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1 **Ton Motor Conformational Switch and Peptidoglycan Role** 2 **in Bacterial Nutrient Uptake**

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15 **ABSTRACT (199 words)**

16 Active nutrient uptake is fundamental for survival and pathogenicity of Gram-negative
17 bacteria, which operate a multi-protein Ton system to transport essential nutrients like metals
18 and vitamins. This system harnesses the proton motive force at the inner membrane to
19 energize the import through the outer membrane, but the mechanism of energy transfer
20 remains enigmatic. Here, we study the periplasmic domain of ExbD, a crucial component of
21 the proton channel of the Ton system. We show that this domain is a dynamic dimer switching
22 between two conformations representing the proton channel's open and closed states. By *in*
23 *vivo* phenotypic assays we demonstrate that this conformational switch is essential for the
24 nutrient uptake by bacteria. The open state of ExbD triggers a disorder to order transition of
25 TonB, enabling TonB to supply energy to the nutrient transporter. We also reveal the
26 anchoring role of the peptidoglycan layer in this mechanism. Herein, we propose a mechanistic
27 model for the Ton system, emphasizing ExbD duality and the pivotal catalytic role of
28 peptidoglycan. Sequence analysis suggests that this mechanism is conserved in other
29 systems energizing gliding motility and membrane integrity. Our study fills important gaps in
30 understanding bacterial motor mechanism and proposes novel antibacterial strategies.

31

32 **INTRODUCTION**

33 Gram-negative bacteria present a unique challenge for the development of novel drugs
34 due to their dual-membrane structure, which effectively protects them by preventing many
35 antibiotics accessing their targets within the cell.¹ This dual-membrane architecture requires
36 specialized transport systems for essential nutrients – like iron, nickel, vitamin B12 and certain

37 carbohydrates². These gram-negative specific systems guarantee efficient transport over both
38 the inner and the outer membrane as well as the periplasm, potentially creating vulnerabilities
39 for therapeutic intervention. One of the key systems involved in this transport is the Ton
40 system, which forms a multi-protein complex embedded in the inner membrane (Figure 1a).
41 This system utilizes the proton motive force (PMF) to physically open a variety of outer
42 membrane transporters – the so called TonB-dependent transporters (TBDTs) – and, hereby,
43 realizes active transport by means of the inner membrane proton channel forming complex
44 ExbB-ExbD and the periplasm crossing protein TonB. While the TBDTs are nutrient-specific,
45 TonB and ExbB-ExbD can be employed to multiple TBDTs (multi-target).³ In addition to this
46 multi-target Ton system, some Ton systems are dedicated to a specific transporter (single-
47 target). This is the case of the Heme Acquisition System (Has) allowing bacteria to acquire
48 heme as an iron source and involving orthologs of ExbB, ExbD with a TonB paralog called
49 HasB.⁴⁻⁶

50 At the inner membrane, the Ton system consists of the proton-channel forming
51 complex ExbB-ExbD and the protein TonB, which are together capable of harnessing the
52 PMF. The structure of the whole complex is unknown. Recent cryo-EM data showed two α -
53 helices situated in a periplasm-facing central pore of the pentameric ExbB channel. These
54 densities were attributed to the most N-terminal part of an ExbD dimer (residues 12-42).⁷
55 However, no information about the organization of the C-terminal, periplasmic part of ExbD
56 could be gained as it remains invisible in all known cryo-EM structures to date most likely due
57 to its dynamics.^{5,8,9} The only structural data is a monomeric, solution NMR structure of the
58 periplasmic domain of ExbD at pH 3 that features an unfolded N-terminus (residues 44-63)
59 followed by a globular fold (residues 64-132).¹⁰ It has been proposed that a PMF-dependent
60 de- and reprotonation of the highly conserved ExbD residue Asp 25 in the helix that is
61 embedded in the pore of the ExbB channel, causes a rotation of ExbD within ExbB¹¹ –
62 analogous to the MotA-MotB system involved in flagellar motility.¹² At the outer membrane,
63 ligand binding to a TBDT results in the propulsion of an N-terminal extension of the TBDT into
64 the periplasm, where it is tightly bound to TonB via a conserved region called the TonB box.¹³
65 TonB consists of an N-terminal α -helix anchoring it into the inner membrane and proposedly
66 to ExbB⁵, a periplasm-spanning intrinsically disordered linker, and a C-terminal, globular
67 domain responsible for TBDT binding.¹⁴ The intrinsically disordered linker contains a proline-
68 rich region, approximately 70 residues in length, with an N-terminal stretch of Glu-Pro
69 repetitions and a C-terminal stretch of Lys-Pro repetitions.¹⁵ These stretches are organized in
70 polyproline II helices and behave like two static rods, which however still retain some
71 flexibility.^{16,17} It is proposed that following TonB's binding to the TonB box, the hypothesized
72 ExbD rotation would generate a pulling force on TonB which unfolds a plug in the TBDT,
73 opening a channel and ensuring selective ligand entry.¹⁸ These dynamics might vary in the
74 Has system due to a couple of distinguishing features: 1.) The disordered linker of HasB does
75 not contain an extensive stretch of Glu-Pro repetitions, consequently lacking two large regions
76 of contrasting charges. 2.) HasB is likely perpetually bound to the TBDT, even in the absence
77 of the ligand. This characteristic can be attributed to the single-target nature of the HasB,
78 eliminating the necessity to accommodate multiple TBDTs.¹⁹

79 Due to the partial localization of the Ton system in the periplasm, a potential role of the
80 periplasmic peptidoglycan layer within the mechanism of the system has been proposed.²⁰ In
81 the related MotA-MotB system, MotB contains a C-terminal peptidoglycan-binding motif that
82 anchors the MotAB stator to the cell wall²¹, while in the analogous Tol-Pal system²², this
83 interaction is facilitated by Pal and TolR^{23,24}. However, the nature of this interaction in the Ton
84 system has remained elusive.

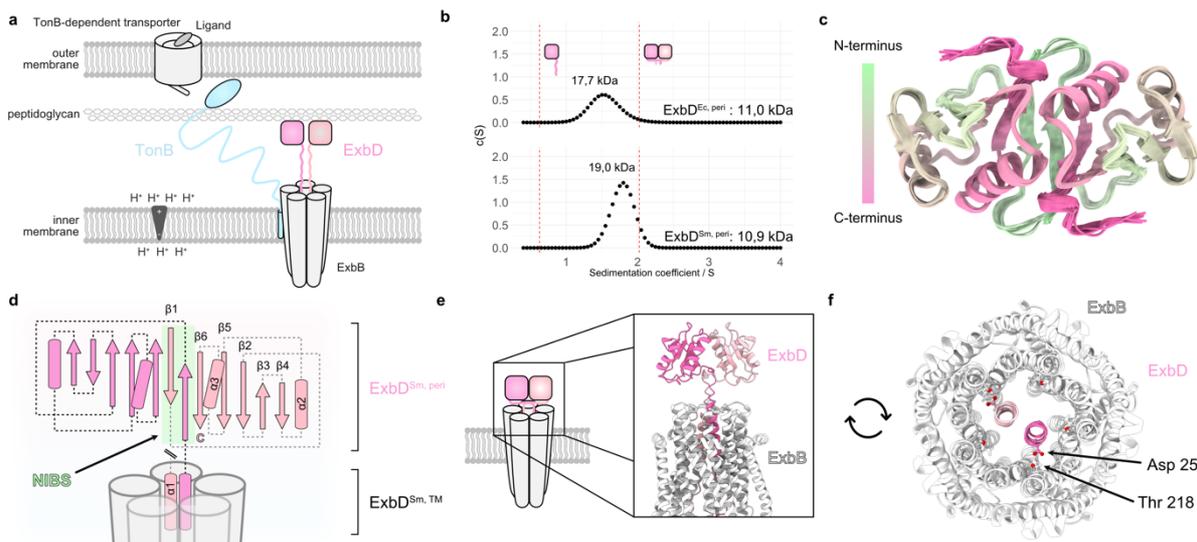
85 Despite being first identified in 1978²⁵, key steps of the Ton system's mechanism
86 remain poorly understood primarily due to incomplete structural information especially on the
87 connection between ExbD and TonB. This is likely due to the disordered and dynamic nature
88 of the periplasmic domains of both proteins, which renders them unsuitable for cryo-EM
89 studies. Uncovering the role and the assembly of these two proteins is of great importance,
90 as it could not only improve our understanding of nutrient transport in gram-negative bacteria
91 but also provide new targets for antibiotics development. To ensure the broad relevance of
92 our results, we investigated two representative systems: the multi-target Ton system from
93 *Escherichia coli* and the single-target Has system from *Serratia marcescens*. These systems
94 were chosen due to the plethora of existing functional data and a partial knowledge of their
95 structures, allowing their comprehensive analysis. In this study, we use NMR spectroscopy to
96 make the dynamic visible: we present the dimeric structure of the periplasmic domain of ExbD
97 in different states, including a sparsely populated one. We demonstrate that this minor state
98 is conformationally selected upon binding to an intrinsically disordered region (IDR) of TonB,
99 which undergoes a disorder-to-order transition. Moreover, mutagenesis and *in vivo* phenotypic
100 assays confirm that this multi-state transition of ExbD is required for its function. Also, we show
101 that the transient interaction of the ExbD dimer to the peptidoglycan layer in the periplasm is
102 crucial for the action of the Ton system as it is transiently bound by the ExbD dimer.

