is lethal (11, 12). The toxicity conferred by these fusions is manifest in the
73 periplasm as evidenced by the fact that overproduction of the periplasmic protease DegP
74 relieves this toxicity (11, 12). In marked contrast, the lethal jamming caused by the
75 LamB-LacZ*42-1* hybrid protein is not relieved by overproduction of DegP (11, 12).
76 Periplasmic toxicity is likely due to the oxidizing nature of the periplasm, which causes
77 LacZ to misfold and aggregate (11, 13, 14). The LamB-LacZ*X90* hybrid protein forms
78 periplasmic disulfide-bonded aggregates that can be disrupted by removing the disulfide
79 bond catalyst DsbA, although this does not relieve maltose sensitivity (see below). LacZ

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80 is also known to misfold in strains expressing the *H*lamB-lacZ* fusion, because it does
81 not confer β-galactosidase activity (12).

82 The same is true of another co-translationally targeted *lacZ* fusion, *malF-lacZ102*,
83 which encodes a hybrid protein comprising a folding-competent LacZ moiety fused to the
84 third transmembrane segment of the integral IM protein MalF (14). MalF topology
85 suggests that LacZ is exposed to the periplasm when this hybrid protein partitions into the
86 membrane. Interestingly, selection for β-galactosidase activity in strains expressing the
87 *malF-lacZ102* fusion returns extragenic (unlinked) mutations that inactivate the *dsbA*
88 gene (15). Because LacZ is only known to fold in the cytoplasm *in vivo*, restoration of β-
89 galactosidase activity in strains expressing *malF-lacZ102* fusion has been attributed to
90 the re-localization of LacZ to the cytoplasm, although it has also been suggested the LacZ
91 may be folding in the periplasm (16). According to the cytoplasmic model, the LacZ
92 moiety in MalF-LacZ*102* partitions into the membrane along with MalF, adopting an
93 inactive, transmembrane structure that is stabilized by intra-LacZ disulfide bonds at the
94 periplasmic face of the IM. In the absence of DsbA, the exergonic folding reaction pulls
95 LacZ through the membrane into the cytoplasm where it can become active (15).

96 Perhaps LacZ contains cryptic stop-transfer sequences that prevent complete
97 translocation of any hybrid protein containing LacZ. Indeed, it is not clear whether the
98 LamB-LacZ*X90* or *H**LamB-LacZ hybrid proteins are fully translocated into the
99 periplasm either. Fractionation of these hybrid proteins has proven difficult, because
100 their misfolding promotes aggregation. The best evidence for full translocation is the fact
101 that DegP overexpression relieves inducer sensitivity in strains expressing these targeted,
102 non-jamming fusions (11, 12); however, this result does not preclude a model whereby



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103 hybrid protein partitions into the IM and confers toxicity through exposed periplasmic
104 domains. Neither the toxicity conferred by high-level production of the MalF-LacZ*102*
105 hybrid protein nor the effects of DegP overproduction in these fusion strains has been
106 systematically examined.

107 If the β-galactosidase activity exhibited by the MalF-LacZ*102* hybrid protein in
108 the absence of DsbA reflects periplasmic localization of LacZ activity, then this strain
109 should grow on lactose in the absence of the lactose permease, LacY. However, fusion
110 toxicity complicates this simple genetic test because low levels of cell lysis caused by
111 fusion toxicity can support growth by releasing soluble β-galactosidase from the
112 cytoplasm (1). On the other hand biochemical experiments demonstrating full
113 translocation of the β-galactosidase portion of the MalF-LacZ*102* hybrid protein are
114 complicated by the fact that the hybrid protein is membrane bound and thus insoluble.

115 In the present study we seek to determine whether LacZ can be fully translocated
116 into the periplasm, and, if so, whether it can fold and what periplasmic factors might be
117 involved. We suggest that the *H*lamB-lacZ* and *malF-lacZ102* fusions provide a useful
118 tool to study protein quality control pathways in the periplasm that help maximize the
119 production of heterologous proteins that require secretion and oxidation in *E. coli*.
120



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121 MATERIALS AND METHODS

122 **Strains and plasmids**

123 Strains and plasmids used in this study are listed in Table 1. All strains are
124 derivatives of NR754, an Ara$^+$ revertant of *Escherichia coli* MC4100. Marked alleles
125 Δ*dsbA*::Kan$^R$, Δ*fkpA*::Cm$^R$, and *lacY*::Tn*9* were moved by transduction with
126 bacteriophage P1 (17). CWB299 is merodiploid, containing both the *H*lamB-lacZ*
127 fusion and a copy of the *lamB* gene with the *H** signal sequence (Fig. 1A). We found
128 that the extra copy of the P*mal* promoter (a divergent promoter) led to a significant
129 decrease in levels of hybrid protein. To facilitate comparison with the other fusion
130 strains, we created a haploid version of *H*lamB-lacZ* (JCM944) by recombineering (18)
131 (Fig. 1F). To generate the *H*lamB-lacZ* deletion series, a fragment of the fusion was
132 cloned into pBR322 (pRSD02), and regions of mature *lamB* sequence were removed
133 using a plasmid-based PCR method (19). Fusions were then amplified from the plasmid
134 by PCR and recombineered into JCM932 as described for JCM944.
135
136 **Media and growth conditions**

