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**Bacterial cell wall integrity surveillance is connected to peptidoglycan biosynthesis by the essential phosphorylation of ReoM**

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29 **ABSTRACT**

30

31 Peptidoglycan (PG) is the main component of bacterial cell walls and the target for many

32 antibiotics. PG biosynthesis is tightly coordinated with cell wall growth and turnover and many

33 of these control activities depend upon PASTA-domain containing eukaryotic-like

34 serine/threonine protein kinases (PASTA-eSTK) that sense PG fragments. However, only a few

35 PG biosynthetic enzymes are actually direct kinase substrates. Here, we identify the conserved

36 ReoM protein as a novel PASTA-eSTK substrate in the Gram-positive pathogen *Listeria*

37 *monocytogenes*. Our data show that the phosphorylation of ReoM is essential as it controls

38 ClpCP-dependent proteolytic degradation of the essential enzyme MurA, which catalyses the first

39 committed step in PG biosynthesis. We also identify ReoY as a second novel factor required for

40 degradation of ClpCP substrates. Collectively, our data imply that the first committed step of PG

41 biosynthesis is activated through control of ClpCP protease activity in response to signals of PG

42 homeostasis imbalance.

43

44 **INTRODUCTION**

45

46 The cell wall of Gram-positive bacteria is a complicated three-dimensional structure that engulfs

47 the cell as a closed sacculus. The main component of bacterial cell walls is peptidoglycan (PG), a

48 network of glycan strands crosslinked together by short peptides (1). PG biosynthesis starts with

49 the conversion of UDP-GlcNAc into lipid II, a disaccharide pentapeptide that is ligated to a

50 membrane-embedded bactoprenol carrier lipid (2). This monomeric PG precursor is then flipped

51 from the inner to the outer leaflet of the cytoplasmic membrane by MurJ- and Amj-like enzymes

52 called flippases (3-5). Glycosyltransferases belonging either to the bifunctional penicillin binding

53 proteins (PBPs) or the recently identified SEDS (shape, elongation, division and sporulation)

54 family then transfer the disaccharide pentapeptides to growing PG strands, which are crosslinked

55 by a transpeptidation reaction catalysed by monofunctional (class B) or bifunctional (class A)

56 PBPs (6-9). Numerous hydrolytic or PG-modifying enzymes are also required to adapt the

57 sacculus to the morphological changes that occur during bacterial cell growth and division (10,

58 11) or to alter its chemical properties for instance for immune evasion (12). Finally, a suite of

59 regulators ensure that spatiotemporal control of PG synthesis is balanced against PG hydrolysis in

60 cycles of bacterial growth and division (13).

61 The activity of several key enzymes along the PG biosynthetic pathway is regulated by PASTA

62 (PBP and serine/threonine kinase associated) domain-containing ekaryotic-like serine/threonine

63 protein kinases (PASTA-eSTKs) (14-16). These membrane-integral enzymes comprise a

64 cytoplasmic kinase domain linked to several extracellular PASTA domains by a single

65 transmembrane region (15). The kinase activity is stimulated by free muropeptides and lipid II

66 (that accumulate during damage and turnover of PG) on interaction with the PASTA domains

67 (17, 18). PknB, a representative of this protein family from *Mycobacterium tuberculosis*,

68 phosphorylates GlmU, a bifunctional uridyltransferase/acetyltransferase important for synthesis  
69 of UDP-GlcNAc, and in so doing reduces GlmU activity (19). *M. tuberculosis* MviN, a MurJ-like  
70 flippase, is also a substrate of PknB and, in its phosphorylated state, P-MviN forms inhibitory  
71 complexes with its binding partner FhaA (20). *M. tuberculosis* PknB also phosphorylates both the  
72 class A PBP PonA1 (21) and the amidase-like PG-hydrolase CwlM, which is essential for growth  
73 (22-24). CwlM is membrane-associated and interacts with MurJ to control lipid II export (24).  
74 However, when phosphorylated, P-CwlM re-locates from the membrane to the cytoplasm (24)  
75 where it allosterically activates MurA 20–40-fold (23). MurA catalyzes the first committed step  
76 of PG biosynthesis by transferring an enoylpyruvate moiety to UDP-GlcNAc; MurA is essential  
77 in *M. tuberculosis* and in all other bacterial species tested (25-28). Finally, the *Listeria*  
78 *monocytogenes* PASTA-eSTK, PrkA, phosphorylates YvcK, which is required for cell wall  
79 homeostasis, and both proteins are important for the survival of this facultative intracellular  
80 pathogen inside the cytosol of infected host cells (29).

81 Numerous additional proteins acting to coordinate cell wall biosynthesis with cell division are  
82 substrates of PASTA-eSTKs in other Gram-positive bacteria (15), including the late cell division  
83 protein GpsB, which is reported to be phosphorylated by PrkC, the PASTA-eSTK of *Bacillus*  
84 *subtilis* (30, 31). We have shown previously that GpsB from *L. monocytogenes* is important for  
85 the last two steps of PG biosynthesis, *i. e.* transglycosylation and transpeptidation, by providing  
86 an assembly platform for the class A PBP, PBP A1 (32-35), and this adaptor function of GpsB is  
87 maintained in at least *B. subtilis* and *Streptococcus pneumoniae* (34). An *L. monocytogenes*  
88  $\Delta$ *gpsB* mutant is impaired in PG biosynthesis and cannot grow at elevated temperatures (32), but  
89 this phenotype is readily corrected by a suppressor mutation, which mapped to *clpC* (28). ClpC is  
90 the ATPase subunit of the ClpCP protease molecular machine that degrades substrate proteins  
91 upon heat stress (36). MurA (*aka* MurAA in *B. subtilis*) is a ClpCP substrate in both *B. subtilis*

92 and *L. monocytogenes* (26, 28) and strongly accumulates in a *L. monocytogenes*  $\Delta clpC$  mutant  
93 (28). Thus, a deficiency in the final two enzymatic steps of PG biosynthesis in the absence of  
94 GpsB is corrected by mutations in *clpC* that increase the amount of the first enzyme of the same  
95 PG biosynthetic pathway.

96 Here we isolate novel *gpsB* suppressor mutations affecting *Listeria* genes previously unstudied.  
97 We demonstrate that both proteins control the ClpCP-dependent degradation of MurA in a PrkA-  
98 dependent and hitherto unprecedented manner. One of them is phosphorylated by PrkA and this  
99 phosphorylation is essential. With these observations, we present the first molecular link between  
100 PrkA-dependent cell wall integrity sensing and control of PG production in low G/C Gram-  
101 positive bacteria, which explains how PG biosynthesis is adjusted in these bacteria to meet PG  
102 production and repair needs.

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## RESULTS

105

### 106 **Novel *gpsB* suppressor mutations in the *lmo1503* (*reoM*) and *lmo1921* (*reoY*) genes**

107 A *L. monocytogenes*  $\Delta$ *gpsB* mutant is unable to replicate at 42°C but forms suppressors  
108 correcting this defect with high frequency (28). In a preliminary selection of *gpsB* suppressors,  
109 the *clpC* and *murZ* genes, important for the stability of the UDP-*N*-acetylglucosamine 1-  
110 carboxyvinyltransferase MurA, were found to be mutated (28). We have characterized three more  
111 *shg* (suppression of heat sensitive growth) suppressor mutants (*shg8*, *shg10* and *shg12*) isolated  
112 from a  $\Delta$ *gpsB* mutant incubated on a BHI agar plate at 42°C. These three *shg* strains grew as fast  
113 as the wild type when cultivated at 37°C or 42°C, whereas the parental  $\Delta$ *gpsB* mutant grew at a  
114 reduced rate at 37°C and did not grow at 42°C (Fig. 1A-B), as shown previously (32).

115 Illumina sequencing of the *shg8*, *shg10* and *shg12* genomes identified one SNP in each strain that  
116 was not found in the parental  $\Delta$ *gpsB* strain. Strain *shg8* carried a mutation in the uncharacterized  
117 *lmo1921* gene (herein named *reoY*, see below) that exchanged H87 into tyrosine; the same gene  
118 was affected by the introduction of a premature stop codon after the 73<sup>rd</sup> codon of *reoY* to yield  
119 strain *shg10*. Strain *shg12* carried a mutation in the ribosomal binding site (RBS) of the *lmo1503*  
120 gene (renamed *reoM*), encoding another protein of unknown function and containing a DUF956  
121 domain, widely conserved in bacteria but with no function ascribed.

122 Whether the mutation in the RBS of *reoM* in strain *shg12* led to its inactivation, expression  
123 effects or a gain of function was not clear. Therefore, the *reoM* gene was deleted from the  
124 genome of the wild type strain and the  $\Delta$ *gpsB* mutant. While deletion of *reoM* had no effect on  
125 growth of wild type bacteria, it suppressed completely the growth defects of the  $\Delta$ *gpsB* mutant at  
126 both 37°C and 42°C (Fig. 1C-D). It is thus likely that the mutation in the *reoM* RBS impairs its

127 expression. Likewise, deletion of *reoY* led to the complete restoration of growth in the  $\Delta gpsB$   
128  $\Delta reoY$  double mutant at both 37°C and 42°C (Fig. 1C-D).

129 Expression of an additional, plasmid-borne copy of *reoM* impaired growth of the  $\Delta gpsB$  mutant  
130 without affecting the growth of wild type bacteria, whilst expression of a second copy of *reoY*  
131 had no effect (Fig. S1A,B). The expression of *reoM* is thus inversely correlated with the growth  
132 of the  $\Delta gpsB$  mutant. To further validate these conclusions, the genomes of the  $\Delta reoM$  and  $\Delta reoY$   
133 single and the  $\Delta gpsB \Delta reoM$  and  $\Delta gpsB \Delta reoY$  double mutants were analysed by Illumina  
134 sequencing. No additional mutations were found in any of these strains, confirming that the *reoM*  
135 and *reoY* deletions were the sole reason for the suppression of the  $\Delta gpsB$  phenotype.

136 Finally, the physiology of the strains harbouring novel *gpsB* suppressor genes was examined. The  
137 cell lengths of strains lacking *reoM* and *reoY* were unaffected by the presence or absence of *gpsB*,  
138 and were the same as the wild type strain, suggesting the absence of cell division defects in the  
139  $\Delta reoM$  or  $\Delta reoY$  mutants (Fig. S2A,B). Furthermore, scanning electron micrographs of  $\Delta reoM$   
140 and  $\Delta reoY$  single mutants revealed that these bacteria had a normal rod-shape, but that the  $\Delta gpsB$   
141  $\Delta reoM$  and  $\Delta gpsB \Delta reoY$  double mutants were partially bent (Fig. S2C), implying the presence  
142 of shape maintenance defects along the lateral cell cylinders.

143

#### 144 **ReoM and ReoY affect the stability of MurA**

145 Suppression of the  $\Delta gpsB$  phenotype can be achieved by the accumulation of the UDP-*N*-  
146 acetylglucosamine 1-carboxyvinyltransferase MurA (28). Consequently, MurA levels were  
147 determined in  $\Delta reoM$  and  $\Delta reoY$  mutant strains by Western blotting. MurA accumulated by at  
148 least eight-fold in comparison to the wildtype in the absence of *reoM* or *reoY* (Fig. 2A),  
149 indicating that the  $\Delta gpsB$  suppression involves MurA accumulation in  $\Delta reoM$  and  $\Delta reoY$   
150 mutants. Remarkably, MurA accumulation in  $\Delta reoM$  and  $\Delta reoY$  mutants reached similar levels as

151 in a mutant lacking *clpC*, which encodes the ATPase subunit of the ClpCP protease (Fig. 2A).  
152 Both MurAA, the *B. subtilis* MurA homologue, and *L. monocytogenes* MurA are degraded by  
153 their respective ClpCP proteases *in vivo* (26, 28). In order to test whether *reoM* and *reoY* exert  
154 their effect on MurA in a ClpC-dependent manner, MurA levels were determined in  $\Delta clpC$   
155  $\Delta reoM$  and  $\Delta clpC \Delta reoY$  double mutants. The MurA levels in  $\Delta clpC$ ,  $\Delta reoM$  and  $\Delta reoY$  single  
156 mutants were the same as in  $\Delta clpC \Delta reoM$  and  $\Delta clpC \Delta reoY$  double mutant strains (Fig. 2B).  
157 Likewise, the MurA level in a mutant lacking *murZ*, previously shown to contribute to MurA  
158 stability (28), is not additive to the MurA level in  $\Delta clpC$  cells (Fig. 2B). Therefore, *reoM*, *reoY*  
159 and *murZ* likely affect the ClpCP-dependent degradation of MurA and each are thus important  
160 for the stability of MurA against targeted proteolytic degradation. Moreover, combinations of  
161  $\Delta reoM$ ,  $\Delta reoY$  and  $\Delta murZ$  deletions did not exert any additive effect on accumulation of MurA  
162 (Fig. S3A,B) further validating the conclusion that these genes all belong to the same pathway.