103

104 RESULTS

105 The periplasmic domain of ExbD forms a homodimer

106 We recombinantly produced the periplasmic domains of ExbD from both organisms
 107 (from *E. coli* including residues 43-141: ExbD^{Ec, peri}, from *S. marcescens* including residues 43-
 108 140: ExbD^{Sm, peri}; all constructs and NMR samples are summarized in Table S1-2). Analytical
 109 ultracentrifugation (AUC) revealed that the periplasmic domain of ExbD of both systems is
 110 organized as a dimer in solution (Figure 1b).



111
 112 **Figure 1. The periplasmic domain of ExbD forms a homodimer.** **a** The Ton system consists of an inner
 113 membrane proton channel – formed by ExbB (grey) and ExbD (pink) – and the periplasm spanning protein TonB.
 114 (blue). The latter links the system to a TonB-dependent transporter in the outer membrane. The globular C-terminal
 115 domain of ExbD (pink boxes) is connected to its helical N-terminus, which is inserted into the ExbB channel, by a
 116 disordered region (pink, wiggly lines). **b** Sedimentation coefficient distribution obtained from analytical
 117 ultracentrifugation of ExbD^{Sm, peri} and ExbD^{Ec, peri} indicates that both proteins exist in a dimeric state at physiological
 118 pH. The red dashed lines represent estimated sedimentation coefficients for monomeric ExbD and dimeric ExbD
 119 as depicted by the cartoons (pink). The theoretical molecular weights of both species are indicated above. **c** The
 120 top view of the NMR structure ensemble of dimeric ExbD^{Sm, peri} reveals that the N-terminal residues 44-49 (green)
 121 form an intermolecular β -strand. **d** Homodimeric ExbD^{Sm, peri} consists of two monomers (pink and light pink), each
 122 with a β -sheet (arrows) and two α -helices (barrels). Part of the dimeric interface is formed by a swapped,
 123 intermolecular and anti-parallel β -sheet called the N-terminal Inter-molecular Beta-Strand (NIBS, green). The NIBS,
 124 composed of residues 44-49, connects the intramolecular β -sheets, creating a continuous β -sheet across the dimer
 125 interface. In the full-length protein, ExbD^{Sm, peri} is N-terminally connected to two α -helices (α 1) that are embedded
 126 in the ExbB channel (grey). **e** Side view and **f** Top view of the AlphaFold2 model of the full ExbB-ExbD complex,
 127 generated using the NMR structure (PDB ID 8PEK, this work) as a template. In this model, the dimeric organization
 128 of the periplasmic domain of ExbD (pink and light pink) imposes the alignment of its helices in similar positions and
 129 orientations, and locking both Asp 25 sidechains in hydrogen bonds with Thr 218 from ExbB. In the top view, the
 130 periplasmic domain of ExbD is not visible due to its location above the clipping plane.

131 We proceeded our structural study with ExbD^{Sm, peri} as NMR fingerprint spectra indicated that
 132 ExbD^{Ec, peri} suffers from line broadening due to conformational exchange dynamics, while the
 133 *S. marcescens* protein revealed high-quality spectral data (Figure S1-S2). The solution
 134 structure of ExbD^{Sm, peri} was determined as detailed in the Methods and resulted in a swapped
 135 homodimer (Figure 1c and Table S3). Each protomer is composed of a four-stranded β -sheet
 136 on one side and two α -helices on the other (Figure 1d). The dimeric interface is established
 137 through i) a network of hydrophobic interactions centered around the sidechain of Tyr 112,
 138 and ii) a swapped, intermolecular and anti-parallel β -sheet, referred to as the N-terminal

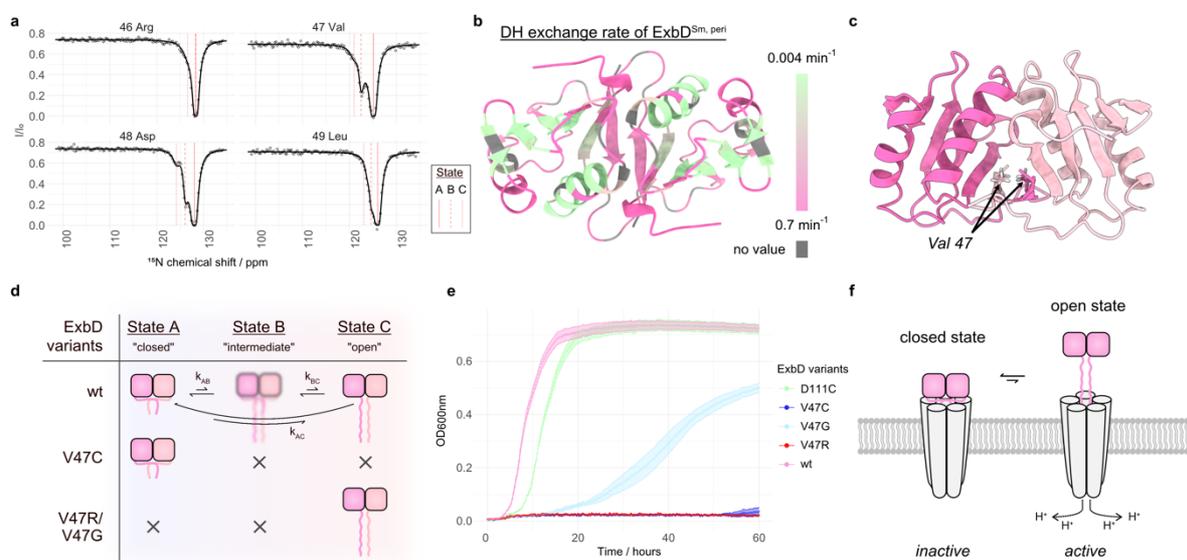
139 Intermolecular Beta-Strand (NIBS). The NIBS, formed by residues 44-49, links the β -sheets
140 of each protomer creating a continuous, intermolecular β -sheet. This structure exhibits
141 structural similarity with a crystal structure obtained for the periplasmic domain of TolR, the
142 ExbD counterpart in the Tol-Pal system.²⁴ However, this dimeric organization has not been
143 observed in the only known ExbD^{Ec, peri} structure that was shown to be monomeric.¹⁰ This
144 structure was solved at pH 3 and its NIBS region is disordered. Here, our experiments were
145 performed at pH 7, and the ordered nature of the NIBS was also confirmed by relaxation
146 measurements, as shown in Figure S3.

147 To contextualize the dimeric structure of ExbD^{Sm,peri} within the whole assembly, we
148 used our solution structure as a template to generate a model of the full ExbB-ExbD complex
149 with AlphaFold2 (Figure 1e, f).²⁶ In the published cryo-EM structures, the ExbB-embedded
150 helices of ExbD are not equivalent, displaying differences regarding their height and rotation
151 towards each other as they are shifted by half a helical turn. Also it has to be noted, that in
152 these structures only one of the two Asp 25 of ExbD is involved in hydrogen bond formation,
153 leaving the other Asp 25 proposedly “ready” to accept a new proton for conduction.^{5,7}
154 However, in the generated model with a dimeric organization of the periplasmic domain of
155 ExbD, the helices are aligned in similar positions and orientations, directing both Asp 25
156 sidechains towards Thr 218 with a distance compatible with the formation of a hydrogen bond.
157 Based on these observations, we postulate that this model, featuring both Asp 25 sidechains
158 engaged in hydrogen bonds, could represent an inactive, proton-impermeable state of the
159 channel.

160

161 ExbD samples different conformational states

162 The absence of the periplasmic domain of ExbD in the cryo-EM reconstructions
 163 indicates that the dimer undergoes extensive conformational dynamics and does not simply
 164 occupy a single state as the NMR structure might imply. To characterize these conformational
 165 dynamics and to understand how the protein can fulfill its function, we explored the dynamical
 166 landscape of the periplasmic domain of ExbD. For this purpose, we analyzed ExbD^{Sm, peri} by
 167 ¹⁵N chemical exchange saturation transfer (CEST) experiments, which allow for the
 168 characterization and quantification of different exchanging conformational states of a protein
 169 – with populations as sparse as less than 1 % – that are in slow exchange (lifetimes ranging
 170 from ~5-50 ms).²⁷ The per-residue CEST profiles for the NIBS residues are characteristic of
 171 an exchange between three conformational states with a major state A (large dip) and two
 172 minor conformational states B and C (smaller dips) (Figure 2a and Figure S4).



