Title

Co-option and Detoxification of a Phage Lysin for Housekeeping Function

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Summary

Temperate phages constitute a potentially beneficial genetic reservoir for bacterial innovation despite being selfish entities encoding an infection cycle inherently at odds with bacterial fitness. These phages integrate their genomes into the bacterial host during infection, donating new, but deleterious, genetic material: the phage genome encodes toxic genes, such as lysins, that kill the bacterium during the phage infection cycle. Remarkably, some bacteria have exploited the destructive properties of phage genes for their own benefit by co-opting them as toxins for functions related to bacterial warfare, virulence, and secretion. However, do toxic phage genes ever become raw material for functional innovation? Here we report on a toxic phage gene whose product has lost its toxicity and has become a domain of a core cellular factor, SpmX, throughout the bacterial order Caulobacterales. Using a combination of phylogenetics, bioinformatics, structural biology, cell biology, and biochemistry, we have investigated the origin and function of SpmX and determined that its occurrence is the result of the detoxification of a phage peptidoglycan hydrolase gene. We show that the retained, attenuated activity of the phage-derived domain plays an important role in proper cell morphology and developmental regulation in representatives of this large bacterial clade. To our knowledge, this is the first observation of phage gene domestication in which a toxic phage gene has been co-opted for a housekeeping function.

Keywords
Lysozyme, GH24, Prophage domestication, Bacterial evolution, Alphaproteobacteria, *Caulobacter*, *Asticcacaulis*

Introduction

Understanding how new genes arise is key to studying the forces that drive diversity and evolution. Although horizontal gene transfer (HGT) is widely regarded as an important mechanism for the exchange of existing genes among bacteria, mobile genetic elements can transfer exogenous genetic material that gives rise to novel genes. These new genes can provide the basis for evolving new traits and propelling evolutionary transitions [1,2]. Temperate bacteriophages mediate genetic transfer by integrating their genomes into bacterial hosts [3–6]. These integrated tracts of genes, called prophages, remain dormant until induced by various signals to produce phage particles and phage-encoded proteins that lyse the cell. In many cases,

prophages contain genes that benefit the host, promoting retention of prophages in many bacterial lineages, even after mutations have inactivated the prophages and they no longer are capable of producing phage [7–9]. Accumulation of host-specific beneficial mutations in prophages has been referred to as "domestication." Many so-called domesticated segments of inactivated prophages unexpectedly contain lytic and phage structural genes, which would intuitively be of little use or even detrimental to the bacterial host [7]. These genes are often used by the bacteria as weapons against competing bacteria and eukaryotic hosts [10–14]. In contrast, we have identified an instance in which a toxic phage gene has not been repurposed as a weapon, but has rather been incorporated into a bacterial housekeeping factor, *spmX*. Here, we report that SpmX resulted from an ancient domestication event at the root of the alphaproteobacterial order Caulobacterales, in which co-option and detoxification of a toxic phage gene gave rise to a novel bacterial gene with roles in developmental regulation and morphogenesis.

SpmX was first identified as a developmental regulator in the model organism *Caulobacter crescentus* [15]. Like most members of Caulobacterales, stalked *C. crescentus* cells divide asymmetrically to produce a "mother" cell with a polar stalk and a motile "daughter" or "swarmer" cell with a polar flagellum. The *Caulobacter* developmental cycle depends on strict coordination of cell growth, chromosome replication and segregation, and division by various regulatory proteins that differ in localization and timing [16]. This network depends on regulatory phospho-signaling factors localized and regulated by polar scaffolds. SpmX is one scaffold that localizes at the old pole during the swarmer-to-stalked cell transition and recruits and potentially activates the histidine kinase DivJ [15]. Intriguingly, SpmX is required for stalk synthesis initiation and elongation in the closely related *Asticcacaulis* species *A. excentricus* and *A. biprosthecum* [17]. Therefore, this gene appears to have evolved multiple roles within this family of dimorphic, stalked bacteria.

Perplexingly, SpmX contains an N-terminal putative phage muramidase domain that is generally toxic to bacteria. This enzymatic domain is typically used by phage to lyse bacteria and release infectious phage particles during the lytic cycle. As a part of SpmX, this domain is critical for SpmX's role in both developmental regulation and stalk biogenesis. In particular, the muramidase domain is necessary for proper localization of SpmX in both *C. crescentus* [15,18] and the *Asticcacaulis* genus [17]. Various studies have shown that SpmX localizes with the polar scaffold PopZ in *C. crescentus* [19,20], and this interaction occurs entirely through the muramidase domain [18]. A previous study was unable to measure enzymatic activity from purified *C. crescentus* SpmX muramidase domain, and consequently concluded that the domain was repurposed for protein interactions and oligomeric assembly [18]. However, given the remarkable sequence similarity of the SpmX muramidase domain to functional phage lysozymes, including the canonical catalytic glutamate, it seems unlikely that its enzymatic activity is entirely lost. Why would this domain be so highly conserved if its new function were merely for non-essential protein-protein interactions?

To better characterize the SpmX muramidase domain and the constraints underlying its conservation, we performed an in-depth bioinformatics study of more than 60 available SpmX genes together with structural determination, biochemical analysis, and comparative cell biology between *Caulobacter* and *Asticcacaulis*. We show that SpmX arose prior to the diversification of Caulobacterales, a large class of stalked bacteria. We establish that the SpmX muramidase domain is a close relative of GH24 (T4-like) autolysin/endolysins that have been laterally exchanged throughout Proteobacteria via prophages. We find that the SpmX domain is still an active muramidase, albeit with attenuated ancestral phage activity, consistent with its remodeled

active cleft. Finally, we demonstrate that the enzymatic activity is necessary for SpmX function in three representative species. We conclude that, close to the time of the genesis of the full-length *spmX* gene, this co-opted domain accumulated mutations that attenuated its hydrolytic activity on peptidoglycan and detoxified it for bacterial use. To our knowledge, this is the first case of phage gene domestication in which a toxic phage gene has been incorporated into a new bacterial gene with housekeeping function.

Results

The SpmX muramidase domain was co-opted from prophage in an early Caulobacterales ancestor. Our first step in characterizing the muramidase domain of SpmX was to determine the prevalence of SpmX and its homologues in the bacterial domain. Simple pBLAST analysis revealed that SpmX, as defined by its three-part architecture with an N-terminal muramidase domain, a charged and proline-rich intermediate domain, and two C-terminal transmembrane (TM) segments (**Fig. 1A**), is taxonomically constrained to Caulobacterales and one member of its sister taxa, Parvularculales. It is conserved as a single-copy gene in all sequenced members (**Table S1**). In all 69 identified *spmX* orthologues, the muramidase domains exhibit high amino acid sequence conservation (**Fig. S1**), the TM segments moderate sequence conservation, and the intermediate domains high variability in length and composition among genera (**Fig. 1A**). Apart from these SpmX orthologues, BLAST searches using SpmX only returned hits for the muramidase domain. These hits came from Gram-negative bacterial genomes that span the entire bacterial domain, particularly proteobacteria, and from viral genomes. Most of these bacterial genes are likely to be in prophage regions, as evidenced by their position in long runs of predicted prophage genes. We did not detect sequences homologous to SpmX TMs in our search, although we occasionally detected homologous phage muramidase domains fused to other, non-homologous TM segments.