163

#### 164 **The effect of ReoM and ReoY on MurA levels is conserved**

165 Homologues of the 90-residue ReoM protein are found across the entire Firmicute phylum, and  
166 include IreB, a substrate of the protein serine/threonine kinase IreK and its cognate phosphatase  
167 IreP from *Enterococcus faecalis* (37), whereas ReoY homologues are present only in the *Bacilli*.  
168 In *B. subtilis*, ReoM corresponds to YrzL (e-value  $3e^{-29}$ ) and ReoY to YpiB ( $4e^{-61}$ ), but neither  
169 protein has been studied thus far. Analysis of the chromosomal structure reveals that *yrzL* is in an  
170 operon with *alaS*, *yrrK* and *yrzB* (38) and expression seems to be regulated by an alanine-  
171 dependent T-box (39). YrrK seems to be an RNase that degrades the 5'-end of 16S rRNA (40). It  
172 is perhaps pertinent to note that just 9 genes downstream are the PBPI (aka PBP4) and YrrS  
173 reading frames, both of which are binding partners of GpsB (34). By contrast, expression of *ypiB*  
174 was reported to be under the control of  $\sigma^H$  (41). To assess whether YrzL and YpiB were also



175 crucial for control of MurAA levels in *B. subtilis*, cellular protein extracts from *B. subtilis*  $\Delta$ yrzL  
176 and  $\Delta$ ypiB mutants were probed by Western blot (Fig. 2C). MurAA accumulated by at least 12-  
177 fold in these strains in comparison to the wildtype. Furthermore, the amount of MurAA was also  
178 increased by 12-fold in the  $\Delta$ clpC mutant. Taken together, these data indicate that ReoM and  
179 ReoY functions are conserved in both species implying that homologues of these proteins exert  
180 similar effects on MurA levels in all other Firmicutes. We thus propose to rename *lmo1503*  
181 (*yrzL*) as *reoM* (regulator of MurA(A) degradation) and analogously *lmo1921* (*ypiB*) as *reoY*.  
182 Several other ClpC substrates are known in *B. subtilis*, including the glutamine fructose-6-  
183 phosphate transaminase GlnS and the acetolactate synthase subunit IlvB (42). The levels of both  
184 proteins were significantly increased in *B. subtilis*  $\Delta$ reoM and  $\Delta$ reoY mutants (Fig. S4),  
185 indicating that ReoM and ReoY are required for degradation of ClpC substrates in general.

186

### 187 **Phosphorylation and dephosphorylation of ReoM by PrkA and PrkP *in vitro***

188 PrkA (encoded by *lmo1820*) and PrkP (*lmo1821*) are the *L. monocytogenes* homologs of *E.*  
189 *faecalis* IreK and IreP, respectively. Consequently, the pairwise interactions and biochemical  
190 properties of ReoM, the PrkA kinase domain (PrkA-KD) and the cognate phosphatase PrkP were  
191 investigated. All isolated proteins electrophoresed as single species in non-denaturing PAGE  
192 (lanes 1, 2, Fig. 3A; lanes 1-4, Fig. 3B). When ReoM was incubated with PrkA-KD, in the  
193 absence of ATP, a slower migrating species was observed and the individual bands  
194 corresponding to ReoM and PrkA-KD disappeared indicating that the slower migrating species  
195 was a ReoM:PrkA-KD complex (lane 3, Fig. 3A). When ReoM was incubated with PrkA-KD and  
196 Mg/ATP under the same conditions, free PrkA-KD was observed but no bands equivalent to  
197 ReoM and the ReoM:PrkA-KD complex remained; instead a new species was present, migrating  
198 faster in the gel than ReoM (lane 4, Fig. 3A), which is likely to be phosphorylated ReoM (P-

199 ReoM). Intact protein liquid chromatography-mass spectrometry (LC-MS) analysis of ReoM  
200 isolated from PrkA-KD after phosphorylation revealed the addition of 79.9 Da in comparison to  
201 the mass of ReoM (10671.5 Da), which corresponds to the formation of a singly-phosphorylated  
202 ReoM product of 10751.4 Da (Fig. 3C, S5). MS/MS spectra obtained during peptide mass  
203 fingerprinting were also consistent with one phosphorylation event per protein chain: the mass of  
204 one ReoM peptide, spanning residues Asp5 to Lys22 with mass of 2151.89 Da, was increased by  
205 79.96 Da after incubation with PrkA-KD and Mg/ATP. Analysis of the *y*- and *b*- ions in the  
206 MS/MS fragmentation spectrum of this peptide was consistent only with Thr7 as the sole  
207 phosphosite in ReoM (Fig. 3D). Finally, mutation of Thr7 to alanine completely abrogated the  
208 phosphorylation of ReoM by PrkA-KD when analysed by LC-MS (Fig. S6).

209 The ability of PrkP, the partner phosphatase to PrkA in *L. monocytogenes*, to interact with and  
210 remove phosphoryl groups from PrkA-KD and P-ReoM was also tested *in vitro*. PrkA and  
211 purified P-ReoM were each incubated with PrkP in the absence and presence of MnCl<sub>2</sub>, since  
212 divalent cations are essential co-factors for the PPM phosphatase family to which PrkP belongs  
213 (43), and the products were analysed by non-denaturing PAGE. Unlike the situation with ReoM  
214 and PrkA-KD, no stable protein:protein complexes were formed either in the presence or absence  
215 of endogenous MnCl<sub>2</sub> (Fig. 3B). The incubation of P-ReoM with PrkP and manganese resulted in  
216 the almost complete disappearance of the band corresponding to P-ReoM (lane 6, Fig. 3B) in  
217 comparison to the same reaction conducted without the addition of MnCl<sub>2</sub> (lane 5, Fig. 3B). The  
218 new band, corresponding to ReoM alone in lane 6, is masked by that for PrkP which migrates  
219 similarly to ReoM (lanes 1 and 4, Fig. 3B) under these electrophoresis conditions, but the  
220 presence of unphosphorylated ReoM and the absence of P-ReoM was confirmed by LC-MS (Fig.  
221 S7). When incubated with PrkP in the presence of manganese ions, the band for PrkA-KD  
222 electrophoresed more slowly than for PrkA-KD in isolation (lanes 3 and 8, Fig. 3B), indicating

223 that PrkA-KD had been dephosphorylated by PrkP. LC-MS analysis of PrkA-KD that had been  
224 incubated with PrkP/MnCl<sub>2</sub> yielded a single major species of 37,413.2 Da, consistent with the  
225 predicted mass of the expressed recombinant construct, and the absence of a peak corresponding  
226 to phosphorylated PrkA-KD, P-PrkA-KD (Fig. S8). Therefore, PrkA-KD is capable of  
227 autophosphorylation even when expressed in a heterologous host, consistent with previous  
228 observations made for similar PASTA-eSTKs from other Gram-positive bacteria (44, 45).  
229 Finally, in the absence of MnCl<sub>2</sub> no change in electrophoretic mobility was observed for P-PrkA-  
230 KD (lane 7, Fig. 3B).

231

### 232 **Phosphorylation of ReoM at threonine 7 is essential for viability**

233 PrkA phosphorylates ReoM on Thr7 and PrkP reverses this reaction. In the absence of molecular  
234 details on the impact of Thr7 phosphorylation we determined the importance of this  
235 phosphorylation *in vivo* by engineering a phospho-ablative T7A exchange in an IPTG-inducible  
236 allele of *reoM* and introduced it into the  $\Delta$ *reoM* mutant background. Deletion, depletion or  
237 expression of wildtype *reoM* had no effect on growth in strains LMSW30 ( $\Delta$ *reoM*) and LMSW57  
238 (*ireoM*, i - is used to denote IPTG-dependent alleles throughout the manuscript) at 37°C.  
239 Likewise, strain LMSW52 (*ireoM T7A*) grew normally in the absence of IPTG. However, the  
240 *reoM* mutant with the T7A mutation did not grow at all in the presence of IPTG, when expression  
241 of the phospho-ablative *reoM T7A* allele was induced (Fig. 4A), suggesting that phosphorylation  
242 of ReoM at Thr7 is essential for the viability of *L. monocytogenes*. Since ReoM influences the  
243 proteolytic stability of MurA, we determined the cellular amount of MurA in strains expressing  
244 the T7A variant of ReoM. For this purpose, strains LMSW57 (*ireoM*) and LMSW52 (*ireoM T7A*)  
245 were initially cultivated in plain BHI broth. At an OD<sub>600</sub> of 0.2, IPTG was added to a final  
246 concentration of 1 mM and cells were harvested 2 hours later. Strain LMSW57 (*ireoM*) showed

247  $\Delta clpC$ -like MurA accumulation (around seven-fold in this experiment) when cultured in the  
248 absence of IPTG, but MurA was present at wild type levels in the presence of IPTG (Fig. 4C).  
249 The strain with the T7A exchange also accumulated MurA to a  $\Delta clpC$ -like extent in the absence  
250 of IPTG. However, around 10% of the wild type MurA levels could be detected in cells  
251 expressing the *reoM* T7A allele (Fig. 4C). These data demonstrate that Thr7 in ReoM is of special  
252 importance for the proteolytic stability of MurA.

253

#### 254 **Lethality of the *reoM* T7A mutations depends on ClpC**

255 That MurA is rapidly degraded in cells expressing *reoM* T7A implies that  
256 phosphorylation/dephosphorylation of ReoM at Thr7 controls ClpCP-dependent MurA  
257 degradation. MurA is an essential enzyme in *L. monocytogenes* (28), and stimulation of ClpCP-  
258 dependent MurA degradation in the *reoM* T7A mutant would provide an explanation for the  
259 lethality of this mutation. In order to address this possibility, we deleted *clpC* in the conditional  
260 *ireoM* T7A background. This strain grew even in the presence of IPTG, a compelling  
261 demonstration that the removal of *clpC* suppressed the lethality of the *reoM* T7A mutation (Fig.  
262 4B). MurA also accumulated to the same degree as in the  $\Delta clpC$  mutant in this strain (Fig. 4C),  
263 suggesting that inactivation of the ClpCP-dependent degradation of MurA overcame the lethal  
264 effect of the T7 mutation in *reoM*. Moreover, this suggests that ClpCP acts downstream of ReoM.  
265 We next wondered whether deletion of *reoY* and *murZ* would have a similar effect and also  
266 deleted these genes in the *reoM* T7A mutant. As can be seen in Fig. 4D, deletion of either gene  
267 also overcame the lethality of *reoM* T7A, indicating that ReoY and MurZ must also act  
268 downstream of ReoM.

269 **Crystal structure of ReoM, a homologue of *Enterococcus faecalis* IreB**

270 Purified ReoM yielded crystals that diffracted to a maximum resolution of 1.6 Å. The structure of  
271 IreB (PDBid 5US5) (46) was used to solve the structure of ReoM by molecular replacement (Fig.  
272 5A). The data collection and refinement statistics for the ReoM structure are summarised in Table  
273 3. ReoM shares the same overall fold as IreB (46), each containing a compact 5-helical bundle (4  
274 standard  $\alpha$ -helices and one single-turned  $3_{10}$ -helix between residues 52 and 54) with short loops  
275 between the secondary structure elements, which are defined above the sequence alignment in  
276 Fig. 5B. Other than IreB (46), there are no functionally-significant structural homologues of  
277 ReoM in the PDB. In both cases the helical bundles associate into homodimers with  $\alpha$ -helices  
278 two and four from each protomer forming the majority of the homodimer interface (Fig. 5A), and  
279 these residues are highlighted in Fig. 5B. In agreement with the IreB structural analysis, 1200 Å<sup>2</sup>  
280 of surface area is buried in the ReoM dimer interface, representing 9% of the total solvent  
281 accessible surface area. The similarity of the monomers and the dimeric assemblies of ReoM and  
282 IreB is underlined by the 1.5 and 1.7 Å r.m.s.d. values, respectively, on global secondary  
283 structure superposition matching 74 C $\alpha$  atoms from each protomer in the comparison.

284 Other than the compact helical bundle of ReoM, there is a ~16 residue-long N-terminal tail, with  
285 B-factors 25% higher than the rest of the protein, prior to the start of  $\alpha$ -helix 1 at residue Ile17.  
286 The equivalent N-terminal region is also disordered in the NMR structure of IreB (46). Despite  
287 the absence of secondary structure, the ReoM model covering this region could be built with  
288 confidence from Asp5 in chain A and Asp2 in chain B (Fig. 5C). Consequently, it is possible to  
289 visualise Thr7, the target for phosphorylation by PrkA, in the flexible N-terminal region in both  
290 chains. The side chain of Thr7 in both chains makes no intramolecular interactions and is thus  
291 amenable to phosphorylation by PrkA. The extended N-terminal regions are at least partially  
292 stabilised by crystal lattice interactions that, in chain B, include Phe9 and Tyr10 forming a

293 network of hydrophobic interactions with other aromatic residues from symmetry equivalent  
294 molecules, including contributions from another copy of Phe9 and Tyr10. Phosphorylation could  
295 force a change in oligomeric state, as observed quite commonly in response regulators in order to  
296 bind more effectively to promoter regions to effect transcription (47). However, analysis of the  
297 oligomeric state of P-ReoM by size exclusion chromatography revealed that the protein behaved  
298 in solution the same as to unphosphorylated ReoM (Fig. S9).