173

174 **Figure 2. The periplasmic domain of ExbD samples different conformational states.** **a** ¹⁵N-Chemical exchange
 175 saturation transfer (CEST) profiles reveal a 3-state exchange of the NIBS in ¹⁵N-labeled ExbD^{Sm, peri}. The
 176 experimental data points and the least-square fit are represented by dots and solid lines, respectively. The fitted
 177 chemical shift values for states A, B, and C are indicated by red lines. Intensity ratios of NMR signals from residues
 178 46 to 49 are shown. **b** Deuterium hydrogen (DH) exchange rates measured by NMR spectroscopy mapped onto
 179 the structure of ExbD^{Sm, peri} (fast exchange: pink, slow exchange: green, no value: grey) show that the NIBS
 180 undergoes faster amide proton exchange than adjacent β -strands, supporting the disordered nature of state C. **c**
 181 Side view of dimeric ExbD^{Sm, peri} where the monomeric units are colored in pink and light pink. The residue Val 47
 182 is located at the dimer interface in the closed state. **d** Wild type (wt) ExbD^{Sm, peri} (pink) samples a major state A,
 183 and the minor states B and C. State A is characterized by a folded NIBS, while state C features a disordered NIBS.
 184 State B represents an intermediate state between these two extremes. The V47C mutant forms a disulfide bridge
 185 that locks ExbD^{Sm, peri} in the closed state, while the V47R and V47G mutants mainly yield the open state. **e** Bacterial
 186 growth curves of ExbD^{Sm, peri} variants wt (pink), V47R (red), V47G (light blue), V47C (blue) and the control D111C
 187 (green). Under *in vivo* conditions that necessitate the activity of the Has system, these mutations substantially
 188 impact bacterial growth, emphasizing that not merely the individual states of ExbD, but the exchange between
 189 them, is vital for the system's proper functioning. **f** In a schematic representation of the ExbB-ExbD complex, the
 190 ExbD dimer exchanges between a closed, main state and a sparsely populated open state. The transition between
 191 the states is characterized by the un- and refolding of the NIBS residues. Herein, only the open state might be
 192 permeable to protons.

193 We utilized the software package ChemEx²⁸ to fit the profiles and extract exchange
 194 parameters, including the chemical shifts of the minor conformations (Table S4). Intriguingly,
 195 upon re-examination of 2D TROSY spectra, we were able to identify the resonances for state
 196 C as low signal-to-noise peaks (Figure S5). Additionally, deuterium-hydrogen exchange

197 spectroscopy of ExbD^{Sm, peri} revealed that the NIBS is more accessible to the solvent than the
198 adjacent β -strands (Figure 2b and Figure S6). This observation, in conjunction with the
199 chemical shifts of state C, indicates that state C is an “open” state characterized by the
200 disordered nature of the NIBS residues, as opposed to the “closed” state A, where those
201 residues form an intramolecular β -sheet, as shown in the NMR structure. It is important to note
202 that the dimeric structure of ExbD^{Sm, peri} is maintained during this opening process. We
203 hypothesize that state B marks an intermediate state between those extrema, as its chemical
204 shifts lie in all cases in between those of state A and state C. We note that a similar
205 conformational exchange process is present in ExbD^{Ec, peri}, however, for this species, the
206 exchange resides in the intermediate NMR timescale (μ s-ms), as observed in the fingerprint
207 spectra (Figure S2).

208 To confirm the exchange driven order-to-disorder transition of the NIBS, we designed
209 Val 47 mutants for both ExbD^{Sm, peri} and ExbD^{Ec, peri}. In the structure of ExbD^{Sm, peri} wild type
210 (wt), this residue at the dimer interface occupies a hydrophobic pocket and is in close spatial
211 proximity to the corresponding Val 47 from the other protomer within the homodimer (Figure
212 2c). The mutant V47C is thought to form a disulfide bridge locking the subunits together and,
213 hence, mimicking a pure closed state. Conversely, V47R and V47G both would lead to a dimer
214 with an unfolded NIBS as the arginine sidechain cannot occupy the hydrophobic pocket and
215 the glycine perturbs the stability of the NIBS – resulting in a mainly open state (Figure 2d).
216 These behaviors were confirmed by NMR spectroscopy, which can be briefly summarized as
217 follows: a) The V47C mutation of ExbD^{Sm, peri} shows only small chemical shift perturbations in
218 comparison to the wt, indicating that the main conformation of ExbD^{Sm, peri} is indeed the closed
219 state. b) The V47C mutation of ExbD^{Ec, peri} results in NMR spectra absent of conformational
220 exchange. c) The V47R and V47G mutants of both ExbD^{Sm, peri} and ExbD^{Ec, peri} exhibit NIBS
221 residues with chemical shifts indicative of a disordered state (Figure S7-9).

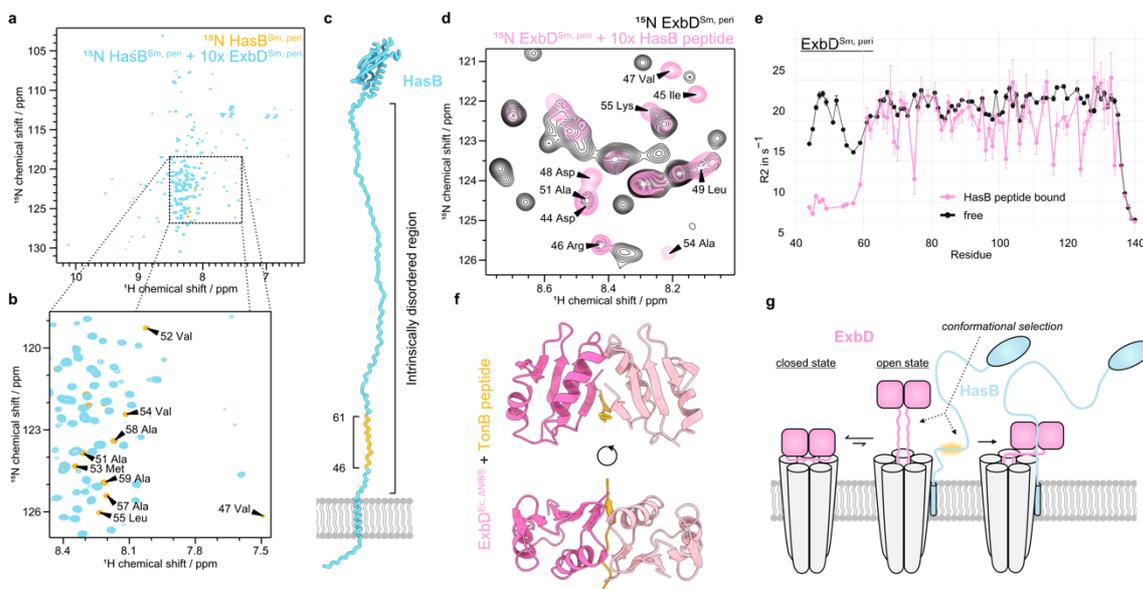
222 Having established ExbD mutants that mainly occupy either the open (V47R/V47G) or
223 closed state (V47C), we proceeded to study the impact of these mutations on the activity of
224 the Has system *in vivo*. For that purpose, we monitored the growth of the Ton system-deficient
225 *E. coli* strain K12 C600 Δ hemA Δ tonB Δ exbBD, which requires external heme as an iron source
226 for growth. This deficiency was compensated by two plasmids, one encoding for the entire
227 Has operon (hasISRADEB) from *S. marcescens* and a second one for ExbB-ExbDSm. This
228 setup allowed for a direct correlation of bacterial growth and the efficiency of heme import and,
229 hence, reflecting the correct functioning of the Has system. We also verified the assembly of
230 the wt and mutant ExbB-ExbD complexes and confirmed that the proteins are expressed at
231 comparable levels in the membrane, ensuring that the observed effects on bacterial growth
232 are attributable to the specific mutations and their impact on the ExbD conformational
233 exchange (Figure S10). Consequently, we could analyse the influence of ExbD adopting
234 exclusively the open or closed state on the functioning of the Has system. Compared to the
235 wt and the control mutant D111C (a residue far from the NIBS), V47C and V47R completely
236 abolish growth, while V47G significantly reduces growth (Figure 2e). These results
237 demonstrate that neither the open state nor the closed state of ExbD alone, but rather the
238 exchange between them, is crucial for the mechanism of the Has system, potentially reflecting
239 distinct steps in the mechanism. Systematic mutations and *in vivo* photo crosslinking
240 experiments have previously suggested the functional role of the ExbD NIBS also in *E. coli*²⁹,
241 further emphasizing the importance of the opening and closing of ExbD as a general
242 mechanism.