Consistent with finding close relatives of SpmX muramidase in prophages, NCBI's Conserved Domain Database (CDD) tool [21,22] clustered SpmX muramidase with glycoside hydroloase (GH) 24 lysozymes in the autolysin/endolysin class (**Fig. S2)**. These enzymes are closely related to classical phage T4 lysozyme-like (T4L-like) peptidoglycan hydrolases. Phages generally use lysozymes of this class to cleave peptidoglycan and lyse cells during the lytic cycle. These lysozymes are distinct from the other well-known phage Lambda lysozyme-like lytic transglycosylases, which are related to known housekeeping bacterial hydrolases with roles in cell growth and division. Lytic transglycosylases are also assigned to the GH24 group but share no sequence similarity with and catalyze a different peptidoglycan cleaving reaction than T4L-like muramidases [23–25]. Thus although core bacterial genomes encode peptidoglycan hydrolases, the muramidase domain of SpmX is most closely related to peptidoglycan hydrolases encoded by prophages.

Unlike its close relatives that have been transferred horizontally through the bacterial domain via prophage, the SpmX muramidase domain coding region has been inherited vertically as part of the *spmX* gene in Caulobacterales. The SpmX gene tree mirrors the phylogeny of Caulobacterales from concatenated gene alignments (**Fig. 1B**). None of the SpmX genes appear in tracks of prophage genes, and the genomic context of *spmX* appears to be well maintained in members of Caulobacterales, with the gene occurring between a putative Mg^{2+} transporter and a putative isovaleryl-CoA dehydrogenase in nearly all species. Together, these findings suggest that SpmX muramidase is a peptidoglycan hydrolase derived from prophage that has become a

139 domain in a bacterial protein that is no longer within prophage and is under direct cellular
140 control. It likely fused with the intermediate and TM domains in a common ancestor of
141 Parvularculales and Caulobacterales. The vertical transmission of SpmX and strong sequence
142 conservation of the muramidase domain suggests an important function in members of
143 Caulobacterales.
144
145 **The SpmX muramidase domain retains the canonical GH24 motif but contains mutations**
146 **in the catalytic cleft known to inactivate phage lysozymes.** To determine if critical enzymatic
147 residues in SpmX muramidase were conserved, we compared the amino acid sequences of SpmX
148 and other lysozymes. By definition, lysozymes catalyze the hydrolysis of β1,4-linked glycosidic
149 bonds in peptidoglycan and chitin [25]. This superfamily includes at least seven distinct groups
150 that are unrelated by sequence similarity but share a common fold in which the catalytic Glu and
151 the beta-hairpin motif in the N-terminal lobe form the catalytic cleft by packing against the C-
152 terminal lobe (**Fig. 2A)** [26]. This beta-hairpin, or GH motif, contains family-specific residues
153 critical for enzyme activity in all members of the lysozyme superfamily [26].
154 We compared the GH motif of SpmX to lysozymes from the T4L and endolysin/autolysin
155 classes with varying degrees of relatedness. **Figure 2B** shows the amino acid conservation in the
156 GH motif of T4L-like, autolysin/endolysin, closely related non-SpmX muramidase, and SpmX
157 muramidase protein sequences. Because the autolysin/endolysin class and the closest non-SpmX
158 relatives are likely to be active phage enzymes, highly conserved residues shared by these groups
159 with T4L delineate positions that are evolutionarily constrained for phage lysozyme activity and
160 stability. For example, D10 is not conserved outside of T4L-like enzymes because the
161 autolysin/endolysin class does not have a salt bridge between D10 and the C-terminal lobe [27].
162 On the other hand, all of the putative phage sequences (**Fig. 2B(i-iii)**) conserve the "catalytic
163 triad" of T4 lysozymes: the catalytic residue E11 and active site residues D20 and T26. While
164 the exact roles of D20 and T26 are not clear, they are posited as being critical for effective
165 catalysis [26–29]. Position D20 is very sensitive to mutation, with only substitutions D20C/A
166 retaining the hydrolytic activity of T4L or P22 phage lysozymes [30]; these substitutions are
167 tellingly well represented amongst the putative phage sequences. Remarkably, SpmX
168 muramidase domains demonstrate strong conservation of residues required for T4L stability and
169 creating the GH fold, but low conservation of residues associated with catalysis, with the
170 exception of the main catalytic residue, E11 (**Fig. 2B(iv)**). The majority of SpmX genes contain
171 the mutation D20L/R, both of which reduced T4L activity to less than 3% of WT in previous
172 studies [27] and which are distinctly unrepresented in the other phage muramidases. Moreover,
173 the T26 position no longer appears to be under constraint in SpmX. The conservation of the GH
174 motif coupled with the apparent inactivation of the catalytic triad across all SpmX genes suggests
175 that muramidase function may not remain fully active.
176
177 **The SpmX muramidase domain has a wider, more dynamic catalytic cleft than related**
178 **phage lysins.** Obtaining the structure of the SpmX muramidase domain (residues 1-150) from
179 *Asticcacaulis excentricus* (SpmX-Mur-*Ae*) allowed us to directly visualize the effect of the D20L
180 and T26X mutations on the catalytic cleft. We solved the X-ray crystal structure by molecular
181 replacement using the atomic coordinates of phage P22 lysozyme (PDB 2ANX) to 1.9 Å
182 resolution (*R*cryst of 21.1%, *R*free of 25.5%,) (**Table S2**). SpmX-Mur-*Ae* exhibited the expected
183 structural similarity with the phage P22 lysozyme (root mean square deviation (rmsd) of 1.7 Å
184 and 40% identity over 141 aligned Cα atoms, Dali Z-score of 21.5). It therefore shares the