299 Unlike the packing arrangement of Thr7 in chain B, the local symmetry surrounding Thr7 in  
300 chain A might provide some information of potential functional significance to the  
301 phosphorylated form of ReoM. Here, a sulphate ion (a component of the crystallisation reagent)  
302 is hydrogen bonded to the sidechain of Thr7 and hence mimics, to some degree, what the  
303 phosphorylated protein may look like (Fig. 5C). The sulphate ion is captured by a positively-  
304 charged micro-environment incorporating residues Lys35, Arg57, His58 and Arg62 from a  
305 symmetry-equivalent molecule. Intramolecular re-arrangements of structures to accommodate  
306 phosphorylated amino acids in positively-charged surface environments is a common response to  
307 phosphorylation, with distances of up to 50 Å between the positions of phosphorylated and non-  
308 phosphorylated Ser14 in rabbit muscle glycogen phosphorylase, for example (47). ReoM could  
309 react to phosphorylation on Thr7 by a substantial movement of the N-terminal tail to interact with  
310 conserved, positively-charged amino acids on the protein surface. We identified a single cluster  
311 of arginines (Arg57 [57%], Arg62 [99%], Arg66 [76%], Arg70 [98%]) in close spatial proximity  
312 with levels of conservation amongst all 2909 ReoM homologues present at NCBI approaching  
313 that of Thr7 (96%). Alanine substitutions were made at these four positions and the respective  
314 mutated alleles were expressed in the *L. monocytogenes*  $\Delta$ reoM background. Whereas the R66A  
315 and R70A mutations were without any effect on growth (data not shown), expression of ReoM  
316 R57A and R62A mutations were as lethal as expression of ReoM T7A (Fig. S10). Thus, Arg57

317 and Arg62 might co-ordinate P-Thr7, stabilising the conformation and position of the flexible N-  
318 terminal region (Fig. S11), leading to a change in the surface electrophysical properties of ReoM  
319 to drive the downstream consequences of its phosphorylation. Despite multiple attempts no  
320 crystals were grown of P-ReoM and, therefore, the molecular consequences of ReoM  
321 phosphorylation remains to be determined.

322

### 323 **Control of MurA stability by the PrkA/PrkP protein kinase/phosphatase pair**

324 To study the contribution of the PrkA/PrkP couple to cell wall biosynthesis in more detail, we  
325 aimed to construct *prkA* and *prkP* deletion mutants, but failed to delete *prkA*, as has been reported  
326 previously (29). However, *prkA* could be deleted in the presence of an IPTG-inducible ectopic  
327 *prkA* copy and the resulting strain (LMSW84) required IPTG for growth (Fig. 6A), demonstrating  
328 the essentiality of this gene. Repeated attempts to delete *prkP* finally yielded a single  $\Delta$ *prkP*  
329 clone (LMSW76). Genomic sequencing of this strain, which grew at a similar rate to wildtype  
330 (Fig. 6A), confirmed the successful deletion of *prkP* but also identified a trinucleotide deletion in  
331 the *prkA* gene (designated *prkA\**), effectively removing the complete codon of Gly18 that is part  
332 of a conserved glycine-rich loop important for ATP binding (48). Presumably, this mutation  
333 reduces the PrkA kinase activity to balance the absence of PrkP. By contrast, *prkP* could be  
334 deleted readily in the presence of a second IPTG-dependent copy of *prkP* and growth of the  
335 resulting strain (LMSW83) did not require IPTG, most likely explained by promoter leakiness in  
336 the absence of IPTG (Fig. 6A). Next, the effect of *prkA* and *prkP* mutations on MurA  
337 accumulation was analyzed by Western blotting. Intermediate MurA accumulation was evident in  
338 the  $\Delta$ *prkP prkA\** strain, while full accumulation of MurA was observed in PrkP-depleted cells.  
339 By contrast, no MurA was detected in cells depleted for PrkA (Fig. 6B). Therefore, PrkA and



340 PrkP inversely contribute to the accumulation of MurA suggesting that phosphorylated ReoM  
341 favors MurA accumulation, while un-phosphorylated ReoM counteracts this process.

342

### 343 **Deletion of *reoM*, *reoY* or *clpC* eliminates *prkA* essentiality**

344 In order to test whether the essentiality of *prkA* could be explained by complete MurA  
345 degradation through ClpCP, we first tested the effect of *clpC* on the essentiality of *prkA*. For this  
346 purpose, *clpC* was removed from the *iprkA* strain and growth of the resulting strain (LMSW91)  
347 was tested. In contrast to the parental *iprkA* strain (LMSW84), which required IPTG for growth,  
348 strain LMSW91 was viable without IPTG (Fig. 7A), confirming that the essentiality of PrkA  
349 depends on ClpC. As we have shown above, ReoM controls MurA stability via ClpC and is  
350 phosphorylated by PrkA (Fig. 2B and 3A). Thus, we wondered whether ReoM was required for  
351 PrkA essentiality and consequently deleted *reoM* from the *iprkA* background to test this  
352 hypothesis. Again, the resulting strain (LMSW89) did not require IPTG for growth in contrast to  
353 the parental *iprkA* strain (Fig. 7A). Finally, we tested the contribution of *reoY* to *prkA* essentiality  
354 in a similar manner and also found that deletion of *reoY* suppressed the essentiality of *prkA* (Fig.  
355 7A). In good agreement with these findings, deletion of *clpC*, *reoM* or *reoY* all stabilized MurA  
356 in PrkA-depleted cells (Fig. 7B), showing that the stimulated degradation of MurA that we  
357 observe in cells depleted for PrkA (Fig. 6B) is dependent on any one of these three proteins.  
358 These results indicate that the essentiality of PrkA is explained by its contribution to proteolytic  
359 stabilization of another essential enzyme, MurA, and since ReoM and ReoY play a crucial role in  
360 this process, their deletion has the same effect on suppression of the  $\Delta$ *gpsB* phenotype as the  
361 deletion of *clpC*.

362



363 **DISCUSSION**

364

365 With ReoM we have identified a missing link in a regulatory pathway that enables Firmicute  
366 bacteria to activate PG biosynthesis under conditions damaging their cell walls. In *L.*  
367 *monocytogenes*, the sensory module of this pathway comprises the membrane integral protein  
368 kinase PrkA and the cognate protein phosphatase PrkP, their newly discovered substrate ReoM  
369 and the associated factors ReoY and MurZ, which together regulate ClpCP activity, the effector  
370 protease that acts on MurA (Fig. 8). It has been demonstrated previously that the kinase activity  
371 of PrkA homologues was activated by muropeptides (17, 49) or the PG precursor lipid II (18).  
372 Muropeptides were released from the cell wall during normal PG turnover, and their release was  
373 intensified when PG hydrolysis prevailed over PG biosynthesis (10, 50), whereas blocking PG  
374 chain elongation by moenomycin treatment caused the accumulation of lipid-linked PG  
375 precursors (51). Thus, both types of molecules accumulated when PG biosynthesis was inhibited  
376 and could represent useful signals for detecting cell wall-damaging situations. We show here that  
377 upon stimulation, PrkA phosphorylated ReoM and P-ReoM no longer activates ClpCP, which  
378 leads to MurA stabilization and activation of PG biosynthesis (Fig. 8).

379 How ReoM and ReoY exert their effect on ClpCP is currently unknown, but a fascinating  
380 possibility would be a function similar to that of an adaptor protein to target a subset of protein  
381 substrates, including MurA, to the ClpCP complex for degradation. Several ClpC adaptors are  
382 known in *B. subtilis* (52, 53), but an adaptor for *BsMurAA* is not among them (26, 52).  
383 Interestingly, the ClpC adaptor protein McsB from *B. subtilis* is also subject to phosphorylation  
384 like ReoM but, unlike ReoM, it targets its substrate CtsR to the ClpCP machinery only when  
385 phosphorylated (54). According to our genetic data, either ReoM or ReoY could act as this  
386 adaptor, leaving a subsidiary function for the other respective protein. Alternatively, both proteins

387 could work in tandem, where each of them is equally needed for ClpCP-dependent MurA  
388 degradation since the phenotypes of *reoM* and *reoY* mutants were identical with respect to MurA  
389 stability. However, overexpression or deletion of *reoM* altered the phenotype of the  $\Delta$ *gpsB*  
390 mutant, but that of *reoY* was without phenotype (Fig. S1, Fig. S2). ReoY, restricted to the *Bacilli*,  
391 also showed a narrower phylogenetic distribution than ReoM, which is found across different  
392 Firmicutes (Fig. 5B). Thus, it seems that ReoM might have a more generalized role, whereas  
393 ReoY could play a subordinate function in control of MurA degradation by ClpCP. The role of  
394 the MurA homologue MurZ in this process is entirely unclear, but our genetic data now place it  
395 downstream of ReoM (Fig. 8). Furthermore, arginine phosphorylation targets proteins to ClpCP  
396 for degradation (55). *L. monocytogenes* MurA contains 17 arginines and MurAA of *B. subtilis*  
397 has been found in complex with the protein arginine phosphatase YwIE (56). The possibility that  
398 MurA proteins could also require arginine phosphorylation to be accepted as a substrate by  
399 ClpCP offers additional control possibilities for ReoM/ReoY/MurZ to modulate MurA levels.

400 A screen for *gpsB* suppressors in *S. pneumoniae* D39 and Rx1 strains did not yield *reoM*  
401 mutations (and these strains do not contain *reoY*, consistent with a subordinate function for this  
402 gene), but instead suppressor mutations were found that affect *phpP*, which encodes a Ser/Thr  
403 protein phosphatase that acts in concert with StkP, the PASTA-eSTK of this organism (57, 58).  
404 Absence or inactivation of PhpP triggered an increase in StkP-dependent protein phosphorylation  
405 levels in the pneumococcus (57, 59). It is tempting to speculate that loss of PhpP activity in this  
406 suppressor also triggers P-ReoM formation that, according to our model, would help to stabilize  
407 MurA and thus suppress the  $\Delta$ *gpsB* phenotype. Interestingly, another *S. pneumoniae* *gpsB*  
408 suppressor was identified that carries a duplication of a ~150 kb genomic fragment (57), a region  
409 that includes the open reading frame for MurA. Suppression of the *gpsB* phenotype in this  
410 instance could also work via MurA accumulation, but this time due to a gene dosage effect.

411 It is becoming increasingly evident that control of PG biosynthesis in response to cell wall  
412 derived signals, via PASTA-eSTKs, is a regulatory capacity common to Firmicutes and  
413 Actinobacteria. CwlM is the critical kinase substrate in the Actinobacterium *M. tuberculosis* that,  
414 when phosphorylated by PknB, binds to and activates MurA (23). Full-length homologues of  
415 CwlM are not present in *L. monocytogenes* or *B. subtilis* and instead these bacteria adjust their  
416 MurA levels by controlling MurA turnover in response to PrkA-dependent phosphorylation of  
417 ReoM. Consequently, both mechanisms activate PG biosynthesis in a PrkA-dependent manner  
418 either by activation or stabilization of MurA. Presumably *B. subtilis*, and other endospore  
419 forming bacteria, must re-start PG biosynthesis at the onset of germination in a similar way.  
420 Germination of *B. subtilis* spores can be triggered by muropeptides in a manner that depends  
421 upon PrkC (49), the PASTA-eSTK of *B. subtilis* (60). Even though *BsPrkC* phosphorylates  
422 multiple substrates (61), whose individual contribution to germination is not known precisely,  
423 phosphorylation of ReoM (*aka* YrzL) could be required to restart PG biosynthesis in germinating  
424 *B. subtilis* cells by stabilizing MurAA. Moreover, an *E. faecalis* mutant lacking the PASTA-  
425 eSTK IreK was more susceptible to the third-generation cephalosporin ceftriaxone but  
426 overexpression of *EfMurAA* overcame this defect (62). This implies the possibility that  
427 unphosphorylated IreB might stimulate MurAA proteolysis in *E. faecalis* as well. Taken together  
428 it seems that observations made in different Firmicutes are in good agreement with the  
429 PrkA→ReoM/ReoY→ClpC→MurA signaling sequence that we propose. The open questions  
430 that remain on the molecular mechanism of ClpCP control by ReoM and ReoY will be addressed  
431 by future experiments.

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## METHODS

### **Bacterial strains and growth conditions**

Table 1 lists all strains used in this study. Strains of *L. monocytogenes* were cultivated in BHI broth or on BHI agar plates. *B. subtilis* strains were grown in LB broth at 37°C. Antibiotics and supplements were added when required at the following concentrations: erythromycin (5 µg/ml), kanamycin (50 µg/ml), X-Gal (100 µg/ml) and IPTG (as indicated). *Escherichia coli* TOP10 was used as host for all cloning procedures (63).

### **General methods, manipulation of DNA and oligonucleotide primers**

Standard methods were used for transformation of *E. coli* and isolation of plasmid DNA (63). Transformation of *L. monocytogenes* was carried out as described by others (64). Restriction and ligation of DNA was performed according to the manufacturer's instructions. All primer sequences are listed in Table 2.

### **Construction of plasmids for recombinant protein expression**

The plasmids for expressing recombinant versions of ReoM, PrkA-KD and PrkP were prepared by first amplifying the corresponding genes (*reoM*, *lmo1820* and *lmo1821*) from *L. monocytogenes* EGD-e genomic DNA using primer pairs Lmo1503F/Lmo1503R, PrkAF/PrkAR, and PrkPF/PrkPR, respectively. The PCR products were individually ligated between the *NcoI* and *XhoI* sites of pETM11 (65). All mutagenesis was carried out using the Quikchange protocol and the correct sequence of each plasmid and mutant constructed was verified by Sanger DNA sequencing (Eurofins Genomics).