137 All fusion strains were cultured overnight at 37°C with aeration in minimal M63
138 media supplemented with 1% glucose (w/v) to repress expression of the fusion and 0.4%
139 LB (v/v) unless otherwise noted (17). All subsequent experiments were carried out at
140 37°C with aeration unless noted otherwise. Chloramphenicol (20 μg/ml), Kanamycin (25
141 μg/ml), and Ampicillin (125 μg/ml) (Sigma) were included in overnight cultures and
142 plates as necessary to select for plasmid retention or recombination.
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144 **Maltose disc diffusion assays**

145 Overnight cultures were resuspended in M63 minimal media. 100 µl aliquots of
146 resuspended cells were then mixed with three milliliters molten M63 top agar and spread
147 evenly on a plate containing M63 bottom agar. Bottom and top agar were supplemented
148 with 0.2% glycerol and 0.4% LB and antibiotics as needed. Filter discs infused with 15
149 µl of 20% maltose (Sigma) were placed in the center of the plates once the top agar
150 solidified. Plates were incubated overnight, and the diameters of zones of inhibition were
151 measured. Measurements exclude the diameter of the filter disc. Representative
152 experiments are shown.

153

154 **β-galactosidase assays**

155 Overnight cultures were resuspended and subcultured 1:50 into LB. Cultures
156 were grown two to three hours to mid-log phase. 1 ml aliquots of culture were pelleted
157 and permeabilized using chloroform and SDS. β-galactosidase assays were performed in
158 triplicate according to Miller (20). Spectrophotometric readings were taken at one-
159 minute intervals over the course of a 15-minute ONPG hydrolysis reaction, and arbitrary
160 units were calculated from measurements of $V_{max}$ after normalization to cell culture
161 density as determined by $OD_{600}$ readings.

162

163 **Growth rate determination**

164 Overnight cultures were washed twice in minimal M63 media and subcultured
165 1:50 in 5 ml M63 minimal media containing either 1% glycerol or 1% lactose. Media
166 was not supplemented with LB. Growth was measured by $OD_{600}$ reading every hour over



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167 an eight hour time period. Growth rates were determined during exponential growth
168 using three data points.
169
170 **Spheroplasting**
171 Overnight cultures were resuspended and subcultured 1:50 into 50 ml liquid LB
172 cultures. Cultures were grown two to three hours until reaching mid-log phase. Cultures
173 were then split and subject to either spheroplasting or lysis in SDS-PAGE sample buffer
174 (21). After pelleting spheroplasts, supernatants containing soluble periplasmic
175 components were filtered and subjected to β-galactosidase assay and SDS-PAGE and
176 Western blot alongside whole cell lysates.
177
178 **Western blotting**
179 Samples resuspended in SDS-PAGE sample buffer were boiled 10 min and
180 subjected to electrophoresis on a 12% polyacrylamide gel. Immunoblotting was
181 performed with MBP (1:30,000 dilution) and CpxR (1:10,000 dilution) antisera, and
182 detection was performed with ECL antibody detection kit (Amersham), and Hyblot CL
183 film (Denville Scientific).
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185 RESULTS

186 **Targeted, non-jamming fusions confer periplasmic toxicity**

187 To determine whether LacZ can be fully translocated into the periplasm, we first
188 set out to see if the non-jamming fusions confer similar phenotypes. We reasoned that if
189 the *malF-lacZ102* fusion also confers maltose sensitivity, some significant portion of
190 LacZ might be exposed to the periplasm in this strain. Using a disc diffusion assay to
191 measure maltose sensitivity, we found that the *malF-lacZ102* fusion does indeed confer
192 maltose sensitivity (Table 2). Because *malF-lacZ102* is plasmid-borne, the size of the
193 zone of clearing cannot be compared directly to those of the other fusion strains, except
194 to say that strains harboring this fusion are certainly $Mal^S$.

195 Previously it was shown that overexpression of the periplasmic protease DegP is
196 sufficient to relieve the $Mal^S$ phenotype conferred by the *H*lamB-lacZ* and *lamB-*
197 *lacZX90* fusions. To ensure that the maltose sensitivity conferred by the *malF-lacZ102*
198 fusion results from periplasmic toxicity rather than some deleterious effect in the IM, we
199 determined the effect of DegP overexpression on the fusion strain. Using maltose disc
200 diffusion assays, we found that DegP overexpression caused strains expressing the *malF-*
201 *lacZ102* fusion to become $Mal^R$ (Table 2). Note that DegP does not relieve the $Mal^S$
202 phenotype of the jamming *lamB-lacZ42-1* fusion, which does not reach the periplasm.
203 The fact that all three non-jamming fusions confer a $Mal^S$ phenotype that can be relieved
204 by DegP overexpression suggests that despite differences in targeting sequences and
205 translocation pathways, the hybrid protein is exposed to the periplasm in all three fusions.
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207 **Toxicity of targeted, non-jamming fusions is caused by misfolded LacZ in the**
208 **periplasm**