185 characteristic structure of T4 lysozymes, with the predicted catalytic glutamate occurring at the
186 C-terminal end of the first alpha-helix and situated in the catalytic cleft formed between the N-
187 and C-terminal lobes (**Fig. 3A**). The active conformation of the distantly related SAR endolysin
188 protein R21 (PDB 3HDE) from bacteriophage P21 (**Fig. S2**) is included in the structural
189 alignment in **Figure 3A** to emphasize the ways in which the SpmX muramidase domain deviates
190 from phage lysozymes: SpmX-Mur-*Ae* contains an extended beta-hairpin in the C-terminal lobe
191 and, more importantly, the canonical GH beta-hairpin in the N-terminal lobe of SpmX-Mur-*Ae*
192 splays away from the catalytic cleft relative to the GH beta-hairpins of the phage lysozymes.
193 There is additional evidence for the flexibility of the GH beta-hairpin in the various
194 conformations observed in the crystallography data, as among the three molecules of SpmX-
195 Mur-*Ae* in the asymmetric unit, this region exhibited the most conformational differences. The
196 overlay of the three chains in **Figure 3B** illustrates how the orientation of the GH beta-hairpin is
197 tilted by about 16° between chains A and B; moreover, chain A appears to have lost some of its
198 beta-strand secondary structure. That SpmX-Mur-*Ae* is capable of both conformations suggests a
199 heightened flexibility in this region compared to other T4L-like lysozymes, which may reduce
200 the ability of the enzyme to coordinate the hydrolysis of peptidoglycan in the catalytic cleft.
201 Sequence comparisons of the GH motif in **Figure 2B** show that SpmX muramidase
202 domains have lost a highly conserved tyrosine residue at position 18. Although T4L enzymatic
203 activity is not sensitive to mutation at this position [27], it is invariant across all the phage
204 lysozyme classes we analyzed. Visualization of Y18 in the P22 lysozyme structure (**Fig. 3C**)
205 shows that it interacts with R14 at the base of the beta-hairpin, possibly a critical interaction for
206 coordinating the beta-hairpin with the catalytic glutamate. In SpmX-Mur-*Ae*, Y18S still appears
207 to make hydrogen-bonding contact with R14; however, most SpmX muramidase domains have
208 non-polar residues at position 18 (**Fig. 2B(iv)),** which may reduce coordination between the
209 catalytic glutamate and the GH beta hairpin. It has been previously shown that the Y18 position
210 is a hot-spot for compensatory mutations that restore activity to inactive catalytic mutants [31],
211 and it is intriguing to imagine that mutations at this position in SpmX muramidase are the result
212 of a remodeled catalytic cleft.
213 **Figure 3** shows the catalytic clefts of both P22 lysozyme (**D**) and SpmX-Mur-*Ae* (**E**). In
214 P22 lysozyme, the E11 carbonyl, D20 carboxyl, and T26 hydroxyl groups point into the aqueous
215 catalytic cleft. In SpmX-Mur-*Ae,* the cleft is slightly reorganized, with the S-methyl thioether of
216 T26M still within 20 Å of E11 and potentially capable of interacting with peptidoglycan. In
217 about two thirds of the SpmX genes, position 26 has either a valine or an isoleucine, which have
218 no polar moieties to contribute to the cleft (**Fig. 2B(iv)**). With the structural data from SpmX-
219 Mur-*Ae*, we can infer that the SpmX muramidase domain has a remodeled catalytic cleft with a
220 correctly positioned catalytic glutamate. However, the increased flexibility between the GH
221 motif and the glutamate, as well as the loss of key coordinating residues might reduce, if not
222 eliminate, hydrolytic activity of SpmX.
223
224 **SpmX retains reduced hydrolytic activity on peptidoglycan.** To determine if the SpmX
225 muramidase domain was capable of binding peptidoglycan, various constructs from *C.*
226 *crescentus, A. excentricus,* and *A. biprosthecum* were purified and incubated with sacculi isolated
227 from the three species. Both muramidase domains and entire soluble domains (muramidase with
228 intermediate domain) from all three species, including the E11A and N105R mutants of SpmX
229 from *C. crescentus* bound sacculi from all three species (**Fig. S3**). Despite previous reports of
230 inactivity [18], we found that purified SpmX muramidase exhibited hydrolytic activity. We used

remazol brilliant blue (RBB) assays, which are standard for measuring lysozyme activity [32], to compare the activity of SpmX muramidase from *C. crescentus* (SpmX-Mur-*Cc*) to that of the P22 lysozyme (P22Lyso) and a P22Lyso mutant with a similarly inactivated catalytic cleft (P22Lyso-D20L) (**Fig. 4A**). The activity curves demonstrate that both SpmX-Mur-*Cc* and P22Lyso-D20L exhibit similarly attenuated hydrolytic activity in comparison to P22Lyso: both reached maximal levels of RBB release near enzyme concentrations of 15 μM while P22Lyso reached the same levels near 5 μM. Mutants in which the catalytic glutamate was replaced with alanine (SpmX-Mur-*Cc*-E11A and P22Lyso-E11A) did not exhibit activity (**Fig. S4A**). Restoring the ancestral D20 (SpmX-Mur-*Cc*-L20D) did not increase SpmX activity *in vitro* (**Fig. S4B**). These data indicate that the "inactivating" substitution D20L attenuates enzymatic activity whereas mutating the catalytic glutamate abolishes it altogether.

We were surprised by the activity of P22Lyso-D20L and SpmX-Mur given the reported inhibition of lytic activity of T4L with D20 mutations in phage plaque assays [27]. We were interested in the biological implications of this *in vitro* activity, and whether the attenuated mutant was active in the periplasmic environment. We therefore designed an experimental system to test the activity of P22Lyso and SpmX-Mur-*Cc* mutants in the periplasmic space of *E. coli*. In this system, we expressed the various muramidase constructs with N-terminal PelB leader sequences (pET22b) for periplasmic expression in Lemo21(DE3) cells, which allow tunable expression of toxic products (see Methods). Lemo21(DE3) cells carrying P22Lyso lysed even in the absence of induction (**Fig. 4B)**. In contrast, Lemo21(D32) cells carrying P22Lyso-D20L lysed only after induction, and strains carrying SpmX-Mur-*Cc* and P22Lyso-E11A never lysed (**Fig. 4C** and **S4C**) despite equivalent periplasmic expression levels (**Fig. S4D**). Different growth conditions and media did not affect the viability of cells expressing SpmX-Mur-*Cc* (**Fig. S4C**). Moreover, SpmX-Mur-*Cc* was active on sacculi isolated from Lemo21(DE3), eliminating the possibility that it cannot cleave *E. coli* peptidoglycan (**Fig. S4E**). It remains unclear why the periplasmic activity of SpmX-Mur-*Cc* does not match its *in vitro* activity, as it appears to have a similar activity to P22Lyso-D20L and yet surpasses the periplasmic levels at which P22Lyso-D20L lyses *E. coli*. It is possible that SpmX-Mur has additional mutations that either make it difficult to fold correctly in the *E. coli* periplasm, or inactivate its activity in the periplasmic environment. However, the periplasmic expression tests in *E. coli* confirm that the D20L mutation attenuates the hydrolytic activity of P22 lysozyme. This tuning of enzymatic activity might have served as a critical detoxifying step in the co-option of the muramidase domain from phage. Moreover, such attenuation helps explain why the D20L substitution is absent from phage despite broad conservation among *spmX* alleles. Because SpmX has retained the ancestral catalytic glutamate and its modified catalytic cleft is capable of hydrolytic activity, we conclude that this attenuated activity is under purifying selection in SpmX and important for SpmX function.