457 **Construction of plasmids for generation of *L. monocytogenes* strains**

458 Plasmid pJR65 was constructed for the inducible expression of *reoM*. To this end, the *reoM* open  
459 reading frame was amplified by PCR using the oligonucleotides JR169/JR170 and cloned into  
460 pIMK3 using NcoI/SalI. The T7A mutation were introduced into *reoM* of plasmid pJR65 by  
461 quickchange mutagenesis using the primer pair SW77/SW78, yielding pSW29. The R57A, R62A  
462 R66A and R70A, mutations were introduced into pJR65 in the same way, but using primer pairs  
463 SW144/SW145, SW146/SW147, SW136/SW137 and SW138/SW139, respectively.

464 Plasmid pJR70 was constructed for inducible *reoY* expression. For this purpose, *reoY* was  
465 amplified using the primer pair JR163/JR164 and cloned into pIMK3 using NcoI/SalI.

466 Plasmid pSW38, for IPTG-inducible *prkA* expression, was constructed by amplification of *prkA*  
467 using the oligonucleotides SW112/SW113 and the subsequent cloning of the generated fragment  
468 into pIMK3 using BamHI/SalI. Plasmid pSW39, for IPTG-controlled expression of *prkP*, was  
469 constructed analogously, but using the oligonucleotides SW110/SW111 as the primers.

470 For construction of plasmid pJR83, facilitating deletion of *reoY*, fragments encompassing ~800  
471 bp up- and down-stream of *reoY* were amplified by PCR with the primer pairs JR197/JR198 and  
472 JR199/JR200. Both fragments were spliced together by splicing by overlapping extension (SOE)  
473 PCR and cloned into pMAD using BamHI/EcoRI.

474 Plasmid pJR126 was generated for deletion of *reoM*. Fragments up- and down-stream of *reoM*  
475 were PCR amplified using the primers JR264/JR265 and JR266/JR267. Both fragments were cut  
476 with BamHI, fused together by ligation and the desired fragment was amplified from the ligation  
477 mixture by PCR using the primers JR264/JR267 and then cloned into pMAD using BglII/SalI.

478 Plasmid pSW36 was constructed to delete the *prkA* gene. Fragments up- and down-stream of  
479 *prkA* were amplified in separate PCRs using the primer pairs SHW819/SHW821 and  
480 SHW820/SHW822. Both fragments were fused together by SOE-PCR and inserted into pMAD

481 by restriction free cloning (66). Plasmid pSW37, facilitating deletion of *prkP*, was constructed in  
482 a similar manner. Up- and down-stream fragments of *prkP* were amplified using the primer pairs  
483 SHW815/SHW817 and SHW816/SHW818 and fused together by SOE-PCR. The resulting  
484 fragment was inserted into pMAD by restriction free cloning.

485 Derivatives of pIMK3 were introduced into *L. monocytogenes* strains by electroporation and  
486 clones were selected on BHI agar plates containing kanamycin. Plasmid insertion at the *attB* site  
487 of the tRNA<sup>Arg</sup> locus was verified by PCR. Plasmid derivatives of pMAD were transformed into  
488 the respective *L. monocytogenes* recipient strains and genes were deleted as described elsewhere  
489 (67). All gene deletions were confirmed by PCR.

490

#### 491 **Genome sequencing**

492 A total of 1 ng of genomic DNA was used for library generation by the Nextera XT DNA Library  
493 Prep Kit according to the manufacturer's recommendations (Illumina). Sequencing was carried  
494 out on a MiSeq benchtop sequencer and performed in paired-end modes (2 x 300 bp) using a  
495 MiSeq Reagent Kit v3 cartridge (600-cycle kit). Sequencing reads were mapped to the reference  
496 genome *L. monocytogenes* EGD-e (NC\_003210.1) (68) by utilizing the Geneious software  
497 (Biomatters Ltd.). Variants, representing putative suppressor mutations, were identified using the  
498 Geneious SNP finder tool. Genome sequences of *shg8*, *shg10*, *shg12* and LMSW76 were  
499 deposited at ENA under study number PRJEB35110 and sample accession numbers ERS3927571  
500 (SAMEA6127277), ERS3927572 (SAMEA6127278), ERS3927573 (SAMEA6127279), and  
501 ERS3967687 (SAMEA6167687) respectively.

502

## 503 **Isolation of cellular proteins and Western blotting**

504 Cells were harvested by centrifugation (13,000 rpm, 1 min in a table-top centrifuge), washed with  
505 ZAP buffer (10 mM Tris.HCl pH7.5, 200 mM NaCl), resuspended in 1 ml ZAP buffer also  
506 containing 1 mM PMSF and disrupted by sonication. Centrifugation was used to remove cellular  
507 debris and the supernatant was used as total cellular protein extract. Sample aliquots were  
508 separated by standard SDS polyacrylamide gel electrophoresis. Gels were transferred onto  
509 positively charged polyvinylidene fluoride membranes by semi-dry transfer. ClpC, GlmS, IlvB  
510 and MurA were immune-stained using a polyclonal rabbit antiserum recognizing *B. subtilis* ClpC  
511 (28), DivIVA (69), GlmS, IlvB (42) and MurAA (26) as the primary antibody and an anti-rabbit  
512 immunoglobulin G conjugated to horseradish peroxidase as the secondary one. The ECL  
513 chemiluminescence detection system (Thermo Scientific) was used for detection of the  
514 peroxidase conjugates on the PVDF membrane in a chemiluminescence imager (Vilber Lourmat).  
515 For depletion of PrkA, PrkA depletion strains were grown overnight in the presence of 1 mM  
516 IPTG and then again inoculated in BHI broth containing 1 mM IPTG to an  $OD_{600}=0.05$  and  
517 grown for 3 h at 37°C. Subsequently, cells were centrifuged, washed and reinoculated in BHI  
518 broth without IPTG at the same  $OD_{600}$  as before centrifugation. Finally, cells were harvested after  
519 3.5 more hours of growth at 37°C and cellular proteins were isolated.

520

## 521 **Microscopy**

522 Cytoplasmic membranes of exponentially growing bacteria were stained through addition of 1  $\mu$ l  
523 of Nile red solution (100  $\mu$ g ml<sup>-1</sup> in DMSO) to 100  $\mu$ l of culture. Images were taken with a Nikon  
524 Eclipse Ti microscope coupled to a Nikon DS-MBwC CCD camera and processed using the NIS  
525 elements AR software package (Nikon) or ImageJ. Scanning electron microscopy was performed  
526 essentially as described earlier (70).

527 **Recombinant protein purification**

528 All proteins were expressed in *E. coli* BL21 (DE3) cells. Cell cultures were grown at 37°C in LB  
529 liquid media supplemented with 50 µg mL<sup>-1</sup> kanamycin to an OD<sub>600</sub> 0.6-0.8 before expression  
530 was induced by the addition of IPTG to a final concentration of 0.4 mM IPTG. The cultures were  
531 incubated at 20°C overnight before the cells from 2 L of cell culture were harvested by  
532 centrifugation at 3500 x g for 30 minutes. The cell pellets were resuspended in 70 mL of buffer A  
533 (50 mM Tris.HCl, pH 8, 300 mM NaCl, 10 mM imidazole) with 500 Kunitz units of DNase I and  
534 1 mL Roche complete protease inhibitor cocktail at 25x working concentration. The cells were  
535 lysed by sonication, centrifuged at 19000 x g for 20 minutes and the supernatant was filtered  
536 using a 0.45 µm filter. The clarified cell lysate was loaded onto a 5 mL Ni-NTA superflow  
537 cartridge (Qiagen), washed with buffer A, and bound proteins were eluted with 50 mM Tris.HCl,  
538 pH 8, 300 mM NaCl, 250 mM imidazole. The His<sub>6</sub>-tag of PrkA-KD was cleaved with His-tagged  
539 TEV protease (1 mg TEV for 20 mg of protein) at 4 °C during an overnight dialysis against a  
540 buffer of 50 mM Tris.HCl, pH 8, 300 mM NaCl, 10 mM imidazole, 1 mM DTT; TEV cleavage  
541 of ReoM was conducted as above except the dialysis was carried out at 20 °C. The proteolysis  
542 reaction products were then passed over a 5 mL Ni-NTA superflow cartridge (Qiagen) to remove  
543 TEV and uncleaved protein. The proteins that did not bind to the Ni-NTA column were  
544 concentrated and loaded onto either a Superdex 75 XK16/60 (GE Healthcare) (ReoM) or a  
545 Superdex 200 XK16/60 (GE Healthcare) (PrkA-KD and PrkP) equilibrated with 10 mM Na-  
546 HEPES, pH 8, 100 mM NaCl for size exclusion chromatography. Fractions from the gel filtration  
547 were analysed for purity by SDS-PAGE, concentrated to 20-40 mg mL<sup>-1</sup>, and small aliquots were  
548 snap-frozen in liquid nitrogen for storage at -80°C.

549



## 550 **X-ray crystallography and ReoM structure determination**

551 For ReoM, 23 mg mL<sup>-1</sup> of protein in 10 mM Na-HEPES pH 8, 100 mM NaCl was subjected to  
552 crystallisation by sparse matrix screening using a panel of commercial crystallisation screens. 100  
553 and 200 nL drops of protein and 100 nL of screen solution were dispensed into 96 well MRC  
554 crystallization plates (Molecular Dimensions) by a Mosquito (TTP Labtech) liquid handling robot  
555 and the crystallisation plates were stored at a constant temperature of 20°C. The crystals that  
556 grew and were subsequently used for diffraction experiments were formed in 0.1 M  
557 phosphate/citrate pH 4.2, 0.2 M lithium sulfate, 20 % w/v PEG 1000 from the JCSG + screen and  
558 were mounted onto rayon loops directly from the crystallization drops and cryo-cooled in liquid  
559 nitrogen.

560 Diffraction data were collected on beamline I03 at the Diamond Light Source (DLS) synchrotron.  
561 Diffraction images were integrated in MOSFLM (71) and scaled and merged with AIMLESS  
562 (72). The initial model was generated by molecular replacement in PHASER (73) using the  
563 dimeric, 20-conformer ensemble model (PDBid 5US5) of IreB solved by nuclear magnetic  
564 resonance (46) as a search model. The final model was produced by iterative cycles of model  
565 building in COOT (74) with refinement in REFMAC (75) until convergence. The diffraction data  
566 collection and model refinement statistics are summarised in Table 3.

567

## 568 **Protein phosphorylation and dephosphorylation**

569 The effect of phosphorylation and dephosphorylation on ReoM and PrkA-KD proteins was  
570 analysed by 20% non-denaturing PAGE. Phosphorylation reactions consisted of 18.5 µM ReoM,  
571 3.7 µM PrkA-KD, 5 mM ATP and 5 mM MgCl<sub>2</sub>, diluted in 10 mM HEPES.HCl pH 8.0 and 100  
572 mM NaCl. Dephosphorylation reactions consisted of 37 µM P-ReoM, 3.7 µM PrkA-KD, 18.5  
573 µM PrkP and 1 mM MnCl<sub>2</sub>, diluted in 10 mM HEPES.HCl pH 8.0 and 100 mM NaCl. In each

574 case controls were loaded at the same concentrations. The reactions were incubated at 37 °C for  
575 20 minutes prior to electrophoresis at 200 V for 2.5 hours on ice.

576  
577 **Isolation of phosphorylated ReoM**  
578 Phosphorylation reactions consisted of 37 μM ReoM, 3.7 μM PrkA-KD, 5 mM ATP and 5 mM  
579 MgCl<sub>2</sub>, diluted in 10 mM HEPES.HCl pH 8.0 and 100 mM NaCl, to a total volume of 5 mL. The  
580 protein mix was loaded onto a PD 10 desalting column to remove excess ATP and protein  
581 fractions were loaded onto a MonoQ 5/50 GL column. Buffer A consisted of 10 mM HEPES.HCl  
582 pH 8.0 and 100 mM NaCl and buffer B was 10 mM HEPES.HCl pH 8.0 and 1M NaCl. Bound  
583 proteins were eluted over 25 mL with a 15-35% gradient of buffer B.