243 In the context of the full ExbB-ExbD complex, we hypothesize that the proton channel
244 would predominantly occupy a main state with ExbD in a closed conformation, while a sparsely
245 populated state would feature ExbD in an open conformation (Figure 2f). The closed state of
246 ExbD would thus represent the inactive, impermeable state of the ExbB-ExbD proton channel,
247 as it should be predominantly closed to prevent continuous proton leakage. Conversely, the
248 open state of ExbD is thought to represent the active, proton-permeable state.

249

250 The open state of ExbD is conformationally selected upon TonB binding

251 To explore the interaction between HasB/TonB and ExbD, we analyzed 2D [^1H - ^{15}N]-
 252 TROSY spectra of ^{15}N -labeled HasB^{Sm, peri} (residues 37-263) and ^{15}N -labeled TonB^{Ec, peri}
 253 (residues 34-239) upon addition of their unlabeled partner ExbD^{Sm, peri} and ExbD^{Ec, peri}
 254 respectively (Figure 3a and Figure S11a).



255

256 **Figure 3. The open state of the periplasmic domain of ExbD is conformationally selected upon binding to**
 257 **TonB/HasB.** **a,b** 2D [^1H - ^{15}N]-TROSY spectra of ^{15}N -labeled HasB^{Sm, peri} alone (orange) and in the presence of
 258 unlabeled ExbD^{Sm, peri} (blue). The presence of ExbD leads to the disappearance (broadening) of the peaks
 259 corresponding to the residues 46-61 of HasB^{Sm, peri} indicating binding of this region to ExbD. **c** Model of HasB
 260 inserted in the inner membrane: The HasB-ExbD region of interaction (orange) is located in an intrinsically
 261 disordered region (IDR) of HasB (blue) close to the inner membrane inserted N-terminal α -helix. **d** 2D [^1H - ^{15}N]-
 262 HMQC spectra of ^{15}N -labeled ExbD^{Sm, peri} without (black) and with (pink) the HasB peptide (corresponding to the
 263 binding region on the HasB side). Binding of the peptide leads to signal intensity decrease and appearance of new
 264 peaks that can be assigned to the NIBS residues. **f** R_2 relaxation rate measurements of the free (black) and HasB
 265 peptide bound state (pink) of ^{15}N ExbD^{Sm, peri} reveal that the binding stabilizes the open state of ExbD^{Sm, peri}
 266 characterized by the disordered nature of the NIBS residues (lower relaxation rates). This suggests a
 267 conformational selection of the open state of ExbD. **e** Deletion of the NIBS residues of ExbD^{Ec, peri} allows for
 268 crystallization with a peptide corresponding to the binding region on the TonB side. Herein, the TonB peptide
 269 (orange) replaces the NIBS residues at the interface in between the two subunits of the ExbD dimer (pink, light
 270 pink). **g** Schematic mechanism: The open state of ExbD (pink, right side) is conformationally selected by
 271 TonB/HasB (blue) leading to a disorder-to-order transition of the IDR of TonB/HasB. The binding motif on the
 272 TonB/HasB side is highlighted in orange.

273 A close examination of the central region of the spectra shows the disappearance of peaks
 274 corresponding to an N-terminal IDR of HasB and TonB (Figure 3b-c and Figure S11b-c),
 275 confirming their interaction with ExbD. To examine the changes on the ExbD side, we further
 276 analyzed 2D [^1H - ^{15}N]-HMQC spectra of ^{15}N -labeled ExbD^{Sm, peri} and ^{15}N -labeled ExbD^{Ec, peri}
 277 with and without the respective HasB/TonB peptides that correspond to the binding regions.
 278 Peptide addition not only causes line broadening – indicative of interaction and conformational
 279 exchange between the peptide bound and free state – but also leads to the emergence of
 280 novel peaks in the center of the spectrum (Figure 3d and Figure S12). Interestingly, these new
 281 peaks can be assigned to the NIBS region, as they show the same chemical shifts as the NIBS
 282 residues in the minor, open conformation (State C) observed in the ^{15}N -CEST experiment

283 (Figure 2a). Additionally, low R_2 relaxation rates confirm the disordered nature of the NIBS
284 residues in the HasB-bound state (Figure 3e).

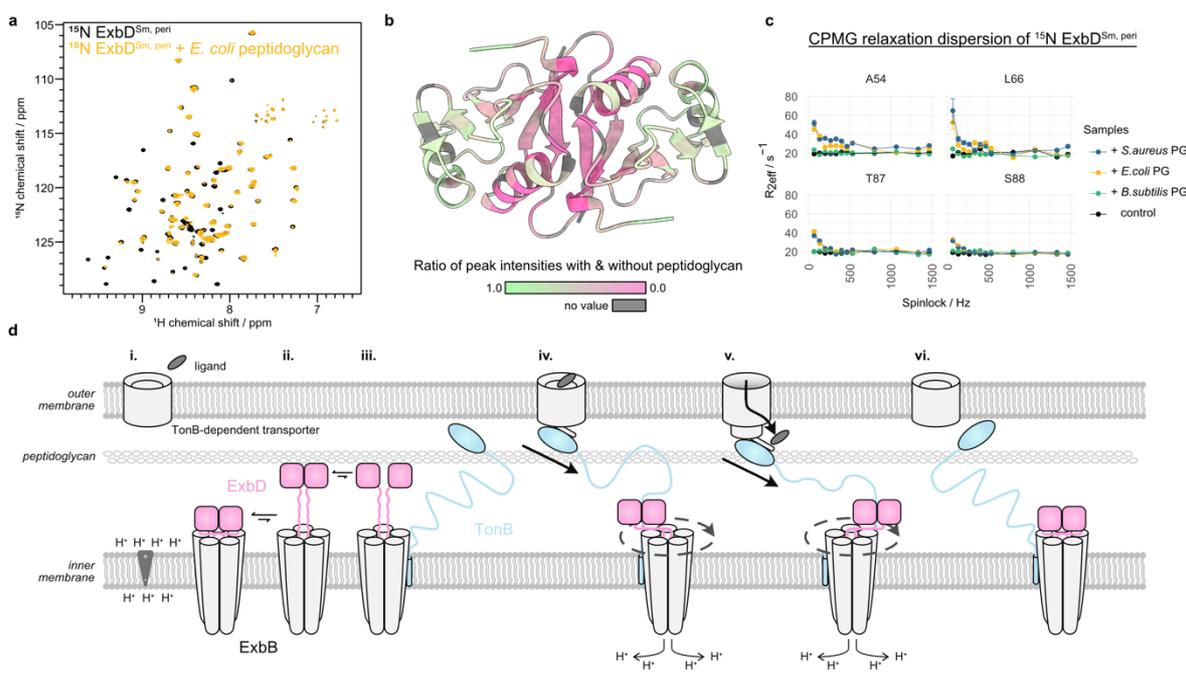
285 Herein, we initially hypothesized that TonB/HasB might bind between the two
286 protomers of ExbD, effectively replacing the NIBS residues at the dimer interface. This idea
287 was driven by our observation that the NIBS residues unfold upon addition of TonB/HasB,
288 implying a potential competition between the NIBS residues and HasB/TonB for the same
289 binding site. To investigate this possibility, we solved the crystal structure of an ExbD construct
290 lacking the N-terminal residues 43-60 (ExbD^{Ec, ΔNIBS}) in complex with the corresponding TonB
291 peptide at 1.5 Å resolution. (Figure 3e and Table S5). The crystal structure confirms that the
292 IDR of TonB undergoes a disorder-to-order transition upon binding to ExbD, replacing the
293 NIBS residues in between the two ExbD protomers. In this arrangement, the TonB peptide
294 forms an intermolecular β -sheet: A parallel β -sheet with the β 6-strand of one protomer, and
295 an antiparallel β -sheet with the β 6-strand of the second one (Figure S13).

296 Our results support that in the context of the full ExbB-ExbD complex, ExbD exchanges
297 between a closed state and a sparsely populated open state. Upon encountering HasB/TonB,
298 this open state is conformationally selected through the binding of HasB/TonB into the groove
299 between the two subunits, which was previously occupied by the now-unfolded NIBS (Figure
300 3g). This would mark a critical first step in the activation of the system.

301

302 ExbD interacts transiently with the periplasmic peptidoglycan layer

303 Since HasB/TonB and ExbD reside in the periplasm in proximity to peptidoglycan, we
 304 explored the role of peptidoglycan in the system. For that purpose, we studied 2D [^1H - ^{15}N]
 305 spectra of ^{15}N -labeled ExbD^{Sm, peri} and ExbD^{Ec, peri} in the presence and absence of different
 306 peptidoglycan sacculi. Upon addition of *E. coli* peptidoglycan (Figure 4a and Figure S14) some
 307 peaks loose intensity, indicating the emergence of peptidoglycan-binding induced
 308 conformational exchange. Notably, this binding is transient since fully peptidoglycan-bound
 309 ExbD would not be visible by solution NMR spectroscopy due to the megadalton size of
 310 sacculi.