Inactivating the muramidase domain impacts SpmX localization *in vivo.* To investigate the role of the conserved catalytic glutamate in SpmX function *in vivo*, chromosomal E11A mutants (E19A in SpmX numbering) were made in *C. crescentus*, *A. excentricus*, and *A. biprosthecum* with eGFP gene fusions (**Fig. 5**). We determined the effects of the E11A mutation on cellular morphology, as the Δ*spmX* strains in all three species have morphological phenotypes (**Fig. 5ABCii**). In *C. crescentus*, Δ*spmX* cells have a characteristic elongated morphology resulting from failed division cycles and often grow stalks prematurely from daughter cells that fail to divide completely (**Fig. 5Aii**) [15]. In *Asticaccaulis*, Δ*spmX* cells lack stalks and do not appear to

277 have other developmental phenotypes (**Fig. 5BCii**) [17]. In *C. crescentus*, the E11A mutant
278 population contained both WT-like cells and cells exhibiting the division defect, but with less
279 severity than in Δ*spmX* (**Fig. 5Aiii**). In both *Asticcacaulis* species, the E11A mutants still grew
280 stalks (**Fig. 5BCiii**). Nevertheless, the *A. biprosthecum* E11A mutant exhibited a significant loss
281 of bilateral stalks (3.5 fold reduction) and an increase in the frequency of cells with a single stalk
282 (**Fig. S5D**).
283 We used GFP fusions of WT and mutant SpmX to monitor changes in SpmX
284 localization. In all species, WT SpmX localizes at the future position of the stalk, either at the
285 pole as in *C. crescentus*, or at sub-polar or bilateral positions in *Asticcacalis*, and is retained at
286 this position (**Fig. 5ABCiii**). Both *C. crescentus* and *A. biprosthecum spmX* E11A mutants
287 exhibited an increase in mislocalized SpmX throughout the cell body compared to WT (**Fig.
288 5ABiv**). Quantification of the fluorescence data indicated that while the overall mean
289 fluorescence of the cells were the same as WT, the SpmX foci were significantly less intense in
290 the mutants (**Fig. S5AB**). We also observed a 3X increase in SpmX E11A in the stalks of *A.
291 biprosthecum* compared to WT (**Fig. S5B**). Although no difference in focal fluorescence
292 intensity was observed in *A. excentricus spmX* E11A mutant cells, more cells had a second
293 SpmX focus at the tip of the stalk than WT cells (**Fig. 5iv, S5C)**, indicating altered localization.
294 Together these data show that the E11A mutation impacts SpmX localization in all three species.
295 N/Q105 has been shown to coordinate peptidoglycan in the active cleft of T4L [33] and
296 the mutation Q105R abolished activity in T4 phage plaque assays [27]. The mutation N105R
297 (N91R in SpmX numbering) in *C. crescentus* and *A. biprosthecum* resulted in similar
298 delocalization phenotypes as E11A (**Fig. 5BCiiv**). Western blots of cells expressing WT SpmX-
299 eGFP and SpmX mutants indicated that the delocalization was not due to clipping (**Fig. S5E**).
300 Overall, these data show that eliminating the catalytic glutamate affects SpmX localization and
301 function. Although it is not clear whether the E11A mutation specifically affects the potential
302 hydrolytic activity or the peptidoglycan-binding ability, the similar phenotype from mutating a
303 predicted peptidoglycan-interacting residue (N105R) underscores the importance of SpmX-
304 peptidoglycan interactions. That the catalytic mutant has an intermediate morphological
305 phenotype in *C. crescentus* and one *Asticcacaulis* species indicates that the muramidase domain
306 may coordinate SpmX functions similarly in the two genera and that this function may rely on its
307 enzymatic activity.
308
309 **Replacing the muramidase domain, or removing it, interferes with native SpmX protein
310 levels *in vivo*.** In an effort to understand the role of the highly conserved muramidase fold in
311 SpmX function, we made chimeras wherein P22 lysozyme replaced the muramidase domain of
312 SpmX in *C. crescentus* (P22Lyso-SpmX). While similarly made constructs with SpmX and the
313 SpmX-E11A mutant exhibited the previously determined morphological and delocalization
314 phenotypes (**Fig. 6Aii-iii**), replacing the SpmX muramidase domain with P22 lysozyme
315 phenocopied the parent Δ*spmX* strain. However, we were unable to detect any sfGFP-fusion
316 products in these chimeras by Western blot (**Fig. 6B**). We confirmed that P22Lyso-SpmX had
317 been correctly inserted in these strains by sequencing, suggesting that the chimeras were
318 expressed but degraded quickly in *C. crescentus*. Therefore the phenotype of this chimera is due
319 to loss of SpmX and not the addition of the P22 lysozyme domain. Inactivation of P22Lyso
320 (E11A) did not change the outcome, suggesting that the toxicity of the phage muramidase was
321 not driving degradation of SpmX. Deletion of the muramidase domain from the *spmX* locus in all
322 three species also resulted in strains that failed to produce detectable amounts of Δmur-SpmX-

323 sfGFP by Western blot (**Fig. S5E**). These results suggest that the SpmX muramidase domain is
324 necessary to produce and/or maintain WT levels of SpmX in all three species, and that phage
325 muramidase P22Lyso, with high sequence similarity (51%) and structural homology (RMSD 1.7
326 Å), is not sufficient to replace it.
327 Given that P22Lyso and SpmX muramidase are fairly distant relatives despite high
328 sequence conservation, we tested the ability of other SpmX muramidase domains to replace that
329 of *C. crescentus*. First, we attempted to restore the phage active site D20 (L28D in SpmX
330 numbering) in the native SpmX gene, but this had no evident effect on SpmX localization or cell
331 morphology (**Fig. 6iv**). Previously, the muramidase domains of *C. crescentus* and *Asticcacaulis*
332 were shown to be interchangeable [17], so we extended the sequence distance to SpmX
333 muramidases from the next closest relative *B. subvibrioides*, which has D20R in the catalytic
334 cleft, and the most distant relative *P. bermudensis,* which shares the D20L mutation. We found
335 that the muramidase domain from *B. subvibrioides* supported the WT phenotype in *C. crescentus*
336 (**Fig. 6v)**, but that the SpmX muramidase domain from *P. bermudensis* did not. We were
337 surprised to see no evidence of delocalization in the *B. subvibrioides* SpmX chimera because the
338 L20R mutant of SpmX in *C. crescentus* showed some delocalization (**Fig. 6vi**). This result
339 suggests that the L20R mutation in the brevundimonads must coexist with other compensatory
340 mutations. The SpmX muramidase domain from *P. bermudensis*, like P22Lyso, must be too
341 distant from *C. crescentus* to support WT levels of SpmX protein. In combination with data from
342 the P22Lyso chimeras, these data indicate that a T4L GH fold alone is not sufficient for SpmX
343 function, and that the SpmX muramidase domain must contain mutations necessary for stable
344 protein levels in Caulobacterales. It also suggests that this domain has additional constraints on it
345 unrelated to potential peptidoglycan interactions.
346
347 **Discussion**
348
349 Bacteriophages shape bacterial evolution in various ways: they increase bacterial diversity by
350 selectively preying on species [2,34], drive horizontal gene transfer of phage and bacterial genes
351 [8,35], and serve as reservoirs of raw material for new bacterial genes [36,37]. Phages are
352 heralded as a major source of genetic material for novel gene emergence in bacteria [2,36,37],
353 but, as we discuss later in this section, very few examples of novel gene emergence from
354 prophage exist in the literature. We have investigated the origin and function of a taxonomically
355 restricted gene from Caulobacterales, *spmX*, and determined that its occurrence is the result of
356 the fusion and domestication of a peptidoglycan hydrolase gene commonly found in prophage.
357 Despite the previously demonstrated role of SpmX as a scaffold in developmental regulation and
358 morphology, the muramidase domain retains high sequence similarity to phage lysozymes,
359 which are toxic to bacteria. The active cleft contains mutations that have attenuated the toxic
360 activity of the domain, presumably making it available for genetic innovation and bacterial use.
361 We show here that the domain remains enzymatically active on peptidoglycan and that reducing
362 or eliminating this activity alters the function of the full-length protein *in vivo*. Thus, the SpmX
363 gene represents a bacterial gene innovation, specific to the Caulobacterales order and with
364 housekeeping function, that originally arose from a prophage gene with antibacterial activity.
365 Previously, it was suggested that the SpmX muramidase domain functions only in
366 protein-protein and self-oligomerizing interactions in SpmX's role as a developmental regulator
367 and scaffold in *C. crescentus* [18]. This conclusion was based on the lack of detectable activity
368 from the purified domain and the inability of the catalytic E11R (E19R in SpmX numbering)