209 The fact that the *malF-lacZ102* fusion confers periplasmic toxicity suggests that
210 the toxicity in all strains may be unrelated to targeting steps downstream of translocation.
211 MalF should not cause the MalF-LacZ*102* hybrid protein to titrate periplasmic
212 chaperones or to interfere with the essential OMP assembly machinery because (1) as an
213 IM protein it should not contain any signal for downstream interactions, and (2) it should
214 tether the hybrid protein to the IM where it cannot interact with OM proteins. To test that
215 the toxicity conferred by the *H*lamB-lacZ* fusion is also a result of localizing LacZ to the
216 periplasm, a series of deletion constructs were made, removing *lamB* sequence from the
217 fusion while leaving the signal sequence intact. These fusions should still be targeted to
218 the translocon, but any downstream targeting signals contained in the *lamB* fragment
219 should be removed by successive deletions. We found no discernible change in the $Mal^S$
220 phenotype of any of the deletion constructs, even when only five amino acids of mature
221 *lamB* were included in the fusion (Table 3). This result supports the conclusion that
222 periplasmic LacZ is the sole source of toxicity in these targeted, non-jamming fusions.

223 Since the LamB-LacZX90 hybrid protein is known to form disulfide-bonded
224 aggregates in the periplasm, we wondered if disulfide bond formation may contribute to
225 the periplasmic toxicity of LacZ. Indeed, LacZ contains a total of 16 cystein residues,
226 any of which could participate in aberrant intra- or inter-molecular disulfide bonds. To
227 test this hypothesis, we deleted the gene encoding the periplasmic disulfide bond catalyst
228 DsbA. We found by disc diffusion assay that the *dsbA*::$Kan^R$ insertion-deletion partially
229 relieves the $Mal^S$ phenotype conferred by the *H* lamB-lacZ* and *malF-lacZ102* fusions;



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230 however, it does not relieve the Mal$^S$ phenotype of cells expressing the *lamB-lacZX90*
231 fusion (Table 2). The *lamB-lacZX90* result is consistent with a previous report showing
232 *dsbA* deletion does not affect maltose sensitivity in this strain (11). Together, these
233 findings suggested that LacZ must be folding-competent in order for *dsbA* disruption to
234 relieve maltose sensitivity. We hypothesized that in the absence of DsbA, LacZ can fold
235 in the periplasm, which reduces its toxicity in turn.
236

237 **In the absence of DsbA, LacZ folds in the periplasm**

238 To determine whether LacZ is folding in the absence of DsbA, β-galactosidase
239 assays were performed on whole-cell lysates derived from the various fusion strains. As
240 expected the Δ*60lamB-lacZ* and *lamB-lacZ42-1* fusions, which lead to retention of LacZ
241 in the cytoplasm, exhibited β-galactosidase activity whether or not DsbA was present
242 (Fig. 2). In the presence of DsbA, none of the targeted, non-jamming fusions conferred
243 β-galactosidase activity; however, strains expressing the *H* lamB-lacZ* and *malF-*
244 *lacZ102* fusions showed significant β-galactosidase activity upon *dsbA* disruption (Fig.
245 2). Coupled with the reduction in maltose sensitivity, this increase in β-galactosidase
246 activity in targeted, non-jamming fusions supports the hypothesis that misfolded LacZ is
247 toxic in the periplasm.

248 We took two complementary approaches to determine whether the β-
249 galactosidase activity exhibited by strains expressing the *H*lamB-lacZ* or *malF-lacZ102*
250 fusions is localized to the periplasm. In the first approach, we took advantage of the fact
251 that otherwise wild-type strains require the inner membrane lactose permease LacY to
252 grow in minimal lactose media. In the absence of LacY, lactose cannot reach the



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253 cytoplasm where LacZ activity normally resides. We reasoned that if LacZ were instead
254 localized and active in the periplasm, it could cleave periplasmic lactose into galactose
255 and glucose, which could be imported by other means, thereby conferring growth in
256 minimal lactose even in the absence of LacY. To this end we tested the ability of strains
257 expressing the *H*lamB-lacZ* and *malF-lacZ102* fusions to grow in minimal lactose in
258 three genetic backgrounds: *lacY*$^{+}$ *dsbA*$^{+}$, *lacY*::Tn*9* *dsbA*$^{+}$, and *lacY*::Tn*9* *dsbA*::Kan$^{R}$.
259 The Δ*60lamB-lacZ* fusion was used as a control exhibiting cytoplasmic β-galactosidase
260 activity. As expected, strains expressing the Δ*60lamB-lacZ* fusion could only grow in the
261 presence of LacY (Fig. 3A). Like *Δ60lamB-lacZ*, neither the *H*lamB-lacZ* nor the *malF-*
262 *lacZ102* fusions conferred a Lac$^{+}$ phenotype in a *lacY*::Tn*9* *dsbA*$^{+}$ background; however,
263 these strains became Lac$^{+}$ in the absence of DsbA (Fig. 3A)—the same condition in
264 which they exhibit β-galactosidase activity. This evidence supports the hypothesis that
265 LacZ can fold in the periplasm in the absence of DsbA. We note that growth in minimal
266 lactose due to cell lysis is not consistent with these findings. If lysis were occurring, we
267 would expect it to be higher in the *dsbA*$^{+}$ background, since the *dsbA*::Kan$^{R}$ allele
268 relieves maltose sensitivity. Yet significant growth is only observed in the low-lysis
269 condition. We conclude that the hydrolysis of lactose in the media by extracellular LacZ
270 cannot explain the observed growth.