311

312 **Figure 4. The periplasmic domain of ExbD interacts with the peptidoglycan layer.** **a** 2D [^1H - ^{15}N]-TROSY
 313 spectra of ^{15}N ExbD^{Sm, peri} without (black) and with (orange) *E. coli* peptidoglycan. The interaction of ExbD^{Sm, peri}
 314 with peptidoglycan leads to the disappearance (broadening) of peaks. **b** Mapping this peak intensity loss in terms
 315 of a peak height ratio (pink to green, grey color means no value) onto the dimeric ExbD^{Sm, peri} structure shows that
 316 the interface within the dimer experiences the largest increase in exchange contribution to the R_2 relaxation rate
 317 upon interaction with peptidoglycan. This suggests that possibly the dimer dissociates upon interaction. **c** This
 318 increase in R_2 relaxation rate due to exchange can be measured by Car-Purcell-Meiboom-Gill (CPMG) relaxation
 319 dispersion experiments and reveals that ExbD^{Sm, peri} selectively interacts with peptidoglycan from *E. coli* (orange),
 320 *S. aureus* (blue) – they all show decaying profiles – but not *B. subtilis* (green), which shows the same flat curve as
 321 the control experiment without peptidoglycan (black). **d** Schematic representation of the full mechanism. In the
 322 resting state in the absence of ligand (i.), closed ExbD (pink) is in exchange with a minor open conformation (ii.).
 323 This extended, open conformation can reach the peptidoglycan layer, in interaction with which its dimer interface
 324 is loosened (iii.). This allows TonB/HasB (blue) to enter in between the ExbD monomers and to subsequently select
 325 the open state of ExbD by binding to it (iv.). Also, the C-terminal domain of TonB/HasB interacts with a ligand (grey)
 326 bound TonB-dependent transporter (TBDT). Proton motive force induced rotation of ExbD within ExbB leads to a
 327 pulling through the IDR of TonB/HasB opening the TBDT and enabling active transport of ligands (v.). Eventually,
 328 the pulling force becomes too large and leads to the dissociation of TonB/HasB from the TBDT, and the return to
 329 the resting state of the system (vi.).

330 Mapping the decrease of peak intensities of ExbD^{Sm, peri} onto the ExbD structure, shows
 331 that the dimeric interface is primarily affected by this exchange (Figure 4b). Consequently, we
 332 propose that upon binding to peptidoglycan the dimeric interface of ExbD is reorganized. This
 333 reorganization would require a prior dissociation of the dimer. Consistent with this, the
 334 disulfide-bridged ExbD^{Sm, peri} V47C mutant, which cannot undergo dimer dissociation, failed to

335 interact with peptidoglycan (Figure S15). To pinpoint the interaction interface on the
336 peptidoglycan side, we further studied the interaction of ExbD^{Sm, peri} with *Staphylococcus*
337 *aureus* and *Bacillus subtilis* peptidoglycan. Compared to the peptidoglycan of *E. coli*, *S. aureus*
338 possesses chemically different peptide stems whereas *B. subtilis* features glycans with a non-
339 acetylated glucosamine moiety.³⁰ Notably, among the peptidoglycans tested here, only the
340 one from *B. subtilis* does not interact with ExbD^{Sm, peri}, which pinpoints the interaction site on
341 the peptidoglycan to the glycan chains (Figure S16). Additionally, peptidoglycan digested with
342 mutanolysin, a muramidase, does not show any interaction with ExbD, further supporting this
343 hypothesis (Figure S17). Furthermore, NMR relaxation dispersion experiments showed an
344 increase in effective R₂ relaxation rates upon the addition of peptidoglycans from *E. coli* and
345 *S. aureus*, but not from *B. subtilis* (Figure 4c and Figure S18-19).

346 DISCUSSION

347 We have elucidated the dimeric structure of the periplasmic domain of ExbD, which
348 was previously unresolved in cryo-EM studies. The observed dimeric form is consistent with
349 the two N-terminal, ExbB-integrated α 1 helices of ExbD present in the cryo-EM maps. We
350 have also demonstrated by NMR spectroscopy that the periplasmic domain of ExbD
351 undergoes extensive conformational exchange on the millisecond timescale, sampling a
352 primary closed state and at least one secondary open state with an unfolded NIBS region.
353 This dynamic nature of the protein might explain its invisibility in the cryo-EM densities. The
354 open state of ExbD is selectively bound by an IDR of the partner proteins TonB/HasB, which
355 undergo a disorder-to-order transition as they bind between the two ExbD protomers, as
356 evidenced by X-ray crystallography. Moreover, we have revealed that the periplasmic domain
357 of ExbD selectively interacts with specific peptidoglycan species, most likely promoting dimer
358 dissociation.

359 Taking our results together, we propose the following mechanism of action for the Ton
360 system: A resting state is characterized by a unliganded TBDT (Figure 4d, stage i). Within the
361 ExbB-ExbD complex, dimeric ExbD predominantly exists in a closed conformation and a
362 sparsely populated open conformation (Figure 4d, stage ii.) as we have shown by NMR
363 spectroscopy. The closed conformation likely represents the inactive, proton impermeable
364 state of the proton channel. The sparsely populated open state of ExbD is triggered by the
365 unfolding of the NIBS region, which subsequently reveals TonB/HasB-binding region between
366 the ExbD protomers. Yet, even in this state, the TonB/HasB-binding region in between the
367 ExbD dimer remains inaccessible. This is due to structural organization of the ExbD-ExbB
368 complex: The N-terminal α 1-helices of each protomer are inserted into the ExbB channel, and
369 the C-terminal periplasmic domain of ExbD forms a tight dimer. However, the adaptation of
370 the sparsely populated open state following the unfolding of the NIBS region brings ExbD into
371 close proximity with the peptidoglycan layer. Here, ExbD binds to the glycan chains of the
372 peptidoglycan. This binding event imposes the reorganization of the dimeric interface of ExbD
373 and, hence, a dimer-to-monomer dissociation – permitting the penetration and interaction of
374 TonB/HasB in between the ExbD protomers (Figure 4d, stage iii). The transient opening and
375 the dynamics of the ExbD is thus a critical prerequisite for TonB/HasB binding and,
376 consequently, the active transport of nutrients.

377 In the Tol-Pal system, dimeric TolR, a ExbD structurally homologous protein, was
378 shown to form a complex with peptidoglycan. However, this complex formation is only possible
379 when the β 6-strand at the dimeric interface is removed yielding a different dimeric interface

380 and organization, proposedly exposing a peptidoglycan binding motif.^{24,31} This is consistent
381 with our observation that the peptidoglycan-interaction strongly affects the ExbD^{Sm, peri} dimer
382 interface. However, in our case, the presence of $\beta 6$ renders the interaction with peptidoglycan
383 more transient as we see an exchange between bound and unbound ExbD (Figure 4a).

384 Upon ligand binding to the TBDT, its N-terminal extension containing the TonB box
385 extends into the periplasm, recruiting the TonB protein. Intriguingly, molecular dynamics
386 simulations of the IDR of TonB have shown that its Glu-Pro and Lys-Pro repeats can form a
387 hairpin structure.¹⁷ This configuration may serve to sterically conceal the ExbD-binding motif
388 of TonB. We hypothesize that with its anchoring points on ExbB within the inner membrane
389 and on the TBDT in the outer membrane, the IDR of TonB undergoes tension as both
390 anchoring points diffuse in their respective membranes. This tension is likely to destabilize the
391 hairpin structure, thereby unmasking the binding motif. This newly accessible motif engages
392 with the open state of ExbD, conformationally selecting its open state. This interaction
393 effectively stabilizes the ExbB-ExbD complex in an active, proton-permeable state (Figure 4d,
394 stage iv). However, in a single-target system such as the Has, the high affinity of HasB for the
395 TBDT (nM K_d versus μ M for TonB-TBDTs) suggests that HasB remains associated to the
396 TBDT even in the absence of the ligand. Here, signaling of ligand-binding is proposedly
397 transmitted via a stabilization of the HasB-TBDT interaction by involving more polar contacts,
398 as shown in our previous study.¹⁹

399 After the stabilization of the ExbB-ExbD complex in the active state, the PMF is
400 translated into a rotation of ExbD within ExbB through proton translocation via the de- and
401 reprotonation of Asp 25 of ExbD (Figure 4d stage iv). This results in a wrapping of the
402 intrinsically disordered linker of HasB/TonB around ExbD, exerting a pulling force on the TonB
403 box of the TBDT. This force was shown to be sufficient in unfolding and removing the plug
404 domain from the TBDT³², culminating in ligand import into the periplasm (Figure 4d, stage v).
405 Finally, due to the exerted force, TonB/HasB dissociates from the TBDT. This marks the return
406 to the resting state of the Ton system (Figure 4d, stage vi). This mechanism extends the wrap-
407 and-pull model recently proposed by Ratliff *et al.*¹⁸

408 Our study uncovers a conserved interaction between HasB/TonB and the open state
409 of ExbD in both single- and multi-target systems. However, we showed that the transition
410 between the open and closed states of ExbD operates at distinct rates in these systems: on
411 the slow NMR time scale (ms) for the single-target Has system and in the intermediate time
412 scale (μ s-ms) for the multi-target Ton system. This faster rate of conformational exchange in
413 the *E. coli* Ton system may indicate a capacity for more rapid adaptation, an attribute beneficial
414 for catering multiple TBDTs (up to 9 have been reported³³), unlike the single-target HasB.