369 mutant to self-oligomerize. The conservation of the GH fold and the catalytic glutamate clearly
370 suggests that the activity of SpmX is important for its domesticated function, and, importantly,
371 we were able to observe muramidase activity *in vitro* with purified protein. It is highly likely that
372 the E11R mutation greatly destabilizes the structure of the muramidase domain. We found that
373 the E11A mutant eluted in multiple fractions during purification, indicating decreased
374 conformational stability. Moreover, the E11R mutant exactly phenocopies the Δmur-SpmX
375 phenotype *in vivo* in that the protein product was no longer detectable in the cells expressing the
376 gene [18]. In this sense, the effect of the E11R mutation is similar to using a distantly related
377 muramidase domain (like P22Lyso) or deleting portions of the muramidase domain entirely.
378 These data indicate that the muramidase domain plays an unanticipated role in maintaining stable
379 levels of SpmX protein across all tested species: without the muramidase domain, the protein is
380 misfolded or misprocessed and is therefore quickly degraded.
381 Inactivating the SpmX muramidase domain resulted in developmental defects in *C.*
382 *crescentus* and significant loss of the bilateral stalk morphology in *A. biprosthecum*. It is curious
383 that inactivating the enzymatic domain did not yield a null phenotype or complete delocalization.
384 Since SpmX-E11A and N105R still bind to sacculi *in vitro*, it is possible that enough
385 peptidoglycan-interactions are maintained in the mutants for partial SpmX function. It is also
386 possible that SpmX recruits another protein with redundant enzymatic activity that cannot be
387 recruited in the Δ*spmX* mutant, as it is already known that SpmX interacts with targeting factors
388 via its C-terminal domains in *Asticcacaulis* [17] and possibly via its transmembrane segments
389 with DivJ [15]. Finally, it is hard to distinguish whether there is a direct relationship between
390 catalytic activity and peptidoglycan binding, and therefore localization, or if cleavage of
391 peptidoglycan by SpmX could indirectly impact SpmX localization. The multiple domains and
392 pleiotropic effects of SpmX make it difficult to assess the effects of an individual domain on its
393 *in vivo* function. However, our data support a model in which the muramidase domain of SpmX
394 is still active, and this activity is used to localize SpmX directly or indirectly.
395 SpmX emerges in the genomic record at the root of Caulobacterales with the attenuating
396 D20L mutation already in the muramidase domain (**Fig. 1B**). The D20L mutation is therefore
397 ancestral and potentially the initial step in the co-option of the domain. The conservation of
398 D20L throughout most of Caulobacterales suggests evolutionary constraint on this position
399 despite no observable phenotype from the SpmX-Mur-*Cc* L20D reversion mutation *in vivo* or *in*
400 *vitro*. There are several modifications to the active cleft, including the loss of selection on the
401 third catalytic triad position, T26, and the invariant residue Y18. This pair is interesting in that
402 Y18 was identified as a hotspot for spontaneous second site revertants of T26 mutants in T4L
403 [31]. It is possible that the scatter we see at these two positions are compensatory mutations
404 retaining attenuated activity, although a clear history of covariation is not clear. In two groups,
405 SpmX has diverged from the ancestral D20L mutation: the marine genera *Oceanicaulis* and
406 *Maricaulis* (D20R/G), and the freshwater/soil genus *Brevundimonas* (D20R) (**Fig. 1B, S1**).
407 Interestingly, D20R is covariant with residue N105 (**Fig. S1**), which is a peptidoglycan-
408 interacting residue in T4 lysozyme [33]. The covariance of peptidoglycan-interacting residues in
409 these diverging genera further underscores the importance of this domain in peptidoglycan
410 interactions, rather than just protein-protein interactions.
411 The SpmX gene has arisen recently enough to see the hallmarks of novel gene emergence
412 and adaptation in a constrained bacterial clade. The gene either arose from a fusion event in the
413 bacterial genome, or the original phage gene contained the transmembrane segments.
414 Domestication of the muramidase appears concomitant with the occurrence of the fused SpmX

domains in the extant genetic record. Maintenance of the muramidase domain since the emergence of SpmX and its activity in current living members of Caulobacterales suggest that its activity was selected for in the ancestral protein and still implicated in its modern function. Given the variability of the intermediate domain throughout Caulobacterales (**Fig. 1A**), this domain is under few constraints and appears to have undergone multiple independent events of elaboration and reduction in this order. This region of charged residues and prolines drives SpmX self-oligomerization *in vitro* [18], and may also facilitate other protein interactions. For example, the intermediate domain is responsible for the targeting of SpmX to sub-polar and bilateral positions in *Asticcacaulis* [17].

In several reported cases bacteria have domesticated phage genes for genetic manipulation and transfer, bacterial warfare, virulence, and secretion. However, these events are distinct from that which created the novel bacterial gene SpmX. Phage genes for DNA replication and recombination have replaced bacterial functional homologues within bacterial genomes several times [38–41]. These genes retain their original function and are now used by bacteria to carry out the same tasks. Gene transfer agents (GTAs) pose an interesting case where structural proteins from domesticated cryptic prophage are used by bacteria to package random DNA from the bacterial genome to presumably share with other bacteria [42]. In Alphaproteobacteria, a specific GTA has been stably maintained across several bacterial orders but the beneficial function is not precisely known [43,44]. Regardless, this domesticated island of phage genes still shuttles DNA around, as it once did in ancestral infectious cycles. Phage tails appear to have been weaponized many times, with multiple domestication events resulting in type VI secretion systems [45,46], tailocins and phage tail-like bacteriocins [12–14], phage tail-like systems with insecticidal properties [11,47,48], and phage tail-like arrays [49]. All of these represent a “guns for hire” acquisition scheme in which phage genes are co-opted without loss of ancestral toxicity and function [2]. Many of these genes reside in genomic islands and confer environmental, niche-specific advantages that directly exploit their ancestral activity for the benefit of the host. Similarly, in two other known cases of phage lysozyme domestication in bacteria, structurally similar, active domains have been fused to colicins [50] or are predicted to be secreted with type III secretion systems [51], presumably for use in bacterial warfare or infection. In one strange case, a phage lysozyme gene has been co-opted in bivalve genomes, which apparently still use the gene for its antibacterial properties [52].