584  
585 **Liquid Chromatography-Mass Spectrometry**  
586 All liquid chromatography-mass spectrometry (LC-MS) analyses were performed using an  
587 Agilent 6530 Q-TOF instrument with electrospray ionisation (ESI) in positive ion mode, coupled  
588 to an Agilent 1260 Infinity II LC system, utilizing mobile phase of 0.1% (v/v) formic acid in LC-  
589 MS grade water (A) and acetonitrile (B). Prior to peptide mapping, 10 μL of purified proteins (~1  
590 mg/ml) were digested using Smart Digest Soluble Trypsin Kit (Thermo Fisher Scientific)  
591 according to the manufacturer's guidelines. Tryptic peptides and intact protein samples were  
592 extracted using HyperSep Spin Tip SPE C18 and C8 tips, respectively (ThermoFisher Scientific)  
593 before analysis. For phosphosite analysis, 10 μL of digest was injected onto an Acclaim RSLC  
594 120 C18 column (Thermo Fisher Scientific, 2.1 x 100mm, 2.2 μm, 120 Å) for reversed phase  
595 separation at 60°C and 0.4 ml/min, over a linear gradient of 5-40% B over 25 min, 40-90% B  
596 over 8 min followed by equilibration at 5% B for 7 min. ESI source conditions were nebuliser  
597 pressure of 45 psig, drying gas flow of 5 L/min and gas temperature of 325°C. Sheath gas

598 temperature of 275°C and gas flow of 12 L/min, capillary voltage of 4000V and nozzle voltage of  
599 300V were also applied. Mass spectra were acquired using MassHunter Acquisition software  
600 (version B.08.00) over the 100-3000 m/z range, at a rate of 5 spectra/s and 200 ms/spectrum,  
601 using standard mass range mode (3200 m/z) with extended dynamic range (2 GHz) and collection  
602 of both centroid and profile data. MS/MS fragmentation spectra were acquired over the 100-3000  
603 m/z range, at a rate of 3 spectra/s and 333.3 ms/spectrum. For intact protein analysis, 10 µL of  
604 desalted protein (~1 mg/ml) was injected onto a Zorbax 300Å Stable Bond C8 column (Agilent  
605 Technologies, 4.6 x 50 mm, 3.5 µM) for reversed phase separation at 60°C and 0.4 mL/min, over  
606 a linear gradient of 15-75% B over 14 min, 75-100% B over 11 min followed by post-run  
607 equilibration at 15% B for 10 min. ESI source conditions were nebuliser pressure of 45 psig,  
608 drying gas flow of 5 L/min and source gas temperature of 325°C were applied. Sheath gas  
609 temperature of 400°C and gas flow of 11 L/min, capillary voltage of 3500V and nozzle voltage of  
610 2000V were also used. Mass spectra were acquired using MassHunter Acquisition software  
611 (version B.08.00) over a mass range of 100-3000 m/z, at a rate of 1 spectra/s and 1000  
612 ms/spectrum in extended mass range (20000 m/z) at 1 GHz. Acquired MS and MS/MS spectra  
613 were analysed using Agilent MassHunter BioConfirm software (version B.10.00) for  
614 identification of phosphorylated residues and subsequent intact mass determination with  
615 processing of raw data using maximum entropy deconvolution.

616

### 617 **Analytical size exclusion chromatography**

618 Purified ReoM and P-ReoM proteins were run individually on a Superdex 200 Increase 10/300  
619 GL column. 100 µl samples at 1.5 mg/mL were injected onto a column equilibrated in 10 mM  
620 HEPES.HCl pH 8.0 and 100 mM NaCl, with a flow of 0.75 mL/min.

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622

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631 structure of ReoM have been deposited at PDBe with accession code 6TIF.

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## AUTHOR CONTRIBUTIONS

635

636 SW, ZJR, JR, CEJ, LM, RJL and SH designed the experiments. SW, ZJR, JR, CEJ and LM  
637 performed the experimental work. SW, ZJR, JR, CEJ, LM, RJL and SH interpreted the data. RJL  
638 and SH wrote the manuscript.

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640

## COMPETING INTERESTS STATEMENT

641

642 All authors declare that NO conflicting interests exist.

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812

813



814 **FIGURE LEGENDS**  
815  
816 **Figure 1:** Suppression of the growth defects of a *L. monocytogenes*  $\Delta gpsB$  mutant by *reoM* and  
817 *reoY* mutations.

818 (A-B) Effect of suppressor mutations on growth of the  $\Delta gpsB$  mutant. Growth of *L.*  
819 *monocytogenes* strains EGD-e (wt), LMJR19 ( $\Delta gpsB$ ), *shg8* ( $\Delta gpsB$  *reoY* H87Y), *shg10* ( $\Delta gpsB$   
820 *reoY* TAA74) and *shg12* ( $\Delta gpsB$  *reoM* RBS mutation) in BHI broth at 37°C (A) and 42°C (B).

821 (C-D) Effect of  $\Delta reoM$  and  $\Delta reoY$  deletions on growth of *L. monocytogenes*. Growth of *L.*  
822 *monocytogenes* strains EGD-e (wt), LMJR19 ( $\Delta gpsB$ ), LMSW30 ( $\Delta reoM$ ), LMSW32 ( $\Delta reoY$ ),  
823 LMJR137 ( $\Delta gpsB$   $\Delta reoM$ ) and LMJR120 ( $\Delta gpsB$   $\Delta reoY$ ) in BHI broth was recorded at 37°C (C)  
824 and 42°C (D). All growth experiments were performed three times and average values and  
825 standard deviations are shown.

826  
827 **Figure 2:** Effect of the *reoM*, *reoY* and *clpC* genes on levels of MurA in *L. monocytogenes* and  
828 MurAA in *B. subtilis*.

829 Western blots showing the levels of MurA (above) and DivIVA (as loading controls, middle) in  
830 respective *L. monocytogenes* strains, and the quantification of MurA signals (below). Non-  
831 relevant lanes were excised from the blots where necessary and average values and standard  
832 deviations were calculated from experiments repeated three times. The wild-type value was  
833 arbitrarily set to 1 and asterisks indicate statistically significant differences compared to wild type  
834 ( $P < 0.05$ , *t*-test) and n. s. means not significant.

835 (A) Effect of *reoM* and *reoY* deletions (single or when combined with *gpsB* deletion) on MurA  
836 levels in *L. monocytogenes* strains EGD-e (wt), LMJR19 ( $\Delta gpsB$ ), LMSW30 ( $\Delta reoM$ ), LMSW32

837 ( $\Delta reoY$ ), LMJR137 ( $\Delta gpsB \Delta reoM$ ) and LMJR120 ( $\Delta gpsB \Delta reoY$ ). Strain LMJR138 ( $\Delta clpC$ ) was  
838 included for comparison.

839 (B) Effect of *reoM*, *reoY* and *murZ* deletions when combined with *clpC* deletion on MurA levels.  
840 MurA levels of *L. monocytogenes* strains EGD-e (wt), LMJR138 ( $\Delta clpC$ ), LMJR104 ( $\Delta murZ$ ),  
841 LMJR171 ( $\Delta clpC \Delta murZ$ ), LMSW30 ( $\Delta reoM$ ), LMSW50 ( $\Delta clpC \Delta reoM$ ), LMSW32 ( $\Delta reoY$ )  
842 and LMSW51 ( $\Delta clpC \Delta reoY$ ). Strain LMJR123 (*ImurA*) grown in the presence or absence of  
843 IPTG was included for comparison.

844 (C) Effect of the *reoM* and *reoY* homologs *yrzL* and *ypiB*, respectively, on MurAA levels of *B.*  
845 *subtilis*. Strains BKE00860 ( $\Delta clpC$ ), BKE22180 ( $\Delta gpsB$ ), BKE22580 ( $\Delta ypiB/reoY$ ) and  
846 BKE27400 ( $\Delta yrzL/reoM$ ) were grown to mid-logarithmic growth phase before total cellular  
847 proteins were isolated. *B. subtilis* 168 (wt) was included as control. That MurAA is detected in  
848 two isoforms had been observed earlier but the reasons for this are not known (26).

849  
850 **Figure 3:** The PrkA/PrkP pair controls the phosphorylation status of ReoM.

851 Non-denaturing PAGE analysis of the phosphorylation (A) and dephosphorylation (B) of ReoM  
852 *in vitro*. The components of each lane in the gel is annotated above the image and the position  
853 and identity of relevant bands is marked to the side.

854 (C) LC-MS analysis of intact ReoM. The deconvoluted mass spectrum for non-phosphorylated  
855 ReoM (black) is overlaid over the equivalent spectrum for mono-phosphorylated ReoM, P-ReoM  
856 (red).

857 (D) LC-MS/MS was used to perform peptide mapping analysis that revealed that Thr7 is the sole  
858 phosphosite of ReoM. The MS/MS fragmentation spectra of the phosphorylated peptide  
859 encompassing Asp5-Lys22 is presented with *b*-ion fragmentation in blue and *y*-ion fragmentation  
860 shown in red, whilst the precursor ion ( $m/z$  1116.86,  $z=2+$ ) is represented by a blue diamond.

861 **Figure 4:** A ReoM T7A exchange affects growth and MurA levels in a ClpC-dependent manner.  
862 (A) Lethality of the *reoM T7A* mutation in *L. monocytogenes*. *L. monocytogenes* strains EGD-e  
863 (wt), LMSW30 ( $\Delta$ *reoM*), LMSW57 (*ireoM*) and LMSW52 (*ireoM T7A*) were grown in BHI  
864 broth  $\pm$  1 mM IPTG at 37°C. The experiment was repeated three times and average values and  
865 standard deviations are shown. (B) Suppression of *reoM T7A* lethality by deletion of *clpC*. *L.*  
866 *monocytogenes* strains EGD-e (wt), LMJR138 ( $\Delta$ *clpC*), LMSW52 (*ireoM T7A*) and LMSW72  
867 (*ireoM T7A*  $\Delta$ *clpC*) were grown in BHI broth  $\pm$  1 mM IPTG at 37°C. The experiment was  
868 repeated three times and average values and standard deviations are shown. (C) Western blot  
869 showing cellular levels of MurA (top) and ClpC (middle) in the strains included in panels A and  
870 B. For this experiment, strains were grown in BHI broth not containing IPTG at 37°C. IPTG (1  
871 mM) was added to the cultures at an OD<sub>600</sub> of 0.2 and the cells were collected 2 hours later.  
872 Irrelevant lanes were removed from both blots. Quantification of MurA signals by densitometry  
873 is shown below the Western blots. Average values and standard deviations calculated from three  
874 independent experiments are shown. Asterisks indicate statistically significant differences  
875 ( $P < 0.05$ , *t*-test). (D) Contribution of ReoY and MurZ to the lethal *reoM T7A* phenotype. *L.*  
876 *monocytogenes* strains EGD-e (wt), LMSW52 (*ireoM T7A*), LMSW72 (*ireoM T7A*  $\Delta$ *clpC*),  
877 LMSW123 (*ireoM T7A*  $\Delta$ *reoY*) and LMSW124 (*ireoM T7A*  $\Delta$ *murZ*) were grown in BHI broth  
878 containing 1 mM IPTG and growth at 37°C was recorded in a microplate reader. Average values  
879 and standard deviations were calculated from an experiment performed in triplicate.

880

881 **Figure 5:** Crystal structure of ReoM.

882 (A) The structure of ReoM depicted as a cartoon with each protomer in the dimer coloured  
883 separately (cyan and orange). The secondary structure elements are numbered according to their  
884 position in the amino acid sequence. Thr7 and some of the key amino acids in the dimer interface

885 and the hydrophobic core are drawn as stick figures. (B) Sequence alignment of ReoM (*Lmo*) and  
886 its homologues from *Bacillus subtilis* (*Bsu*), *Streptococcus pneumoniae* (*Spn*), *Clostridium*  
887 *difficile* (*Cdi*) and *Staphylococcus aureus* (*Sau*) with the sequence of IreB from *Enterococcus*  
888 *faecalis* (*Efa*) underneath. Amino acid sequence numbers pertain to ReoM and the site of  
889 phosphorylation in ReoM (Thr7) and the twin phosphorylations in IreB (minor site: Thr4; major  
890 site: Thr7) are highlighted. Invariant amino acids are shaded black, residues in the ReoM dimer  
891 interface have an asterisk above, and the secondary structure elements are defined by cylinders  
892 above the alignment. Arginine residues mutated in this study are indicated by an hashtag above  
893 the alignment. (C) The final  $2F_{\text{obs}} - F_{\text{calc}}$  electron density map, contoured at a level of  $0.42 \text{ e}^-/\text{\AA}^3$ , of  
894 the N-terminal region in the immediate vicinity of Thr7 in chain A of the ReoM dimer indicates  
895 that the protein model could be built with confidence even though this region contains no  
896 secondary structure elements.

897

898 **Figure 6:** Effect of *prkA* and *prkP* mutations on growth and MurA levels of *L. monocytogenes*.

899 (A) Contribution of PrkA and PrkP to *L. monocytogenes* growth. *L. monocytogenes* strains EGD-  
900 e (wt), LMSW76 ( $\Delta prkP$  *prkA*\*), LMSW83 (*iprkP*) and LMSW84 (*iprkA*) were grown in BHI  
901 broth containing or not containing 1 mM IPTG at 37°C in a microtiter plate reader. The  
902 experiment was repeated three times and average values and standard deviations are shown. (B)  
903 Contribution of PrkA and PrkP to MurA stability. Western blots showing cellular levels of MurA  
904 (top) and DivIVA (middle) in the same set of strains as in panel A and quantification of MurA  
905 signals by densitometry (below). Average values and standard deviations calculated from three  
906 independent experiments are shown. Asterisks indicate statistically significant differences  
907 ( $P < 0.05$ , *t*-test).

908

909 **Fig. 7:** PrkA essentiality depends on *reoM*, *reoY* and *clpC*.