271 In the second approach, we determined whether β-galactosidase activity could be
272 released along with other soluble periplasmic proteins during spheroplast formation.
273 Because the MalF moiety tethers the MalF-LacZ102 hybrid protein to the IM, only the
274 *H*lamB-lacZ* fusion was used in this experiment. As expected, we found that cells
275 expressing the Δ*60lamB-lacZ* fusion released less than 1% of whole-cell β-galactosidase



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276 activity upon spheroplast formation (Fig. 3B). Conversely, cells expressing the *H* lamB-*
277 *lacZ* fusion released 52% of whole cell β-galactosidase activity upon spheroplast
278 formation (Fig 3B). To ensure that release of β-galactosidase activity into the soluble
279 fraction was not due to cell lysis, we attempted to detect cytoplasmic protein in
280 spheroplast supernatants by western blotting. While we identified the cytoplasmic
281 protein CpxR in whole-cell samples, it was absent from spheroplast supernatants,
282 indicating that the IM was still intact and cytoplasmic proteins were not released by
283 spheroplasting (Fig. 3C). Why roughly half of whole-cell β-galactosidase activity is
284 retained in spheroplasts is unclear, although other studies have reported partial release of
285 periplasmic proteins upon spheroplast formation (22). It is possible that a fraction of the
286 hybrid protein is tightly associated with the IM or that translocation efficiency is reduced
287 in the absence of DsbA. In any case, it is clear that in the absence of DsbA strains
288 expressing the *H*lamB-lacZ* fusion harbor folded, active LacZ in the periplasm.
289

290 **FkpA contributes to LacZ folding**
291 We thought it likely that some periplasmic factor must be assisting LacZ folding.
292 Because previous studies have implicated the periplasmic chaperones *skp* and *fkpA* in the
293 folding of heterologous proteins (23–29), we chose to see what effect disruption of these
294 genes might have on the β-galactosidase activity of a strain expressing the *malF-lacZ102*
295 fusion in the absence of DsbA. While disruption of *skp* did not decrease β-galactosidase
296 activity, we found that disruption of *fkpA* reduced it by a factor of 2.6 (Fig. 4). This
297 result firmly supports the conclusion that the LacZ moiety in non-jamming targeted
298 fusions is fully translocated into the periplasm where it can fold in the absence of DsbA.
299



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300 DISCUSSION

301 Three lines of evidence support the conclusion that LacZ is fully translocated into
302 the periplasm in targeted, non-jamming fusions. These three lines of evidence
303 correspond to three periplasmic proteins, each of which alters the phenotypes conferred
304 by the targeted, non-jamming fusions. First we showed that *malF-lacZ102* confers
305 maltose sensitivity like the *H* lamB-lacZ* and *lamB-lacZX90* fusions, and that the
306 periplasmic chaperone/protease DegP can relieve this sensitivity. As DegP is itself
307 localized to the periplasm and is a key component of the Cpx and $\sigma^E$ stress responses in
308 this compartment (30–32), we conclude that in all cases the toxicity conferred by
309 targeted, non-jamming fusions is periplasmic and that LacZ is most likely fully
310 translocated by extension.

311 The second line of evidence supporting full translocation concerns the periplasmic
312 disulfide bond catalyst DsbA, which we found promotes the misfolding and toxicity of
313 the H* LamB-LacZ and MalF-LacZ*102* hybrid proteins. In the absence of DsbA, the
314 LacZ moiety in these proteins can fold and become active in the periplasm, reducing its
315 toxicity. Of course disruption of *dsbA* has no phenotypic effect on the *lamB-lacZX90*
316 fusion, because the *X90* mutation prevents LacZ from folding in any compartment. This
317 led us to conclude that it is misfolded LacZ in particular that is toxic in the periplasm.
318 This hypothesis was confirmed when we found that a derivative of the *H* lamB-lacZ*
319 fusion encoding only the signal sequence and first five amino acids of LamB fused to
320 LacZ was just as toxic as the full-length fusion. Interestingly, it has been reported that
321 the signal sequence alone may not be sufficient for targeting and that more than 27 amino
322 acids of mature LamB are needed in addition to the signal sequence to promote SecB-



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323 dependent export of the LamB-LacZ hybrid protein (33, 34). It has also been suggested
324 that the signal sequence may not play a role in targeting proteins to the Sec translocon *per*
325 *se* (35). Our findings suggest that a signal sequence is both necessary and sufficient for
326 targeting via the SRP pathway.