The domestication of the muramidase domain in SpmX is distinct from the above cases of “guns for hire” because the phage gene has been incorporated into a novel bacterial gene with new function in basic cellular processes. The SpmX muramidase domain, although active, is no longer used to lyse bacterial cells. Instead, the active domain plays a role in localizing the SpmX protein for its function in developmental regulation and morphogenesis and has become part of a core gene in Caulobacterales. The co-option of phage genes for housekeeping function is likely a common event in nature, but identifying such genes may require a careful search. We suggest that the characteristics of SpmX may recommend future strategies for their detection: searching for phage gene homologues that have long histories of vertical inheritance and signs of bacterial innovation.

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470
471 **Figure Legends**
472
473 **Figure 1. The SpmX is vertically inherited in Caulobacterales. (A)** Schematic of SpmX
474 architecture, including the conserved muramidase domain (see **Fig. S1** for alignments), the
475 variable intermediate domain, and two C-terminal transmembrane (TM) segments. Bar indicates
476 amino acid sequence conservation among *spmX* alleles. **(B)** Phylogenetic trees of representative
477 species from Caulobacterales and other Alphaproteobacteria for concatenated housekeeping gene
478 alignments (left) and for SpmX (right), with branch colors indicating the amino acid identity at
479 position 20 of SpmX (D20L in yellow, D20R in red, and D20G in green). The concatenated
480 housekeeping tree is fully supported with posterior probability of 1.0 for all clades. Asterisks
481 indicate clades in the SpmX tree with posterior probabilities > 0.95.
482
483 **Figure 2. The SpmX muramidase domain retains the canonical GH motif but contains**
484 **inactivating mutations in the catalytic cleft. (A)** P22 lysozyme (PDB 2ANX) as a model
485 lysozyme colored with rainbow gradient from blue N-terminus to red C-terminus. The catalytic
486 glutamate appears in fuchsia and the GH beta-hairpin in light blue. **(B)** HMM logos of GH
487 lysozymes made using WebLogo 3 [53]. Logos were constructed from protein sequences of **(i)**
488 T4 lysozyme-like genes (n = 94), **(ii)** representative autolysins/endolysins from the Conserved
489 Domain Database including P22 lysozyme (n = 20) but excluding SpmX genes, **(iii)** closest
490 BLAST hits from non-SpmX muramidases (n = 60), and **(iv)** SpmX muramidases (n = 66), and
491 organized in a cladogram to resemble the sequence cluster tree diagram in **Figure S2**. Amino
492 acids are color-coded according to chemical properties, with uncharged polar residues in green,
493 neutral residues in purple, basic residues in blue, acidic residues in red, and hydrophobic residues
494 in black. The height of each letter is proportional to the relative frequency of a given identity and
495 the height of the stack indicates the sequence conservation at that position. T4L numbering is
496 used for ease of comparison. Asterisks mark positions critical for enzymatic activity and open
497 circles mark positions associated with GH motif stability [26,27].
498
499 **Figure 3. The structure of SpmX muramidase domain has a wider, more dynamic catalytic**
500 **cleft than related phage lysins. (A)** Structural alignment of P22 lysozyme (PDB 2ANX, the
501 model used for molecular replacement) in purple, R21 endolysin from P21 (PDB 2HDE, a
502 distantly related GH24 T4L lysozyme) in navy blue, and SpmX-Mur-*Ae* in gold (PDB 6H9D).
503 The catalytic glutamate is shown in red. **(B)** Structural alignment of the three SpmX-Mur-*Ae*
504 molecules, chains A (green), B (light blue), and C (dark blue), from the asymmetric unit. The
505 surface of chain B is shown in partially transparent light blue. The double-headed arrow
506 indicates the tilt of about 16° between the GH beta-hairpins of chains B and A. **(C)** Overlays of

507 ribbon diagrams and surfaces of P22 lysozyme (2ANX, left) and SpmX-Mur-*Ae* (6H9D, right)
508 illustrating the conformation of the critical residues E11 (red), D20 (dark blue), R14 (yellow),
509 and Y18 (orange). T4L numbering is used for ease of comparison. These structures have been
510 rotated 180° around the y axis from their representation in (A, B, D, E)**. (D)** Surface
511 representation of P22 lysozyme (2ANX) with inset showing ribbon diagram and conformation of
512 catalytic cleft with the canonical E11/D20/T26 catalytic triad. **(E)** Surface representation of
513 SpmX-Mur-*Ae* (6H9D) with inset showing ribbon diagram and conformation of remodeled
514 catalytic cleft with E11/D20L/T26M.
515
516 **Figure 4. The D20L mutation attenuates P22 hydrolytic activity. (A)** Remazol brilliant blue
517 assays on *C. crescentus* sacculi using purified P22 lysozyme, P22 lysozyme D20L mutant, and
518 *C. crescentus* SpmX muramidase. Active enzymes release peptidoglycan monomers covalently-
519 bound to RBB into the supernatant that are detected by absorbance at 595 nm. Error bars are ±
520 standard deviation. Lines are drawn to help guide the eye toward basic trends. Data points are
521 from various days and sacculi preparations, but with internal normalization to Hen Egg White
522 Lysozyme (HEWL). **(B** and **C)** Growth curves of Lemo21(DE3) *E. coli* expressing P22
523 lysozyme (blue), P22 lysozyme D20L mutant (green), and *C. crescentus* SpmX muramidase
524 (red). Proteins were expressed from pET22b with a N-terminal PelB signal sequence. In **(B)**,
525 strains were grown in 5 mM rhamnose without IPTG for maximal repression of basal expression
526 from the plasmids. In **(C)**, strains were grown without rhamnose and induced with 400 μM IPTG
527 at the indicated time. **(D)** Phase/fluorescent overlays show live/dead staining of (i)
528 Lemo21(DE3) cells expressing P22Lyso-D20L and (ii) SpmX-Mur-*Cc* after four hours of
529 induction. Green, membrane permeable SYTO 9 stains DNA in live cells and red, membrane
530 impermeable propidium iodide nucleic acid dyes labels released nucleoids and DNA from lysed
531 bacteria. The rounding of the *E. coli* in (i) is characteristic of spheroplast formation and lysis by
532 hydrolytic activity on the cell wall. Scale bars are 5 μm.
533
534 **Figure 5. Inactivating the muramidase domain partially delocalizes SpmX *in vivo*.**
535 Phase and fluorescent images of (**A**) *C. crescentus*, (**B**) A. *biprosthecum,* and **(C)** *A. excentricus.*
536 In the top panel, phase images with derived schematics emphasizing stalks and morphologies are
537 shown for (**i**) WT and (**ii**) Δ*spmX* cells. In **Aii**, *C. crescentus* cells exhibiting characteristic
538 Δ*spmX* divisional defects are marked with asterisks and a cell growing stalks from both poles has
539 its stalks marked with red arrowheads. Phase and fluorescent images of cells expressing (**iii**)
540 SpmX-eGFP, (**iv**) SpmX-E11A-eGFP, or (**v**) SpmX-N105R-eGFP from the native chromosomal
541 locus are shown in the lower panels. In **Aiv** and **Av**, cells with divisional defects are marked with
542 white asterisks. In **Biii** and **Biv**, cells with one lateral or subpolar stalk are marked with white
543 arrowheads. In **Civ**, cells with foci at the tips of stalks are marked with white arrowheads. All
544 scale bars are 5 μm.
545
546 **Figure 6. Replacing the muramidase domain interferes with native SpmX protein levels *in***
547 ***vivo* (A)** Phase and fluorescent images of strains in which the native *spmX* allele was replaced
548 with the following gene fusions in the Δ*spmX* parent strain (i): (ii) WT *spmX-sfGFP*, (iii) *spmX-*
549 *E11A-sfGFP*, (iv) *spmX-L20D-sfGFP*, (v) *MurBs*-Δ*mur-SpmX-sfGFP* where *MurBs* is the
550 muramidase domain from *Brevundimonas subvibrioides* SpmX*,* and (vi) *spmX-L20R-sfGFP*. All
551 scale bars are 5 μm.. **(B)** Western blot comparing the Δ*spmX* parent strain to SpmX mutants and