415 In homologues of ExbD of other PMF-dependent motors involved in membrane
416 integrity (Tol-Pal system) and gliding motility (AglQRS system)¹¹, the NIBS residues are highly
417 conserved, suggesting a common mode of operation involving multiple states due to the
418 unfolding of the NIBS. This extends to the ExbD-binding motif of TonB homologues, indicating
419 a conserved mechanism of energy transduction from the proton channel to the outer
420 membrane (Figure S20-21). We speculate that stabilizing the open state of ExbD or its
421 homologues with a drug binding in between the protomers could not only inhibit the vital
422 functioning of these motors but also lock the proton channel complexes into an active, proton
423 permeable state, creating a fatal proton leakage in the inner membrane.

424 METHODS

425 Strains and plasmid construction

426 Strains, plasmids, and oligonucleotides are shown in Table S6. To produce ExbD
427 mutants, the pBADexbBD_{Sm} or the pBAD24exbBDhis6_{Sm} plasmids were respectively amplified
428 by PCR with oligonucleotides couples ExbDV475'/ ExbDV47C, ExbDV475'/ ExbDV47G,
429 ExbDV475'/ ExbDV47R and ExbDE1115'/ ExbDD111C. The PCR product was digested with
430 *DpnI*, self-ligated and transformed in XL1-Blue. Recombinant clones were isolated and verified
431 by sequencing.

432 Protein expression and purification

433 Protein samples used in this study are summarized in Table S1. All ExbD variants and
434 TonB^{Ec, peri} contain an N-terminal His-tag followed by a TEV cleavage site. The corresponding
435 genes were synthesized and cloned by ProteoGenix into pet-30a(+) vectors, which were used
436 to transform *E. coli* BL21 (DE3). For protein expression, glycerol stocks of transformed bacteria
437 were used to inoculate 10 mL Luria-Bertani (LB) medium, which was grown for 8 h at 37 °C.
438 In a second step, 100 mL M9 medium containing 4 g/L ¹³C-glucose and 1 g/L ¹⁵NH₄Cl as the
439 only carbon and nitrogen sources were added and the culture was incubated overnight at 30
440 °C. The next morning, 1 L fresh M9 medium was inoculated with the overnight culture to a
441 starting optical density at 600 nm (OD) of 0.1. The bacterial cultures were grown to an OD of
442 0.7 at 37 °C and the expression was induced by the addition of 0.5 mM IPTG (isopropyl β-D-
443 1-thiogalactopyranoside). Protein expression was conducted for 5 h at 37 °C (ExbD variants
444 and TonB^{Ec, peri}) or for 20 h at 30 °C (HasB_{37-263, Sm}). For non-NMR studies, all protein
445 expression steps were conducted in LB medium instead of M9 medium. The bacterial cells
446 were harvested by centrifugation at 7000xg for 15 min at 37 °C, resuspended in Buffer A (20
447 mM sodium phosphate, 30 mM imidazole, 500 mM NaCl, pH 7.4) containing one EDTA-free
448 protease inhibitor pill (Roche) per 25 mL and stored at -80 °C.

449 For expression of deuterated protein used for the detection of hydrogen bonds by NMR
450 spectroscopy, bacteria were adapted to and grown in fully deuterated M9 medium containing
451 ¹³C,²D₇-glucose and ¹⁵ND₄Cl as the only carbon and nitrogen sources.

452 For protein purification, the bacterial cell pellets were thawed and 1.5 μL benzonase
453 (Millipore) were added per 25 mL of cell suspension to digest nucleic acids. Cells were
454 disrupted by sonication with a Vibracell 72405 sonicator (2s on, 1 s off, 80% amplitude and a
455 total time of 20 min). Insoluble cell debris was removed from the soluble protein-containing
456 supernatant by centrifugation at 30.000xg at 4 °C for 30 min and by sterile filtration through a
457 0.22 μm membrane. By means of an Akta system, the lysate was loaded onto a pre-
458 equilibrated (Buffer A: 20 mM sodium phosphate, 30 mM imidazole, 500 mM NaCl, pH 7.4)
459 nickel affinity chromatography column (two stacked 5 mL HisTrap Chelating HP, Cytiva),
460 washed with 5 column volumes (CV) Buffer A and eluted with a linear gradient of 0-100 %
461 Buffer B (20 mM sodium phosphate, 500 mM imidazole, 500 mM NaCl, pH 7.4) over 5 CV.
462 The protein containing fractions as judged by SDS-PAGE were united and diluted (at least
463 10x) in TEV buffer (50 mM Tris-HCl, 1 mM dithiothreitol, pH 8.0). 1 mg of TEV protease was
464 added per 100 mg of target protein and cleavage of the His-tag was conducted at 34 °C for 2
465 h. Then the mixture was loaded onto a HisTrap HP column to remove TEV-His₆ and un-

466 cleaved proteins. The target protein containing fractions from the washing step were pooled,
467 concentrated to about 1.5 mL and injected with a 2 mL loop onto a pre-equilibrated (Buffer C:
468 50 mM sodium phosphate, 50 mM NaCl, pH 7.0) size-exclusion chromatography column
469 (HiLoad 16/60 Superdex 75 pg, Cytiva or HiPrep 16/60 Sephacryl S-100 HR, Cytiva) and
470 eluted with 1.5 CV Buffer C. The fractions containing the target protein were pooled, aliquoted,
471 flash-frozen in liquid nitrogen and stored at -20 °C. For X-ray crystallography sample
472 preparation, Buffer D (50 mM Tris-HCl, 50 mM NaCl, pH 7.0) was used for size-exclusion
473 chromatography.

474 For the His-tag lacking protein HasB^{Sm, peri}, the nickel affinity column steps were
475 replaced by a cation-exchange chromatography step. For that purpose, bacterial cells were
476 resuspended in Buffer E (50 mM Tris HCl, 100 mM NaCl, pH 8.8) after harvest. For purification,
477 the disrupted lysate was loaded onto a pre-equilibrated (Buffer E: 50 mM Tris HCl, 100 mM
478 NaCl, pH 8.8) cation-exchange chromatography column (SP Sepharose HP 16/60, Cytiva),
479 washed with 10 CV Buffer E and eluted with a linear gradient of 0-100 % Buffer F (50 mM Tris
480 HCl, 1000 mM NaCl, pH 8.8) over 15 CV. The protein containing fractions as judged by SDS-
481 PAGE were united, concentrated and submitted to size-exclusion chromatography as
482 mentioned above.

483 To acquire intermolecular NMR restraints, a sample containing 50% ¹⁵N, ¹³C-ExbDSm,
484 ^{peri} and 50% ¹⁴N, ¹²C-ExbD^{Sm, peri} (50% labeled – 50% unlabeled) was prepared. For that
485 purpose, the two batches of purified protein were mixed in equimolar quantities, denatured in
486 8 M Urea and subsequently refolded by dialyzing to Buffer C (3x 1 L Buffer C). Fingerprint
487 NMR spectra showed no difference between native and refolded proteins.