327 We believe the H* signal sequence may present an ideal method for targeting
328 exogenous proteins for folding in the oxidizing environment of the *E. coli* periplasm.
329 While SecB-dependent targeting sequences like those derived from MBP are sufficient to
330 target hybrid protein to the translocon, they risk lethal jamming. The H* signal sequence
331 avoids jamming by directing hybrid protein to the co-translational SRP pathway, which
332 should allow for greater induction and higher yield of exogenous proteins. Also, the fact
333 that the signal sequence alone seems to be sufficient for targeting means that little if any
334 targeting sequence need remain after signal sequence cleavage.

335 The third line of evidence supporting full-translocation concerns the effect of
336 FkpA on LacZ folding. FkpA is one of four known peptidyl-prolyl *cis-trans* isomerases
337 in the periplasm (36). It functions as a chaperone *in vivo*, promoting assembly of OMPs.
338 FkpA function overlaps at least in part with Skp function, and both seem to be involved
339 in assembly of the essential OMP LptD (37), which is in turn responsible for inserting
340 lipopolysaccharide (LPS) into the OM of Gram-negative bacteria like *E. coli* (38, 39). In
341 the present study we found that disruption of *fkpA* leads to a two- to three-fold reduction
342 in whole-cell β-galactosidase activity in cells expressing the *malF-lacZ102* fusion in the
343 absence of DsbA. This finding is consistent with previous studies, which have implicated
344 FkpA in the folding of exogenous proteins in the periplasm, and, like our findings with
345 DegP and DsbA, it confirms that LacZ is fully translocated into the periplasm. Whether



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346 the PPIase activity of FkpA contributes to LacZ folding remains unclear, although LacZ
347 does contain proline residues. We note that FkpA has previously been shown to prevent
348 protein aggregation in the periplasm independent of its PPIase function (40).

349 The fact that a periplasmic factor contributes to LacZ folding is not surprising
350 given the obstacles the periplasm presents to folding. LacZ is much larger than most
351 periplasmic proteins, and it must tetramerize to form active enzyme (41). In the
352 cytoplasm folding is driven by energy-dependent folding factors like GroEL and DnaJ/K;
353 however, there are no homologs of these generalized folding factors in the periplasm, nor
354 are there nucleotide triphosphates in the periplasm to drive energy-dependent folding
355 (42). As previously discussed, the periplasm is also an oxidizing environment, and this is
356 true even in the absence of DsbA as evidenced by the fact that *dsbA* is not essential under
357 aerobic growth conditions. Given that *dsbA* disruption only partially relieves the $Mal^S$
358 phenotype of the *H*lamB-lacZ* and *malF-lacZ102* fusions, we think it likely that some
359 significant fraction of translocated LacZ is still misfolding even in the absence of DsbA.
360 LacZ must have at least two fates in the periplasm then, folded or misfolded, with FkpA
361 and DsbA contributing respectively to these fates.

362 We believe that this newly-discovered periplasmic β-galactosidase activity will be
363 extremely useful in designing screens for periplasmic folding factors. It is likely that
364 additional factors contribute to LacZ folding, since the *malF-lacZ102* fusion still confers
365 β-galactosidase activity in the absence of FkpA. Screening for folding factors in an *fkpA*
366 null background will help overcome the functional redundancy of periplasmic
367 chaperones. Identifying such periplasmic folding factors and efficiently targeting

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368 recombinant proteins to the periplasm are keys to the effective expression of exogenous

369 proteins requiring disulfide bonds in *E. coli*.

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371 ## ACKNOWLEDGMENTS

372 This work was supported by National Institute of General Medical Sciences grant
373 GM34821 awarded to T.J.S. J.B. and D.B. were supported by National Institute of
374 General Medical Sciences grant GMO41883.

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502 **Table 1** Strains, plasmids, and phage