910 (A) Effect of *reoM*, *reoY* and *clpC* deletions on *prkA* essentiality. *L. monocytogenes* strains EGD-  
911 e (wt), LMSW84 (*iprkA*), LMSW89 (*iprkA*  $\Delta$ *reoM*), LMSW90 (*iprkA*  $\Delta$ *reoY*) and LMSW91  
912 (*iprkA*  $\Delta$ *clpC*) were grown in BHI broth  $\pm$  1 mM IPTG at 37°C in a microtiter plate reader. The  
913 experiment was repeated three times and average values and standard deviations are shown. (B)  
914 *clpC*, *reoM* and *reoY* deletions overcome MurA degradation in PrkA-depleted cells. Western blot  
915 showing MurA levels in *L. monocytogenes* strains EGD-e (wt), LMJR138 ( $\Delta$ *clpC*), LMSW30  
916 ( $\Delta$ *reoM*), LMSW32 ( $\Delta$ *reoY*), LMSW84 (*iprkA*), LMSW89 (*iprkA*  $\Delta$ *reoM*), LMSW90 (*iprkA*  
917  $\Delta$ *reoY*) and LMSW91 (*iprkA*  $\Delta$ *clpC*, top). PrkA wild type strains were grown in BHI broth at  
918 37°C to mid-exponential growth phase before protein isolation. A parallel DivIVA Western blot  
919 was used as loading control (middle). Quantification of MurA signals by densitometry (below).  
920 Average values and standard deviations calculated from three independent experiments are  
921 shown. Asterisks indicate statistically significant differences ( $P < 0.05$ , *t*-test).

922

923 **Fig. 8:** ReoM links cell wall integrity sensing with peptidoglycan biosynthesis.

924 Model illustrating the role of ReoM as substrate of PrkA and as regulator of ClpCP. Cell wall  
925 damage is sensed by PrkA through recognition of free muropeptides upon which PrkA  
926 phosphorylates ReoM. In its unphosphorylated form, ReoM is an activator of ClpCP-dependent  
927 degradation of MurA, the first enzyme of peptidoglycan biosynthesis, and ReoY and MurZ  
928 contribute to this process. By phosphorylating ReoM, PrkA prevents ClpCP-dependent MurA  
929 degradation so that MurA accumulates and peptidoglycan biosynthesis can occur. Please note that  
930 there is a lesser degree of conservation in the fourth PASTA domain of PrkA.

931

932 **Table 1:** Plasmids and strains used in this study

name	relevant characteristics	source*/ reference
<b>Plasmids</b>		
pIMK3	$P_{help}$ - <i>lacO lacI neo</i>	(64)
pMAD	<i>bla erm bgaB</i>	(67)
pJR127	<i>bla erm bgaB ΔclpC</i> ( <i>lmo0232</i> )	(28)
pSH246	<i>bla erm bgaB ΔgpsB</i> ( <i>lmo1888</i> )	(32)
pJR68	<i>bla erm bgaB ΔmurZ</i> ( <i>lmo2552</i> )	(28)
pJR65	$P_{help}$ - <i>lacO-reoM lacI neo</i>	this work
pJR70	$P_{help}$ - <i>lacO-reoY lacI neo</i>	this work
pJR83	<i>bla erm bgaB ΔreoY</i> ( <i>lmo1921</i> )	this work
pJR126	<i>bla erm bgaB ΔreoM</i> ( <i>lmo1503</i> )	this work
pSW29	$P_{help}$ - <i>lacO-reoM T7A lacI neo</i>	this work
pSW36	<i>bla erm bgaB ΔprkA</i> ( <i>lmo1820</i> )	this work
pSW37	<i>bla erm bgaB ΔprkP</i> ( <i>lmo1821</i> )	this work
pSW38	$P_{help}$ - <i>lacO-prkA lacI neo</i>	this work
pSW39	$P_{help}$ - <i>lacO-prkP lacI neo</i>	this work
pSW55	$P_{help}$ - <i>lacO-reoM R66A lacI neo</i>	this work
pSW56	$P_{help}$ - <i>lacO-reoM R70A lacI neo</i>	this work
pSW58	$P_{help}$ - <i>lacO-reoM R57A lacI neo</i>	this work
pSW59	$P_{help}$ - <i>lacO-reoM R62A lacI neo</i>	this work
<b><i>B. subtilis</i> strains</b>		
168	wild type, lab collection	
BKE00860	$ΔclpC$	(76)
BKE22180	$ΔgpsB$	(76)
BKE22580	$ΔypiB$ ( <i>reoY</i> )	(76)
BKE27400	$ΔyrzL$ ( <i>reoM</i> )	(76)
<b><i>L. monocytogenes</i> strains</b>		
EGD-e	wild-type, serovar 1/2a strain	(68)
LMJR19	$ΔgpsB$ ( <i>lmo1888</i> )	(32)
LMJR104	$ΔmurZ$ ( <i>lmo2552</i> )	(28)
LMJR123	$ΔmurA$ ( <i>lmo2526</i> ) <i>attB::P<sub>help</sub>-lacO-murA lacI neo</i>	(28)
LMJR138	$ΔclpC$ ( <i>lmo0232</i> )	(28)
<i>shg8</i>	$ΔgpsB reoY$ H87Y	this work
<i>shg10</i>	$ΔgpsB reoY$ TAA74	this work
<i>shg12</i>	$ΔgpsB reoM$ RBS mutation	this work
LMJR96	$ΔgpsB attB::P_{help}$ - <i>lacO-reoM lacI neo</i>	pJR65 → LMJR19
LMJR102	$attB::P_{help}$ - <i>lacO-reoM lacI neo</i>	pJR65 → EGD-e
LMJR106	$ΔgpsB attB::P_{help}$ - <i>lacO-reoY lacI neo</i>	pJR70 → LMJR19
LMJR120	$ΔgpsB ΔreoY$	pJR83 ↔ LMJR19
LMJR137	$ΔgpsB ΔreoM$	pJR126 ↔ LMJR19
LMJR171	$ΔclpC ΔmurZ$	pJR127 ↔ LMJR104
LMSW30	$ΔreoM$ ( <i>lmo1503</i> )	pJR126 ↔ EGD-e
LMSW32	$ΔreoY$ ( <i>lmo1921</i> )	pJR83 ↔ EGD-e
LMSW50	$ΔclpC ΔreoM$	pJR127 ↔ LMSW30
LMSW51	$ΔclpC ΔreoY$	pJR127 ↔ LMSW32
LMSW52	$ΔreoM attB::P_{help}$ - <i>lacO-reoM T7A lacI neo</i>	pSW29 → LMSW30
LMSW57	$ΔreoM attB::P_{help}$ - <i>lacO-reoM lacI neo</i>	pJR65 → LMSW30
LMSW72	$ΔreoM attB::P_{help}$ - <i>lacO-reoM T7A lacI neo ΔclpC</i>	pJR127 ↔ LMSW52
LMSW76	$ΔprkP prkA^*$	pSW37 ↔ EGD-e
LMSW80	$attB::P_{help}$ - <i>lacO-prkA lacI neo</i>	pSW38 → EGD-e
LMSW81	$attB::P_{help}$ - <i>lacO-prkP lacI neo</i>	pSW39 → EGD-e
LMSW83	$ΔprkP attB::P_{help}$ - <i>lacO-prkP lacI neo</i>	pSW37 ↔ LMSW81
LMSW84	$ΔprkA attB::P_{help}$ - <i>lacO-prkA lacI neo</i>	pSW36 ↔ LMSW80

<b>name</b>	<b>relevant characteristics</b>	<b>source*/ reference</b>
LMSW89	$\Delta prkA$ attB::P <sub>help</sub> -lacO-prkA lacI neo $\Delta reoM$	pJR126 ↔ LMSW84
LMSW90	$\Delta prkA$ attB::P <sub>help</sub> -lacO-prkA lacI neo $\Delta reoY$	pJR83 ↔ LMSW84
LMSW91	$\Delta prkA$ attB::P <sub>help</sub> -lacO-prkA lacI neo $\Delta clpC$	pJR127 ↔ LMSW84
LMSW117	$\Delta reoM$ $\Delta reoY$	pJR126 ↔ LMSW32
LMSW118	$\Delta reoY$ $\Delta murZ$	pJR68 ↔ LMSW32
LMSW119	$\Delta reoM$ $\Delta murZ$	pJR68 ↔ LMSW30
LMSW120	$\Delta reoM$ attB::P <sub>help</sub> -lacO-reoM R66A lacI neo	pSW55 → LMSW30
LMSW121	$\Delta reoM$ attB::P <sub>help</sub> -lacO-reoM R70A lacI neo	pSW56 → LMSW30
LMSW123	$\Delta reoM$ attB::P <sub>help</sub> -lacO-reoM T7A lacI neo $\Delta reoY$	pSW29 → LMSW117
LMSW124	$\Delta reoM$ attB::P <sub>help</sub> -lacO-reoM T7A lacI neo $\Delta murZ$	pSW29 → LMSW119
LMSW125	$\Delta reoM$ attB::P <sub>help</sub> -lacO-reoM R57A lacI neo	pSW58 → LMSW30
LMSW126	$\Delta reoM$ attB::P <sub>help</sub> -lacO-reoM R62A lacI neo	pSW59 → LMSW30

933 \* The arrow (→) stands for a transformation event and the double arrow (↔) indicates gene  
 934 deletions obtained by chromosomal insertion and subsequent excision of pMAD plasmid  
 935 derivatives (see experimental procedures for details).

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939 **Table 2: Oligonucleotides used in this study.**

name	sequence (5'→3')
JR163	GCGCCCATGGCTAAGGCATCCATTTCAATAGACGAGAAG
JR164	GCGCGTCGACTTATTCTTTTTCCGTATCCATTTGCTGTA
JR169	GCGCCCATGGATTCAAAAGATCAAACAATGTTTTACAACCTTC
JR170	GCGCGTCGACTCATTCTCACCAATTTTCGTTATTTTTTCAG
JR197	GCGCGGATCCCAATTATTTTCGAATGGTGC GGTTGTC
JR198	TCCTTATTCGTGCGACCATCTTTCTCAGTCCCTTCCTG
JR199	GGAAAGATGGTTCGACGAATAAGGAATAAATCCTAGTTAGTAGGG
JR200	CGCGGAATTTCCAAGACTCAACCTCTTTCACTC
JR264	GCGCAGATCTGGCAAATACAGCATTGAACTATGTG
JR265	GCGCGGATCCAATCGAAGCACCTCATTCCTTC
JR266	GCGCGGATCCATGAGAATAATGGGTTTAGATGTCCGGC
JR267	GCGCGTCGACGCTAGGAATGTAGCAAGGATTTCTTC
SHW815	GATCTATCGATGCATGCCATGGGCTAAATGACCAAGGAATTACCG
SHW816	CGCGTCGGGCGATATCGGATCCTTTCTTCCGCGTTTTGGTAACG
SHW817	CAATCATCATTTTTAAAAGCACCTCACTATTTTTTCAG
SHW818	TGCTTTTAAAATGATGATTGGTAAGCGATTAAGC
SHW819	GATCTATCGATGCATGCCATGGAGATAGAGGCAGAATAAGACATC
SHW820	CGCGTCGGGCGATATCGGATCCGGTATTTACAACCACTACGTCG
SHW821	CGTTCTTATTTTCATGAAGCATCCCTCCCTTTC
SHW822	TGCTTCATGAAATAAGAACGGAGGAAATGTGCTG
SW77	GTA AACATTGCTTGATCTTTTGAATCCATGGGTTTCAC
SW78	GATCAAGCAATGTTTTACAACCTTCGGCGATGATTC
SW110	GCGCGCGGATCCATGCATGCAGAATTTAGAACAGATAG
SW111	GCGCGCGTTCGACTCATGAAGCATCCCTCCCTTTC
SW112	GCGCGCGGATCCATGATGATTGGTAAGCGATTAAGCG
SW113	GCGCGCGTTCGACTTAATTTGGATAAGGGACTGTACCTTC
SW136	CTAAACGAGCTATCATACTTCTAGCATCCTTGTGAC
SW137	GTATGATAGCTCGTTTAGAACGAGATGAAATTATCGAG
SW138	AATTTTCATCTGCTTCTAAACGACGTATCATACTTCTAGC
SW139	GTTTAGAAGCAGATGAAATTATCGAGGAACCTTGTC AAAG
SW144	CCTTGTGAGCAGGAATATAAGCAGGATCGCCTG
SW145	TATATTCTGCTCACAAGGATGCTAGAAGTATGATAC
SW146	GTATCATACTTGCAGCATCCTTGTGACGAGGAATATAAG
SW147	GGATGCTGCAAGTATGATACGTCGTTTAGAACGAG
Lmo1503F	GCTATACCATGGATTCAAAAGATCAAACAATGTTTTACAAC
Lmo1503R	CGATATCTCGAGTCATTTCTCACCAATTTTCGTTATTTTTTCAG
PrkAF	GCTATACCATGGCAATGATGATTGGTAAGCGATTAAGCG
PrkAR	CGATATCTCGAGTCATTTTTTCTTTTTCTTATCTTTTTTCTCCTCAGG
PrkPF	GCTATACCATGGCAATGCATGCAGAATTTAGAACAGATAGAG
PrkPR	CGATATCTCGAGTCATGAAGCATCCCTCCCTTTC

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942 **Table 3:** Summary of the data collection and refinement statistics for ReoM.