488 Analytical ultracentrifugation (AUC)

489 For sedimentation velocity experiments, ExbD^{Sm, peri} and ExbD^{Ec, peri} at concentrations
490 of 20 μM, 100 μM and 1000 μM were loaded into 3 mm or 1.2 cm centerpieces and centrifuged
491 overnight at 42000 rpm in a Beckman Coulter Optima AUC centrifuge operating with an AN60-
492 Ti rotor. Fitting of the data using a continuous size distribution c(S) model was conducted via
493 SEDFIT 15.1 with a confidence level (F-ratio) of 0.95. From the fit, sedimentation coefficients
494 at zero concentration in the buffer (50 mM sodium phosphate, 50 mM NaCl, pH 7.0) could be
495 calculated as well as molecular weights estimated.³⁴ Hydrodynamic parameters of the closed-
496 state ExbD^{Sm, peri} were calculated with Hydropro.³⁵ For the closed-state the NMR-structure
497 determined in this study was used, the sedimentation coefficient of monomeric ExbD was
498 calculated from PDB ID 2PFU.¹⁰

499 Solution NMR spectroscopy and resonance assignment

500 All used NMR samples are summarized in Table S2. All spectra were acquired using
501 Topspin4 on either a 600 MHz Avance III HD or a 800 MHz Avance NEO spectrometer
502 equipped with cryogenically cooled triple resonance ¹H[¹³C/¹⁵N] probes (Bruker Biospin). For
503 pulse calibration and setting up standard experiments NMRLib was used.³⁶ For spectral
504 fingerprinting, either 2D ¹⁵N-¹H SOFAST-HMQC or BEST-TROSY spectra were recorded.³⁷
505 For backbone assignments, generally 3D BEST-HNCA/-HNcoCA/-HNCO/-HNCACB/-
506 HNcoCACB and BEST-TROSY HNcaCO spectra were acquired with 25% non-uniform
507 sampling (NUS).³⁸ Sidechain assignments were conducted by the acquisition of 3D

508 HBHAcoNH and HCCH-TOCSY as well as 2D hbCBcgc dHD and 2D hbCBcgc dceHE spectra.
509 For structural restraints, 3D ¹⁵N-edited NOESY-HSQC, ¹³C^{aromatic}-edited NOESY-HSQC and
510 ¹³C^{aliphatic}-edited NOESY-HSQC, and, for intermolecular structural restraints, ¹³C-, ¹⁵N-filtered
511 3D NOESY-¹³C-HSQC and ¹³C-, ¹⁵N-filtered 3D NOESY-¹⁵N-HSQC spectra were recorded at
512 a B₀ field strength of 800 MHz. A mixing time of 120 ms was used for all NOESY spectra. For
513 the detection of hydrogen bonds, a 3D BEST-TROSY HNCO spectrum with a total N-CO
514 INEPT transfer period of 133 ms was acquired.³⁹ Spectral data were referenced to 2,2-
515 Dimethyl-2-silapentane-5-sulfonate (DSS), processed with NMRPipe⁴⁰ and in the case of NUS
516 reconstructed using SMILE⁴¹. Visualization of spectra, peak picking and resonance
517 assignments were conducted using CcpNmr version 2.5⁴² and version 3⁴³.

518 The final resonance assignment of ExbD^{Sm, peri} was deposited to the Biological
519 Magnetic Resonance Data Bank (BMRB) under the accession code 34826.

520 ¹⁵N-R₁ and -R₂ relaxation rates were measured by pseudo-3D experiments (¹H, delay,
521 ¹⁵N). For ¹⁵N-R₁ measurements the relaxation delays were 0, 20 70, 150, 200, 300, 400, 550,
522 700, 900, 1100, 1500, 1800 and 2000 ms in random order with a recycle delay of 5.5 s between
523 scans. For ¹⁵N-R₂ measurements the relaxation delays were 0, 66.67, 133.33, 200, 266.67,
524 400, 533.33, 800, 1066.67, 1333.33 and 1466.67 ms in random order with a recycle delay of
525 4.5 s between scans. Peak heights and errors were extracted with the non-linear N-
526 dimensional spectral modeling (nlinLS) program as part of the NMRPipe suite⁴⁰, and fitted to
527 a monoexponential function. Hereby, the errors were estimated by running 21 Monte Carlo
528 trials for each fit. For ¹⁵N[¹H] heteronuclear nuclear Overhauser effect (nOe) measurements,
529 two HSQC spectra without (reference) and with a ¹H saturation pulse (1 ms, 250 Hz), and with
530 a pre-saturation delay of 3 s and a recycle delay of 10.5 s were recorded. Peak heights and
531 errors were extracted from the two spectra with nlinLS and heteronuclear nOe values
532 calculated as the ratio of peak heights with and without saturation pulse.

533 Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion profiles were measured by
534 BEST-TROSY pseudo-3D experiments (¹H, ¹⁵N spinlock strength, ¹⁵N) at a B₀ field strength of
535 600 and 800 MHz. For these experiments the spinlock strengths were 0, 66.67, 133.33, 200,
536 266.67, 400, 800, 1066.67, 1333.33 and 1466.67 Hz in random order during a total relaxation
537 delay T_{relax} of 30 ms. Peak heights and errors were extracted from the spectral data with nlinLS
538 and used to calculate the spinlock dependent R_{2eff} rate following equation 1.

539
$$R_{2eff} = \frac{-\ln(I/I_0)}{T_{relax}}$$

540 (1)

541 The spinlock dependent R_{2eff} rates were fitted numerically to a two-state exchange with
542 ChemEx²⁸ by minimizing the target function χ^2 (See equation 2).

543
$$\chi^2 = \sum_i \left(\frac{I_i^{expt} - I_i^{calc}}{\sigma_i^{expt}} \right)^2$$

544 (2)

545 Chemical exchange saturation transfer (CEST) profiles were measured by two
546 pseudo-3D experiments (^1H , ^{15}N carrier position, ^{15}N) with ^{15}N spinlock strengths of 22.7 Hz
547 and 55.9 Hz applied for a duration T_{ex} of 400 ms at a B_0 field strength of 800 MHz. The CEST
548 dimension had in both cases a spectral width of 2854.25 Hz, and for 22.7 Hz spinlock strength
549 increments of 20.3 Hz and for 55.9 Hz spinlock strength increments of 40.6 Hz. The exact
550 spinlock strength was determined by an incremented nutation experiment.⁴⁴ Peak heights and
551 errors were extracted from the spectral data with nlinLS, and fitted to a three-state exchange
552 with ChemEx by minimizing the target function χ^2 (See equation 2). The errors were calculated
553 based on the scatter observed in the CEST profiles using the ChemEx function “scatter”.

554 For the Deuterium-Hydrogen exchange experiments, we used a ExbD^{Sm, peri} sample
555 that was previously exchanged to D_2O After 2 weeks, this sample was lyophilized overnight,
556 then redissolved in Buffer C (50 mM sodium phosphate, 50 mM NaCl, pH 7.0). The first SO-
557 FAST HMQC was recorded after 2 min 50 s with subsequent 99 time points of 2 min and 39
558 s. A final reference spectrum was recorded after 24 h. The peak intensities were extracted
559 with nlinLS and fitted with fitXY.tcl of the NMRpipe suite⁴⁰ to the complement of an exponential
560 decay of the form $F(t)=A[1-e^{-(k*t)}]$, where A is a normalization factor depending on the
561 maximal peak height, t the exchange time and k the exchange rate.

562 For examining the interaction between ExbD protein species and peptidoglycan,
563 peptidoglycan sacculi from *E. coli*, *B. subtilis* or *S. aureus* (InvivoGen) were washed twice with
564 Buffer C and added to a 100 μM protein-containing NMR sample. Simultaneously, a reference
565 sample – maintaining the same protein concentration but devoid of peptidoglycan – was also
566 prepared.

567 NMR structure calculation of the periplasmic domain of ExbD

568 NMR structure calculation was performed by the ARIAweb server using the standard
569 torsion angle simulating annealing protocol.⁴⁵ Firstly, the unambiguous intermolecular NMR
570 restraints were used to assign peaks in the 3D ^{15}N -edited NOESY-HSQC, $^{13}\text{C}^{\text{aromatic}}$ -edited
571 NOESY-HSQC and $^{13}\text{C}^{\text{aliphatic}}$ -edited NOESY-HSQC spectra. The rationale behind this was
572 that peak lists of the intermolecular NMR restraints cannot directly be used during the structure
573 calculation protocol because their sparsity makes them un-calibratable. Additionally, further
574 unambiguous peaks in the NOESY spectra were assigned. Torsion angles were predicted
575 based on assigned chemical shift values using TALOS-N.⁴⁶ Secondary structure information
576 was also used as “structural rules” in ARIA to initially prevent incorrect assignments of
577 intermolecular NOE as intramolecular. The CcpNmr project file containing all assigned
578 chemical shifts, NOESY peak lists and hydrogen bonds was uploaded to the ARIAweb server.
579 Here, 9 iterations of automatic peak assignment and calibration of the 3D ^{15}N -edited NOESY-
580 HSQC, $^{13}\text{C}^{\text{aromatic}}$ -edited NOESY-HSQC and $^{13}\text{C}^{\text{aliphatic}}$ -edited NOESY-HSQC spectra was
581 conducted in the following way: Manual assignments were used and flagged as reliable,
582 diagonal peaks were filtered, and structural rules enabled. The error of chemical shifts based
583 on the resonance assignment was used in addition to a ^1H chemical shift tolerance of 0.04/0.02
584 ppm in the indirect/direct dimensions and a $^{13}\text{C}/^{15}\text{N}$ chemical shift tolerance of 0.3 ppm. For
585 spin diffusion correction, a molecule correlation time of 10 ns was adopted. During structure
586 calculation, a C2 symmetry was applied along with NCS restraints⁴⁷, where the latter were
587 used only until iteration 4. Dihedral angles derived from the TALOS-N predictions were
588 supplied together with hydrogen bonds. In all 9 iterations 50 structures were calculated and
589 the 7 best structures (based on their total energy) were used for analysis. Torsion angle