| Strain, plasmid, or phage | Genotype or relevant description | Reference(s) or source(s) |
|---|---|---|
| NR754 | MC4100 F⁻ *araD*⁺ Δ(*argF-lac*) *U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 thi* | (43, 44) |
| CWB299 | MC4100 Φ(H**lamB*'-'*lacZ*) Hyb42-1 [λp1(209)] H**lamB* | (12) |
| JCM911 | JCM913 pKD46 | This study |
| JCM912 | NR754 Φ(*lamB*'-'*lacZ*) Hyb42-1 [λp1(209)] | This study; (1) |
| JCM913 | NR754 Φ(*lamB*Δ*60*'-'*lacZ*) Hyb42-1 [λp1(209)] | This study; (5) |
| JCM914 | NR754 Φ(*lamB-lacZX90*) Hyb42-1 [λp1(209)] | This study; (11) |
| JCM932 | NR754 Φ(P*lamB* Δ*230*::Kan$^R$ *lamB*'-'*lacZ*) Hyb42-1 [λp1(209)] pKD46 | This study |
| JCM944 | NR754 Φ(*H*lamB*'-'*lacZ*) Hyb42-1 [λp1(209)] | This study |
| RSD106 | NR754 pNG102 | This study |
| RSD107 | NR754 Φ(*H*SS+90lamB*'-'*lacZ*) Hyb42-1 [λp1(209)] | This study |
| RSD108 | NR754 Φ(*H*SS+49lamB*'-'*lacZ*) Hyb42-1 [λp1(209)] | This study |
| RSD109 | NR754 Φ(*H*SS+43lamB*'-'*lacZ*) Hyb42-1 [λp1(209)] | This study |
| RSD110 | NR754 Φ(*H*SS+27lamB*'-'*lacZ*) Hyb42-1 [λp1(209)] | This study |
| RSD111 | NR754 Φ(*H*SS+5lamB*'-'*lacZ*) Hyb42-1 [λp1(209)] | This study |
| RSD112 | JCM913 pDegP | This study |
| RSD113 | JCM912 pDegP | This study |
| RSD114 | JCM914 pDegP | This study |
| RSD115 | JCM944 pDegP | This study |
| RSD116 | RSD106 pDegP | This study |
| RSD117 | JCM913 *dsbA*::Kan$^R$ | This study |
| RSD118 | JCM912 *dsbA*::Kan$^R$ | This study |
| RSD119 | JCM914 *dsbA*::Kan$^R$ | This study |
| RSD120 | JCM944 *dsbA*::Kan$^R$ | This study |
| RSD121 | RSD106 *dsbA*::Kan$^R$ | This study |
| RSD122 | JCM913 *lacY*::Tn*9* | This study |
| RSD123 | JCM944 *lacY*::Tn*9* | This study |
| RSD124 | RSD117 *lacY*::Tn*9* | This study |
| RSD125 | RSD120 *lacY*::Tn*9* | This study |
| RSD126 | RSD121 Δ*skp yafC*::Tn*10* | This study |
| RSD127 | RSD121 Δ*fkpA*::Cm$^R$ | This study |
| pNG102 | Amp$^R$ LacZ⁻ P*malmalE'* Φ(*malF-lacZ*) Hyb102 | (14) |
| pDegP | Cm$^R$ pACYC184::*degP* | (45) |
| pKD4 | Amp$^R$ Kan$^R$ | (18) |
| pKD46 | Amp$^R$ P*araB* λ-Red recombinase *γ β exo* | (18) |
| pCWB36 | *H*lamB* | (12) |
| pRSD02 | pBR322::*'H*lamB-lacZ'* Tet$^R$ | This study |
| λp1(209) | ::(+Mu')*trp'BA'* – W209 *lac 'O Z U118Y A'* | (43) |

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**Table 2** Inducer sensitivity profiles of fusion strains

| Fusion | Zone of Inhibition (mm) | | |
|---|---|---|---|
| | Wild Type[a] | pDegP[b] | *dsbA*::Kan$^R$ |
| *Δ60 lamB-lacZ* | 0 | 0 | 0 |
| *lamB-lacZ42-1* | 25 | 19 | 23 |
| *lamB-lacZX90* | 23 | 0 | 24 |
| *H* lamB-lacZ* | 11 | 0 | 4 |
| *malF-lacZ102* | 28 | 0 | 2 |

505

506 [a]The wild type heading indicates strains with fusions present in the NR754 background without

507 plasmids or markers. See fusion strains JCM913, JCM912, JCM914, JCM944, and RSD06.

508 [b]The pDegP heading indicates fusion strains containing a high copy *degP* plasmid. See fusion

509 strains are RSD112-116.

510 [c]The *dsbA*::Kan$^R$ heading refers to strains RSD117-121.

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**Table 3** Inducer sensitivity of the *H*lamB-lacZ* deletion series

| Strain | Mature LamB sequence[a] | Zone of inhibition (mm) |
|---|---|---|
| JCM944 | 173 | 7 |
| RSD107 | 90 | 7 |
| RSD108 | 49 | 6 |
| RSD109 | 43 | 7 |
| RSD110 | 27 | 8 |
| RSD111 | 5 | 7 |

512

513 [a]The number of mature LamB residues included in addition to the *H**LamB signal sequence.