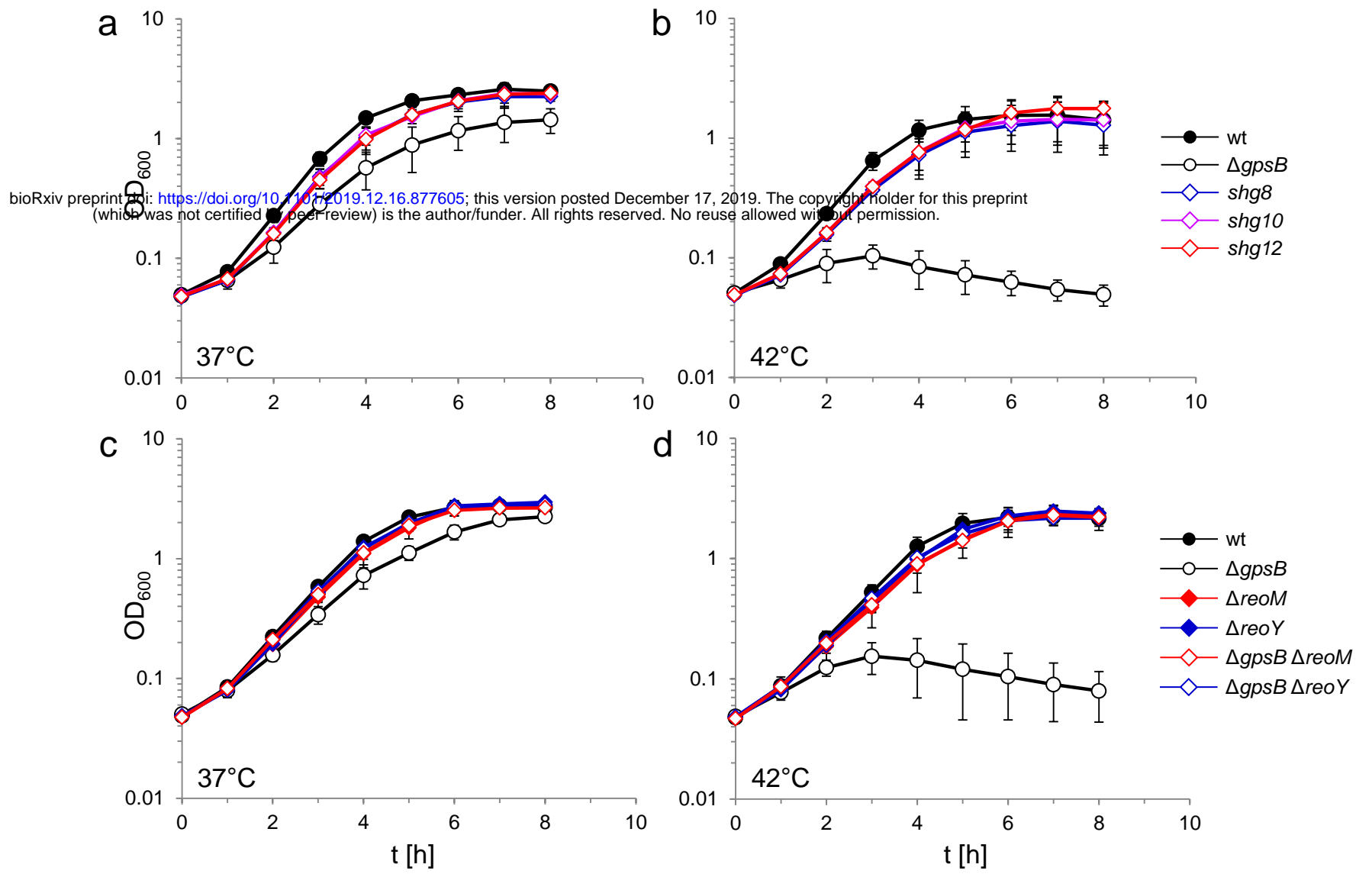
<b>Data collection</b>	
Beamline	Diamond I03
Wavelength (Å)	0.976
Resolution (Å)	74.45-1.60 (1.63-1.60)*
Space group	P 2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>
<i>a</i> , <i>b</i> , <i>c</i> (Å)	38.79, 58.62, 74.45
$\alpha$ , $\beta$ , $\gamma$ (°)	90, 90, 90
R <sub>pim</sub>	0.064 (0.533)
CC (1/2) (%)	98.6 (62.0)
$\langle I \rangle / \langle \sigma(I) \rangle$	8.2 (2.2)
Completeness (%)	99.8 (99.8)
Redundancy	4.8 (4.9)
Total observations	111229 (5581)
Unique reflections	23059 (1129)
<b>Refinement</b>	
R <sub>work</sub> (%)	15.3
R <sub>free</sub> (%)	21.4
Solvent content (%)	38.0
# atoms	
Protein	1399
Ligand/ion	20
Water	94
<b>B-factors (Å<sup>2</sup>)</b>	
Protein	26.4
Ligand/ion	50.5
Water	37.7
R.m.s deviations	
Bonds (Å)	0.015
Angles (°)	1.79

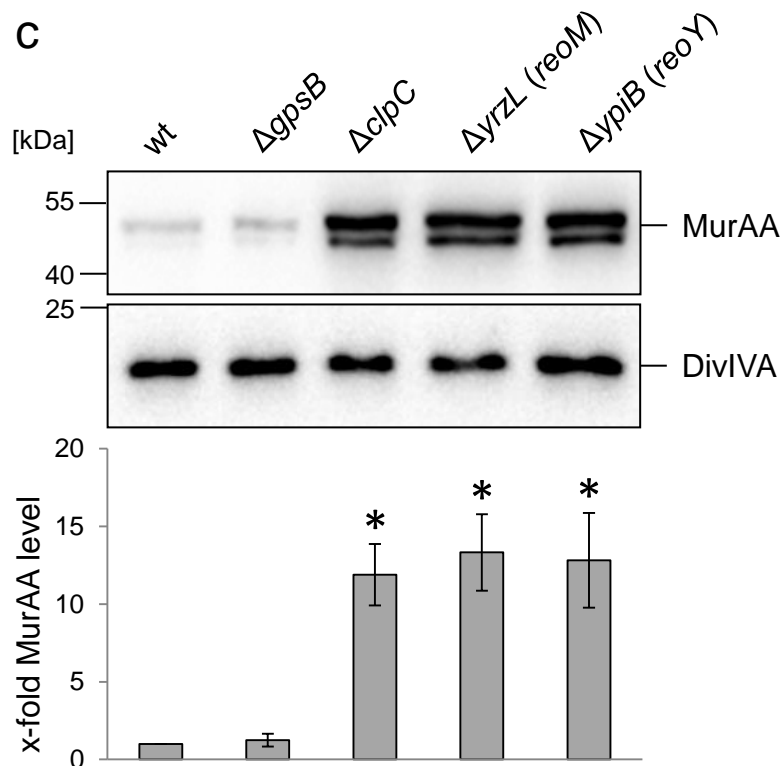
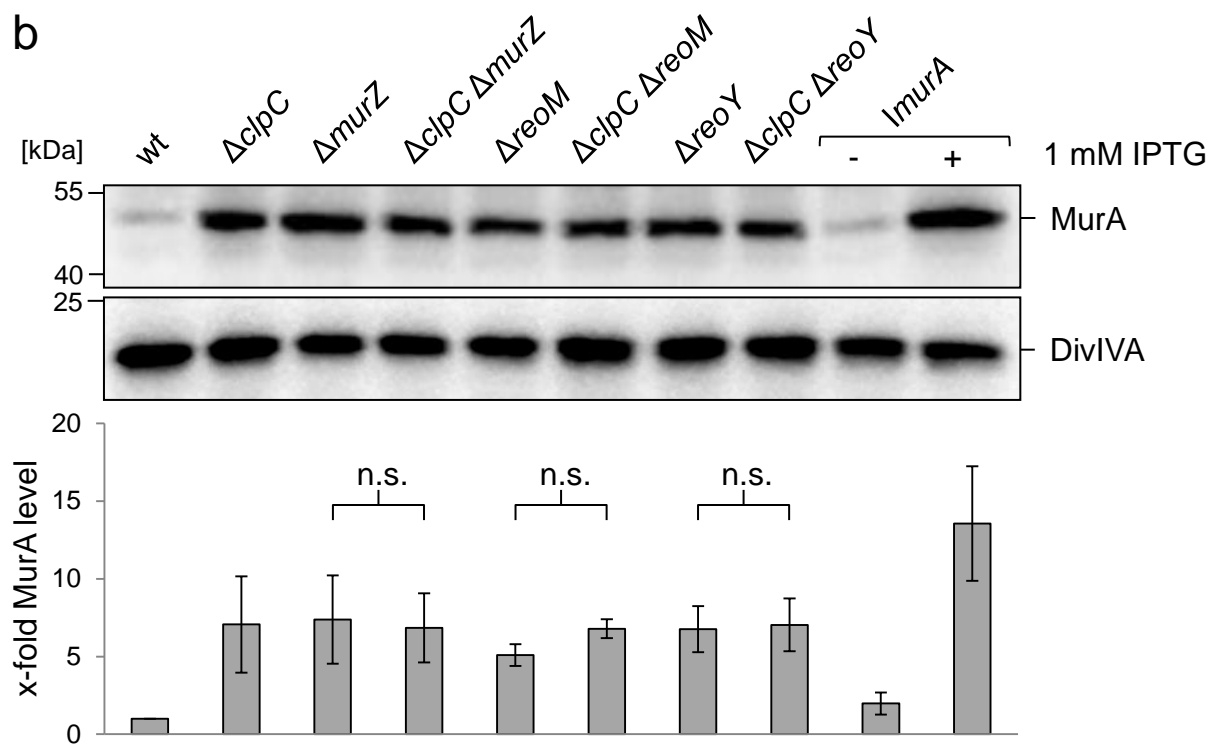
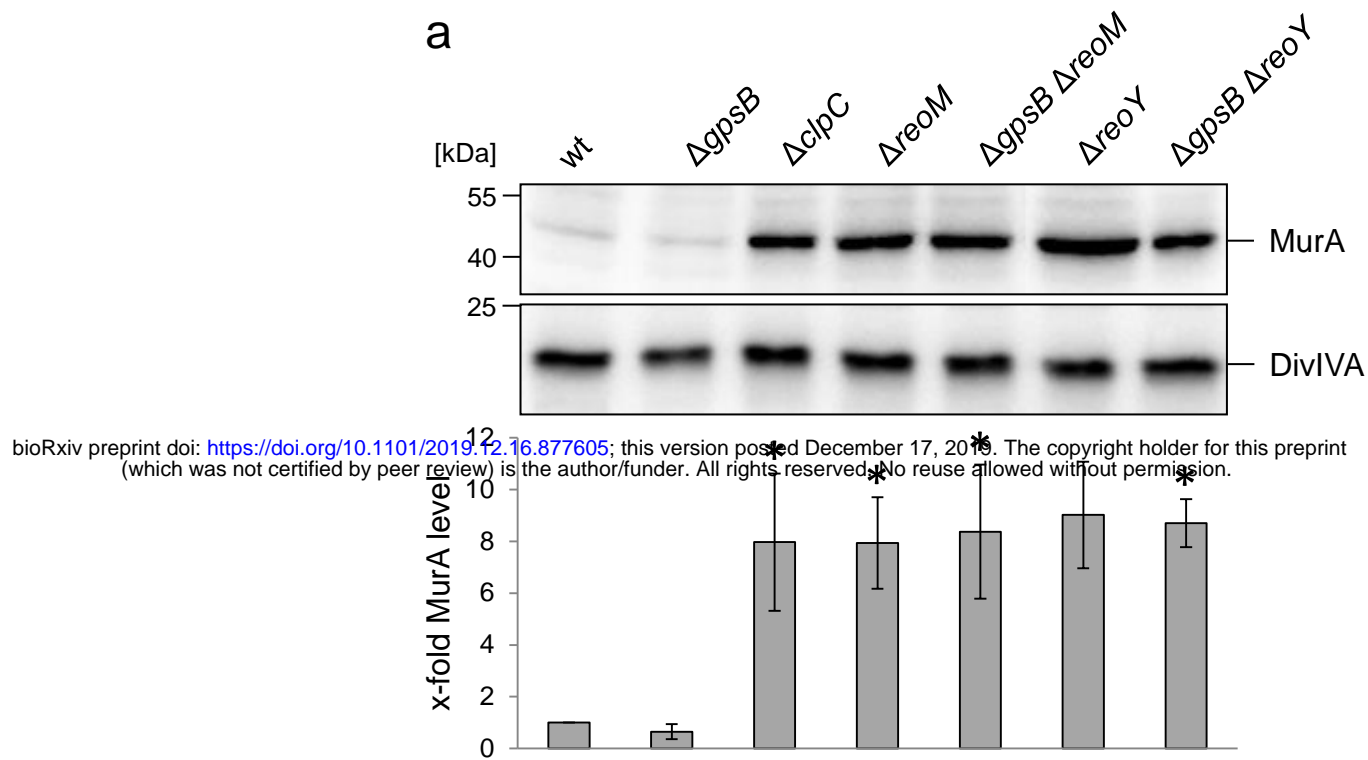
943 \*Where values in parentheses refer to the highest resolution shell.