552 chimeras inserted at the *spmX* locus. In all cases, the primary antibody is directed against the C-
553 terminal GFP fusion.
554
555 **Tables**
556
557 **Table S1. *spmX* genes**

Gene ID	Genome ID	Genome Name	Aas
2579190773	2576861677	*Asticcacaulis benevestitus DSM 16100, ATCC BAA-896*	825
2515453797	2515154077	*Asticcacaulis benevestitus DSM 16100, ATCC BAA-896*	825
651318754	651285000	*Asticcacaulis biprosthecum C19, ATCC 27554*	826
649817473	649633007	*Asticcacaulis excentricus CB 48*	810
2579161412	2576861669	*Asticcacaulis sp. AC402*	861
2575314089	2574180013	*Asticcacaulis sp. AC460*	803
2573897243	2571042840	*Asticcacaulis sp. AC466*	863
2579118533	2576861656	*Asticcacaulis sp. YBE204*	851
2597242146	2596583650	*Asticcacaulis taihuensis CGMCC 1.3431*	775
2525860150	2524614798	*Brevundimonas aveniformis DSM 17977*	453
2562052386	2561511096	*Brevundimonas bacteroides DSM 4726*	507
2534536731	2531839707	*Brevundimonas diminuta 470-4*	291
2635997287	2634166514	*Brevundimonas diminuta XGC1*	291
2580800557	2579778742	*Brevundimonas naejangsanensis B1*	291
2525302517	2524614659	*Brevundimonas naejangsanensis DSM 23858*	296
2602030628	2600255394	*Brevundimonas nasdae TPW30*	393
2597236398	2596583649	*Brevundimonas sp. 374*	381
2646174451	2645727605	*Brevundimonas sp. AAP58*	493
647574062	647533115	*Brevundimonas sp. BAL3*	526
2628481828	2627853674	*Brevundimonas sp. KM4*	381
648120868	648028010	*Brevundimonas subvibrioides ATCC 15264*	514
2671799930	2671180176	*Brevundimonas viscosa CGMCC 1.10683*	420
637088217	637000061	*Caulobacter crescentus CB15*	431
643620503	643348526	*Caulobacter crescentus NA1000*	431
2522234411	2522125057	*Caulobacter crescentus OR37*	429
2585155721	2582581280	*Caulobacter henricii CF287*	419
2585146921	2582581279	*Caulobacter henricii OK261*	398
2585198327	2582581293	*Caulobacter henricii YR570*	419
646754844	646564519	*Caulobacter segnis ATCC 21756*	413
2511123337	2510917020	*Caulobacter sp. AP07*	425
2528897460	2528768059	*Caulobacter sp. JGI 0001010-J14*	422
2528898539	2528768060	*Caulobacter sp. JGI 0001013-D04*	421
641563661	641522612	*Caulobacter sp. K31*	417
2587919405	2585428106	*Caulobacter sp. OV484*	439
2686817300	2684623106	*Caulobacter sp. UKL13*	439
2593324358	2590828858	*Caulobacter sp. UNC279MFTsu5.1*	421

2565991034	2565956508	*Caulobacter sp. UNC358MFTsu5.1*	424
2522923390	2522572103	*Caulobacter sp. URHA0033*	413
2677566563	2675903340	*Caulobacter vibrioides T5M6*	426
2525553380	2524614726	*Hellea balneolensis DSM 19091*	439
2519259833	2519103046	*Henriciella marina DSM 19595*	535
644903978	644736375	*Hirschia baltica ATCC 49814*	597
2520004565	2519899518	*Hirschia maritima DSM 19733*	577
2578549711	2576861496	*Hyphomonas adhaerens MHS-3*	386
2578539236	2576861493	*Hyphomonas hirschiana VP5*	421
2578553211	2576861497	*Hyphomonas jannaschiana VP2*	385
2578549047	2576861495	*Hyphomonas johnsonii MHS-2*	409
638143841	637000135	*Hyphomonas neptunium ATCC 15444*	421
2578534396	2576861492	*Hyphomonas oceanitis SCH89*	407
2578543740	2576861494	*Hyphomonas polymorpha PS728*	418
2583291548	2582580773	*Hyphomonas sp. 22II1-22F38*	390
2583281302	2582580770	*Hyphomonas sp. 25B14_1*	392
2583297317	2582580774	*Hyphomonas sp. BH-BN04-4*	407
2583284155	2582580771	*Hyphomonas sp. CY54-11-8*	386
2583256535	2582580764	*Hyphomonas sp. L-53-1-40*	398
2583287429	2582580772	*Hyphomonas sp. T16B2*	392
638141228	637000157	*Maricaulis maris MCS10*	638
2635165820	2634166316	*Maricaulis salignorans DSM 16077*	633
2554749235	2554235119	*Maricaulis sp. JL2009/Euryhalocaulis caribicus*	464
2523851441	2523533582	*Oceanicaulis alexandrii DSM 11625*	643
638892666	638341138	*Oceanicaulis alexandrii HTCC2633*	596
2609050000	2608642188	*Oceanicaulis sp.*	514
2582569245	2579779170	*Oceanicaulis sp. HL-87*	514
648155025	648028050	*Parvularcula bermudensis HTCC2503*	344
2574131646	2571042909	*Phenylobacterium composti DSM 19425*	438
642759179	642555147	*Phenylobacterium zucineum HLK1*	445
2524841340	2524614539	*Ponticaulis koreensis DSM 19734*	687
2522300904	2522125083	*Robiginitomaculum antarcticum DSM 21748*	502
2522254887	2522125070	*Woodsholea maritima DSM 17123*	853