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515 **Figure 1**. Construction of JCM944. (A,B,D,F) Note that the λ insertion is λp1(209). (A)
516 CWB299. CWB299 is an *H*lamB* merodiploid, containing a copy of *H*lamB* in addition to the
517 *H*lamB-lacZ* fusion. (B) JCM911. Construction of a haploid *H*lamB-lacZ* strain began with a
518 strain containing the Δ*60lamB-lacZ* fusion and pKD46. JCM911 was recombineered with the
519 linear DNA fragment depicted in (C). The fragment was obtained by PCR amplification of the
520 $Kan^R$ cassette present on pKD4 with primers having 5' homology to *malK* and *lamB*. The primers
521 were designed to generate a $Kan^R$ insertion-deletion removing 27 nucleotides upstream of the
522 *lamB* ORF and 200 nucleotides downstream (Δ*230*::$Kan^R$). The result of the recombineering
523 reaction is JCM932 depicted in (D). The Δ*230*::$Kan^R$ insertion-deletion is polar on the *lac* operon,
524 preventing expression of *lacY*. As a result JCM932 cannot grow on minimal melibiose at 42°C.
525 JCM932 was then recombineered with the linear DNA fragment depicted in (E). The fragment
526 was obtained by PCR amplification of *malK* and *H*lamB* from pCWB36. Recombinants that
527 replaced the Δ*230*::$Kan^R$ insertion-deletion and restored *lacY* expression were selected on
528 minimal melibiose at 42°C and subsequently screened for $Kan^S$. (F) JCM944 was identified as a
529 $Mel^+$ $Kan^S$ recombinant.
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531 **Figure 2** Effect of DsbA on whole cell β-galactosidase activity. Overnight cultures were washed
532 and subcultured 1:50 into liquid LB cultures. Cultures were incubated with aeration at 37°C for 2-
533 3 hours until reaching mid-log phase. β-galactosidase activity was determined from $V_{max}$ of whole
534 cell lysates in a kinetic Miller assay. Δ60 indicates strains JCM913 (*dsbA*$^{+}$) and RSD117
535 (*dsbA*::Kan$^{R}$). 42-1 indicates strains JCM912 (*dsbA*$^{+}$) and RSD118 (*dsbA*::Kan$^{R}$). H* indicates
536 strains JCM944 (*dsbA*$^{+}$) and RSD120 (*dsbA*::Kan$^{R}$), and 102 indicates strains RSD06 (*dsbA*$^{+}$)
537 and RSD121 (*dsbA*::Kan$^{R}$). Error bars show standard deviation calculated from three
538 independent biological replicates.
539



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540 **Figure 3** Effect of DsbA on periplasmic β-galactosidase activity. (A,B) Error bars show standard
541 deviations calculated from three independent biological replicates. (A) Bypassing *lacY*.
542 Overnight cultures were washed and subcultured in minimal media containing glycerol or lactose
543 as the sole carbon source. Δ60 indicates strains containing the *Δ60lamB-lacZ* fusion in a wild
544 type background (JCM913, white), a *lacY*::Tn*9* background (RSD122, grey), or a *lacY*::Tn*9*
545 *dsbA*::Kan$^R$ background (RSD124, black). H* indicates strains containing the *H*lamB-lacZ* fusion
546 in a wild type background (JCM944, white), a *lacY*::Tn*9* background (RSD123, grey), or a
547 *lacY*::Tn*9* *dsbA*::Kan$^R$ background (RSD125, black). 102 indicates strains containing the *malF-*
548 *lacZ102* fusion in a wild type background without *lacY* (RSD06, grey) or a *dsbA*::Kan$^R$
549 background (RSD121, black). (B,C) Overnight cultures were washed and subcultured 1:50 into
550 liquid LB cultures. Cultures were incubated with aeration at 37°C for 2-3 hours until reaching mid-
551 log phase. Δ60 refers to RSD117 (*dsbA*::Kan$^R$), and H* refers to RSD120 (*dsbA*::Kan$^R$). (B)
552 Releasing soluble periplasmic β-galactosidase activity. β-galactosidase activity was determined
553 from $V_{max}$ of whole-cell lysates or filtered spheroplast supernatants in a kinetic Miller assay.
554 Periplasmic β-galactosidase activity is reported as a fraction of whole-cell β-galactosidase
555 activity. (C) Spheroplasting lysis control. Whole-cell lysates or filtered spheroplast supernatants
556 were subject to SDS-PAGE and Western blotting. MBP is used as a marker for soluble
557 periplasmic proteins. CpxR is used as a marker of soluble cytoplasmic proteins.
558

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559 **Figure 4** Effect of periplasmic chaperones on periplasmic β-galactosidase activity. Overnight
560 cultures were washed and subcultured 1:50 into liquid LB cultures. Cultures were incubated with
561 aeration at 30°C for 2-3 hours until reaching mid-log phase. β-galactosidase activity was
562 determined from $V_{max}$ of whole cell lysates in a kinetic Miller assay. β-galactosidase activity is
563 normalized to RSD121. Error bars show standard deviation calculated from three independent
564 biological replicates. 102 indicates RSD121. Δ*skp* indicates RSD126. *fkpA*::Cm$^R$ indicates
565 RSD127.