590 simulating annealing was performed with 30000 high-temperature steps, 30000 cool1 steps
591 and 30000 cool2 steps, and a log-harmonic potential was applied using automatic restraints
592 weighting during the cool2 step of the simulated annealing.⁴⁸ The 10 best structures of the last
593 iteration were refined in an explicit shell of water molecules.⁴⁹ The structure calculation
594 protocol was iteratively repeated and some manual changes were applied to the NOE
595 assignments in between runs. In the end, a consensus structure was calculated following the
596 methodology reported.⁵⁰ Briefly, 20 independent ARIA runs were carried out using the same
597 input data but different random number seeds, generating varying random initial
598 conformations and velocities for the molecular dynamics simulated annealing protocol.
599 Subsequently, cross-peaks that remained active (i.e., those retaining at least one assignment
600 possibility) were collected at the conclusion of 12 out of the 20 ARIA runs. For each active
601 cross-peak, assignment possibilities from each individual ARIA run were combined to
602 generate a new list of consensus distance restraints. Lastly, a single iteration ARIA run was
603 conducted using the consensus distance restraints as input, resulting in the final consensus
604 structure ensemble.

605 The final structural ensemble of ExbD^{Sm, peri} and the restraints were deposited to the
606 Protein Data Bank (PDB) under the accession code 8PEK.

607 **Bacterial growth test**

608 Strain *E. coli* K12 C600 Δ hemA Δ exbBD Δ tonB(pAMhasISRADEB) was transformed
609 with plasmid pBADexbBD_{Sm} or its derivatives bearing mutation in *exbD*. A few colonies were
610 first inoculated in 3 ml of LB medium at 37°C with the corresponding antibiotics, and 40 μ M
611 dipyrityl, 4 μ g/ml arabinose. Once the culture reached an OD_{600nm} of ca. 1.2-1.5, it was diluted
612 and inoculated in 48 well Greiner plates, in the same medium to which was added 1 μ M He-
613 BSA, as a heme source. The initial OD_{600nm} of the cultures was 0.001. Each well contained
614 300 μ l of growth medium. Triplicates of each strain were made, and the plate was incubated
615 at 37°C with vigorous shaking (500 rpm) in a Clariostar Plus Microplate reader. OD_{600nm} was
616 recorded every 15 minutes for 60 hours.

617 **Isolation of the ExbBD_{sm} complex**

618 Strain *E. coli* K12 C600 Δ hemA Δ exbBD Δ tonB(pAMhasISRADEB) was transformed
619 with the pBAD24exbBDhis6_{Sm} plasmid or its variants pBAD24exbBDV47His6_{Sm},
620 pBAD24exbBDV47Ghis6_{Sm}, pBAD24exbBDV47Rhis6_{Sm}, and pBAD24exbBDD111His6_{Sm}.
621 For the expression of each variant, 200 ml of culture in LB at 37°C (in the presence of ampicillin
622 (100 μ g/ml), spectinomycin (75 μ g/ml), delta-aminolevulinic acid (25 μ g/ml) was induced with
623 40 μ g/ml arabinose at OD_{600nm} of 0.5 and the culture continued for 3 hours. Cells were
624 harvested, and the pellet washed and resuspended in 100 mM Tris-HCl pH 8.0, 1 mM EDTA,
625 in the presence of lysozyme (50 μ g/ml); after one freeze-thaw cycle, the suspension was
626 sonicated, MgSO₄ was added at a final concentration of 4 mM, DNase was added, and the
627 suspension centrifuged at 16000 g for 45 minutes. The pellet was resuspended, and
628 solubilized in 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 20 mM Imidazole, 10% glycerol, 0,8%
629 LMNG, at a final concentration of 160 OD_{600nm} equivalent/ml. After 45 minutes, the suspension
630 was centrifuged (16000 g, 45 minutes), and the supernatant incubated with Ni-Agarose. After
631 3 hrs, of incubation, the beads were washed 4 times with 20 mM Tris-HCl pH 8.0, 100 mM
632 NaCl, 20 mM Imidazole, 10% glycerol, 0,005% LMNG, the bound proteins eluted in the same

633 buffer in the presence of 200 mM Imidazole. The equivalent of 8.5 OD_{600nm} was loaded on
634 each gel lane.

635 **Crystallization and diffraction collection**

636 For the structural analysis of the ExbD–TonB complex, 250 μ L of 2 mM (20 mg/mL)
637 ExbD^{Ec, Δ NIBS} with 10 mM TonB peptide (KKAQPISVTMVTPADLEPPQAKK) in Buffer G (50
638 mM Tris-HCl, 50 mM NaCl, pH 7.0) were prepared. Firstly, preliminary screening of
639 crystallization conditions was performed using the vapor diffusion technique with a
640 MosquitoTM nanoliter-dispensing system (TTP Labtech, Melbourn, United Kingdom) in
641 accordance with established protocols.⁵¹ Specifically, sitting drops were prepared by
642 combining 400 nL of protein-peptide mixture with crystallization solutions (comprising 672
643 commercially available conditions) in a 1:1 ratio and then equilibrating the mixture against a
644 150- μ L reservoir in 96-well plates (Greiner Bio-one, GmbH, Frickenhausen, Germany). The
645 crystallization plates were subsequently maintained at 18 °C in an automated
646 RockImager1000 (Formulatrix, Bedford, MA, United States) imager to monitor the growth of
647 crystals. The best crystals were obtained in 10% w/v glycerol and 3 M ammonium sulfate. The
648 crystals were cryoprotected by soaking them into the crystallization solution supplemented
649 with 20% glycerol as a cryoprotectant before freezing in liquid nitrogen. Subsequently,
650 diffraction data were acquired at cryogenic temperatures (100 K) on the PROXIMA-1 beamline
651 at the SOLEIL synchrotron facility (St Aubin, France) and processed with XDS⁵² through
652 XDSME (<https://github.com/legrandp/xdsme>)⁵³.

653 **X-ray structure determination and refinement**

654 AlphaFold2 multimer⁵⁴ was used to generate initial models of the ExbD-TonB peptide
655 complex. On that basis, crystal structures were determined by molecular replacement with
656 Phaser⁵⁵. The final models were generated via an iterative process that involved manual
657 model building using Coot⁵⁶ and refinement in reciprocal space with REFMAC⁵⁷ and Phenix⁵⁸
658 All data collection details as well as model refinement statistics are summarized in Table S3.

659 The ExbD-TonB peptide complex structure was deposited to the Protein Data Bank
660 (PDB) under the accession code 8P9R.

661 **Digestion of peptidoglycan**

662 Five mg *E. coli* peptidoglycan (InvivoGen) were washed twice with Buffer C and finally
663 resuspended in 800 μ L. An aliquot of 25 μ L of a 5 mg/mL stock solution of mutanolysin (Sigma
664 Aldrich) were added and the mixture was incubated for 2 days at 37 °C. Considering fully
665 glycosidically-digested non-crosslinked peptidoglycan (GlcNAc-MurNAc-L-Ala-D-Glu-DAP-D-
666 Ala; molecular weight = 2060 g/mol), this yields a final concentration estimated at 2.94 mM
667 peptidoglycan fragments. The enzymatic reaction was terminated by heating the mixture at 95
668 °C for 10 min. After this, the mixture was filtered through a filter with a 10 kDa cutoff to remove
669 larger peptidoglycan fragments and any remaining mutanolysin.

670

671 **Figure creation**

672 All depictions of protein structures were generated with ChimeraX.⁵⁹

673

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823 **AUTHOR CONTRIBUTIONS**

824 N.I-P and M.Z conceived the study. M.Z. produced the protein samples and performed NMR
825 measurements. M.L. assisted in the wet lab. M.Z. and N.I-P conceived and analyzed NMR
826 experiments. M.Z. and B.B. performed the NMR structure calculation. A.M. solved the X-ray
827 structure. I.G.B helped in peptidoglycan data analysis. P.D. conducted the *in vivo* experiments.
828 M.Z. designed the figures and wrote the manuscript with contributions of all authors. N.I-P.
829 guided in the writing process. All authors approved the manuscript.

830 **COMPETING INTERESTS**

831 The authors declare no competing interests.

832 Supplementary Information is available for this paper.