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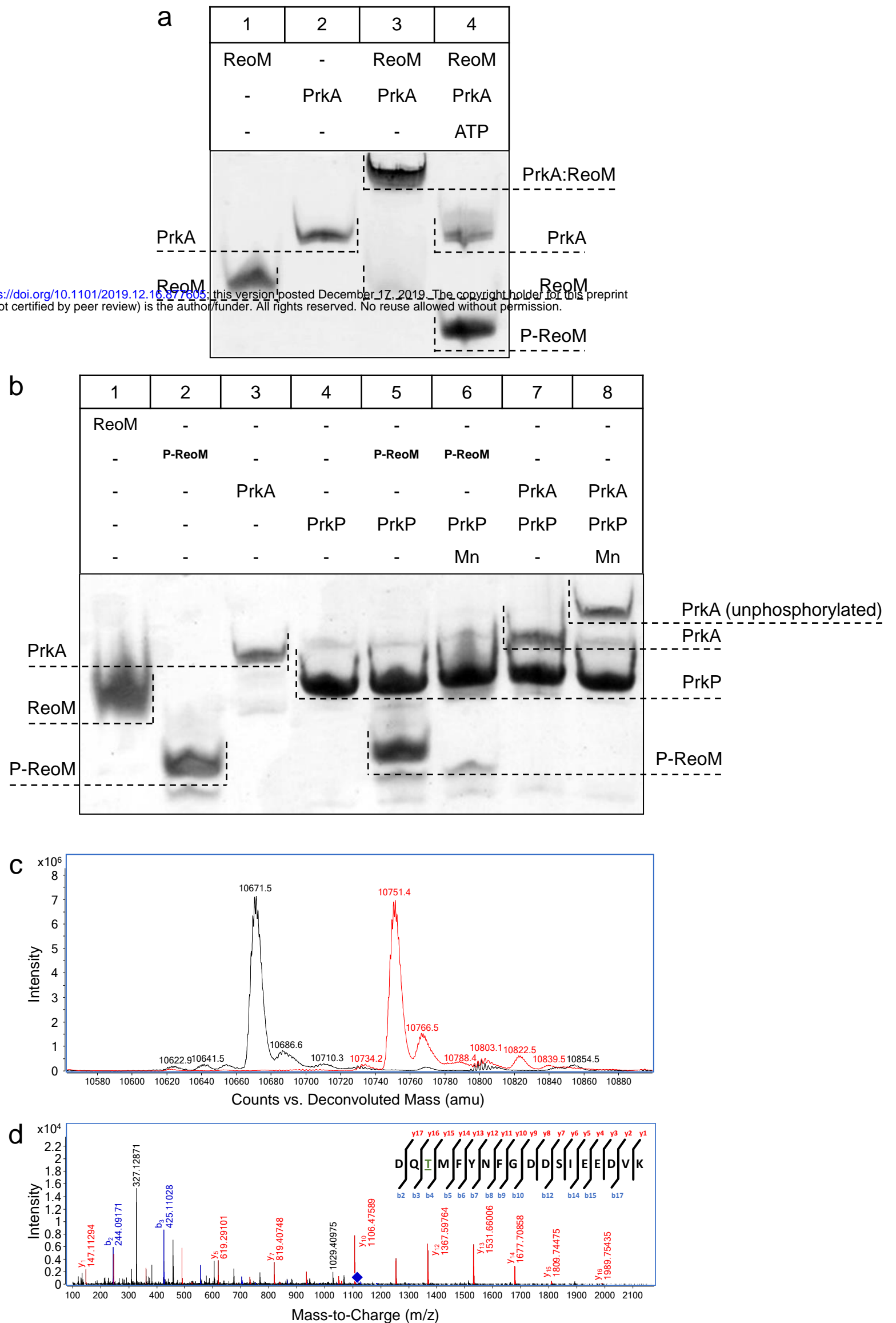
945

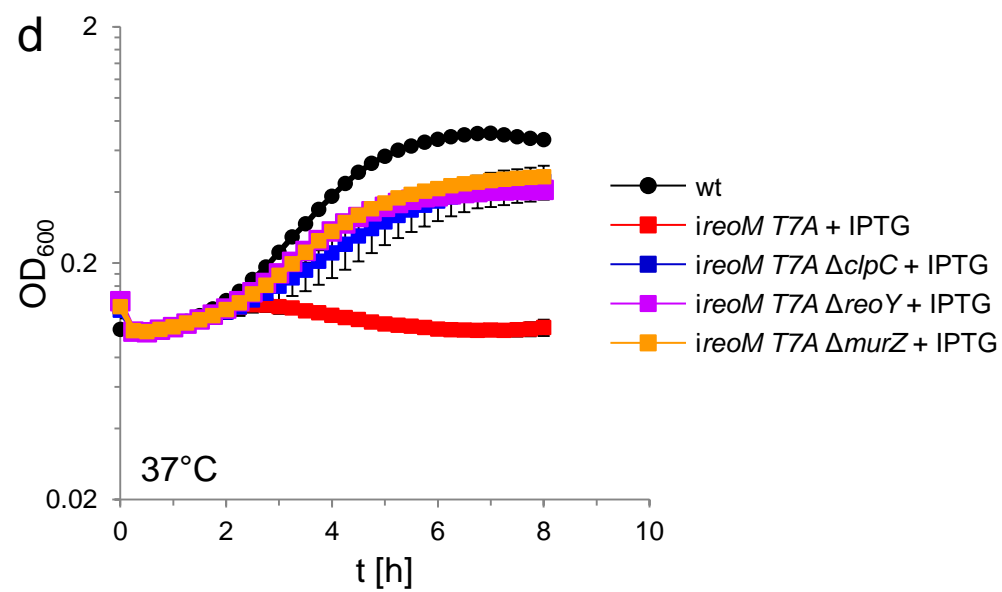
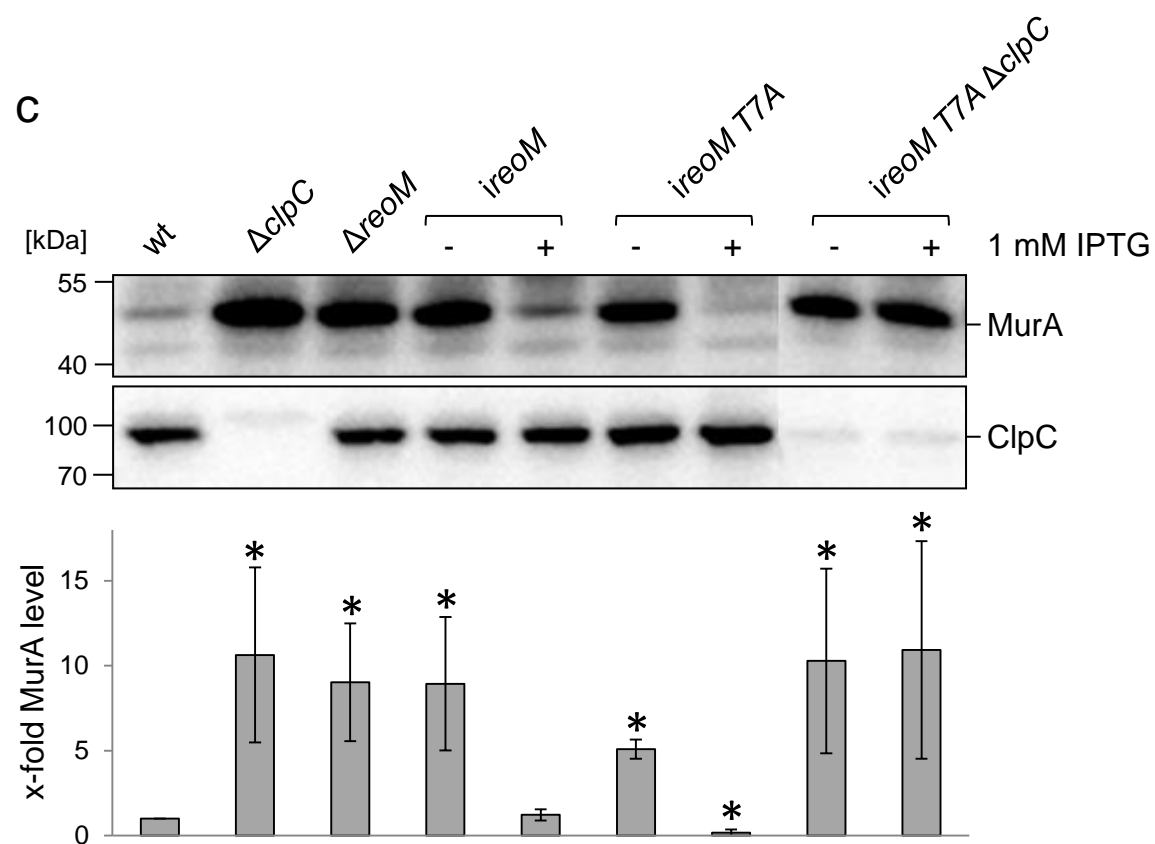
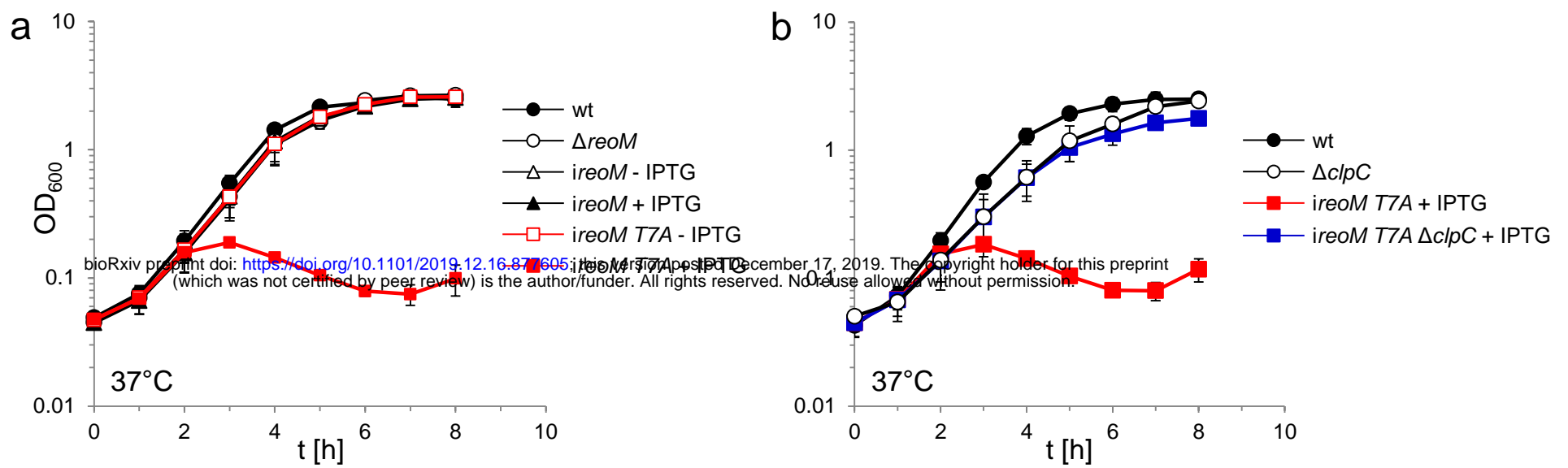
946

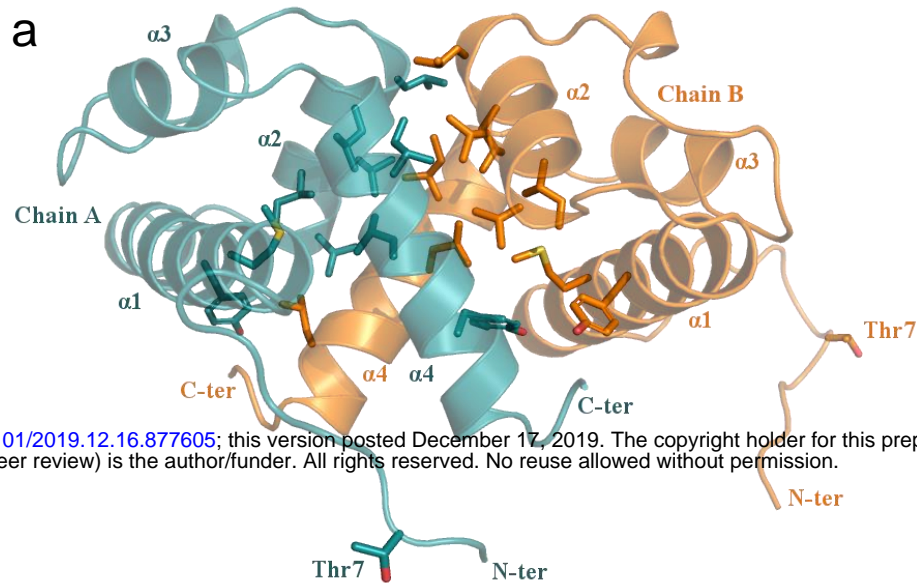




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