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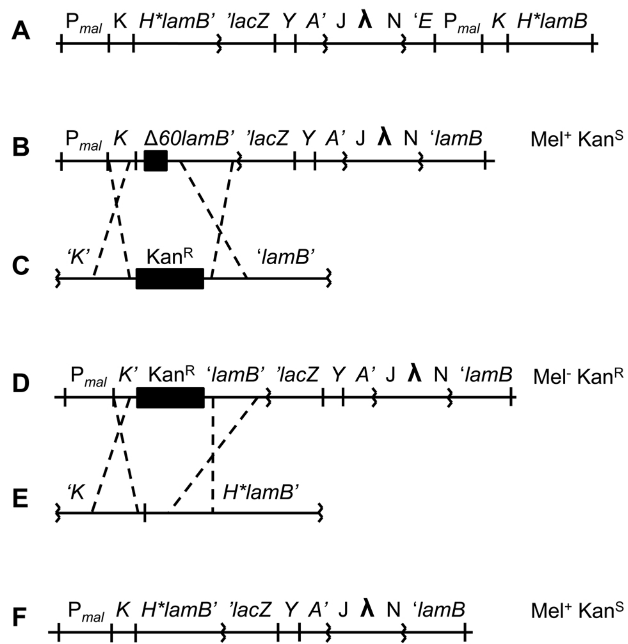


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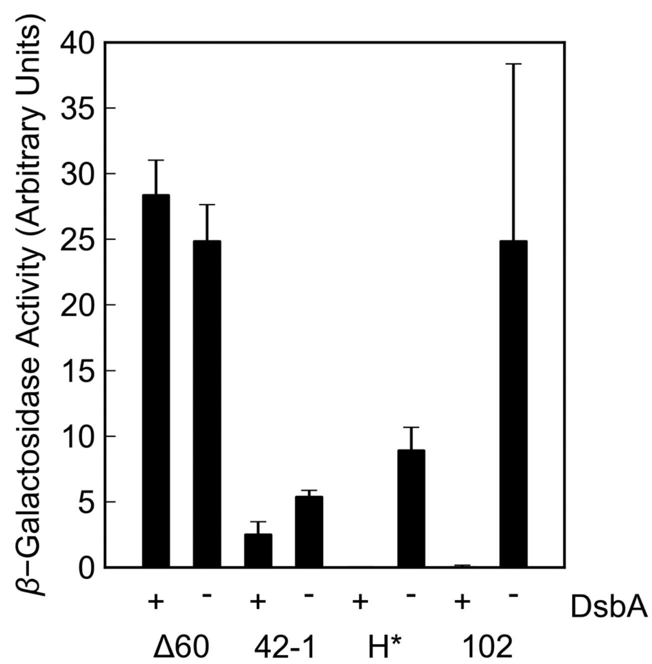


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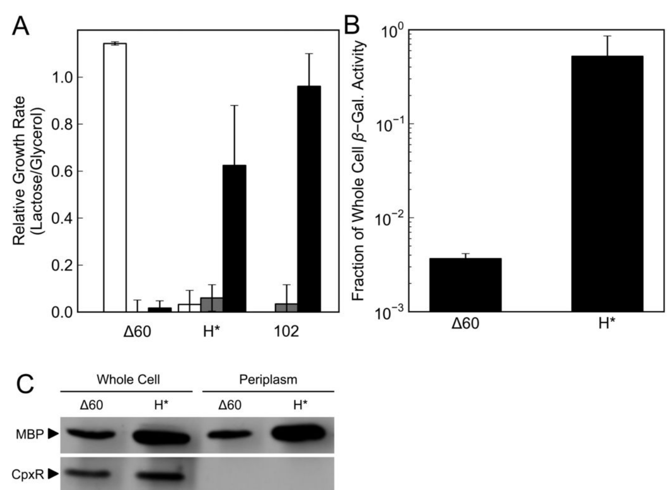


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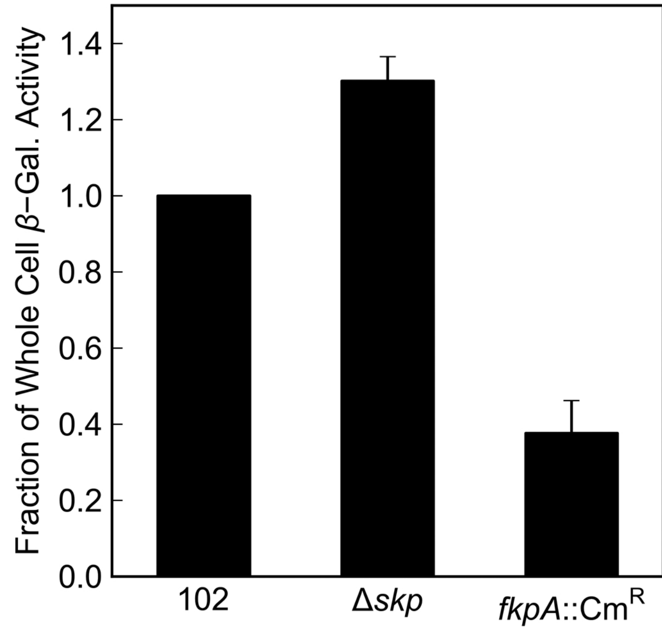


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