Table S2. Data collection and refinement statistics

Data collection	
Name of dataset	3SpmX_B1_1
X-ray source	ID30a-1 (ESRF)
Scan range (°)	76
Oscillation (°)	0.1
Space group	$P3_221$
Unit-cell parameters	
a, Å	100.44
b, Å	100.44
c, Å	96.62

α, °		90
β, °		90
γ, °		120
Resolution (last shell), Å		1.90 (2.01-1.90)
Completeness (last shell), %		95.1 (95.0)
I/ ∫(*I*) (last shell)		33.99 (3.1)
Rsym† (last shell), %		2.0 (43.6)
No of unique reflections		42611
Wilson B factor, (Å²)		44.4
Refinement and model statistics		
Resolution (last shell), Å		1.90 (1.95-1.90)
R-factor‡, R-free§		0.211, 0.256
Molecules/asymmetric unit		3
rmsd from target[i]		
Bond lengths, Å	0.008	
Bond angle, °		1.182
Average B-factor, Å²		86.34
Mean B factor (Å²)		41.7
Ramachandran plot**		
Core, %	97.5	
Allowed, %		2.5
Disallowed, %		0

†Rsym = (Σ(ABS(*I*(h,i)-(*I*(h)))) / (Σ(*I*(h,i))).
‡R-factor = ΣjjFoj - jFcjj/ΣjFoj where Fo and Fc are the observed and calculated structure factor amplitudes, respectively.
§R-free is the R-factor calculated with 5% of the reflections chosen at random and omitted from refinement.
[i]rmsd of bond lengths and bond angles from ideal geometry.
**Performed by Procheck.

Table S3. Plasmids

YB#	**Plasmid Name/Contents (Resistance)**	**Source or Reference**
Expression Plasmids – Structural Studies		
	pTB146-SUMO	Bernhardt Lab, [54]
8120	pTB146-SUMO-*spmX-Mur*(*1-150)-Ae* (Amp)	This study
Expression Plasmids – Activity and Binding		
1170	pET28a (Kan)	Novagen
8107	pET28a-*spmX-Mur(1-150)-Cc* (Kan)	This study
8111	pET28a-*spmX-Mur(1-150)-Ae* (Kan)	This study
8114	pET28a-*spmX-Mur(1-150)-Ab* (Kan)	This study
8889	pET28a-*spmX-Mur-Cc-E19A* (Kan) "E11A"	This study
8890	pET28a-*spmX-Mur-Cc-L28D* (Kan) "L20D"	This study
8891	pET28a-*spmX-Mur-Cc-N91R* (Kan) "N105R"	This study
8898	pET28a-*P22Lyso* (Kan)	This study
410	pET28a-*P22Lyso-E11A* (Kan)	This study
7378	pET28a-*P22Lyso-D20L* (Kan)	This study
Periplasmic Expression Plasmids		
3562	pET22b (Amp)	Novagen
7379	pET22b-*spmX-Mur(1-150)-Cc* (Amp)	This study
7380	pET22b-*spmX-Mur-Cc-E19A* (Amp) "E11A"	This study

7381	pET22b-*spmX-Mur-Cc-L28D* (Amp) "L20D"	This study
7382	pET22b-*P22Lyso* (Amp)	This study
7383	pET22b-*P22Lyso-E11A* (Amp)	This study
7384	pET22b-*P22Lyso-D20L* (Amp)	This study
In vivo Allelic Exchange and Insertional Plasmids		
1621	pNPTS138 (Kan)	M.R.K. Alley, unpublished
8247	pNPTS138-Δ*spmXAe*	This study
8246	pNPTS138-Δ*spmXAb*	This study
8252	pNPTS138-*spmXCc-E19A* "E11A"	This study
8251	pNPTS138-*spmXAe-E19A* "E11A"	This study
8250	pNPTS138-*spmXAb-E19A* "E11A"	This study
7395	pNPTS138-Δ*mur(1-150)-spmXCc*	This study
7396	pNPTS138-Δ*mur(1-150)-spmXAe*	This study
7398	pNPTS138-Δ*mur(1-150)-spmXAb*	This study
5111	pGFPC-1 (Spc/Str)	[55]
	pGFPC-2 (Kan)	[55]
7407	pGFPC-1-*spmXAb*(last 600 bp) (Spc/Str)	[17]
7408	pGFPC-1-*spmXAe*(last 600 bp) (Spc/Str)	[17]
8271	pGFPC-2-*spmXCc*(last 600 bp) (Kan)	This study
	pMCS-2 (Kan)	[55]
7409	pMCS-2-*spmXCc-sfGFP* (Kan)	This study
7410	pMCS-2-*spmXCc-E19A-sfGFP* (Kan) "E11A"	This study
7417	pMCS-2-*P22Lyso*-Δ*mur-spmXCc-sfGFP* (Kan)	This study
7418	pMCS-2-*P22Lyso-E11A*-Δ*mur-spmXcc-sfGFP* (Kan)	This study
1041	pMCS-2-*MBs(1-140)*-Δ*mur-spmXCc-sfGFP* (Kan)	This study
1042	pMCS-2-*MPb(1-168)*-Δ*mur-spmXCc-sfGFP* (Kan)	This study

Table S4. Strains

YB#	**Strain Genotype (Resistance)**	**Construct, Source, or Reference**
***E. coli* Strains**		
	Alpha-Select	Bioline
	BL21(DE3) RIL	Agilent
	BL21(DE3) RIL pTB146SUMO-*spmXMur*(*1-150)-Ae* (Amp)	This study
104	BL21(DE3)	Novagen
8126	BL21(DE3) pET28a-*spmX-Mur(1-150)-Cc*	This study
8129	BL21(DE3) pET28a-*spmX-Mur(1-150)-Ae*	This study
8132	BL21(DE3) pET28a-*spmX-Mur(1-150)-Ab*	This study
8881	BL21(DE3) pET28a-*spmX-Mur-Cc-E19A*	This study
8882	BL21(DE3) pET28a-*spmX-Mur-Cc-L28D*	This study
8899	BL21(DE3) pET28a-*spmX-Mur-Cc-N91R*	This study
8141	BL21(DE3) pET28a-*P22L*	This study
1043	BL21(DE3) pET28a-*P22L-E11A*	This study
1044	BL21(DE3) pET28a-*P22L-D20L*	This study
8880	Lemo21(DE3) (Cm)	NEB

1045	Lemo21(DE3) pET22b-*spmX-Mur(1-150)-Cc* (Cm/Amp)	This study
1046	Lemo21(DE3) pET22b-*spmX-Mur-Cc-E19A* (Cm/Amp)	This study
1047	Lemo21(DE3) pET22b-*spmX-Mur-Cc-L20D* (Cm/Amp)	This study
	Lemo21(DE3) pET22b-*P22L* (Cm/Amp)	This study
1049	Lemo21(DE3) pET22b-*P22L-E11A* (Cm/Amp)	This study
1050	Lemo21(DE3) pET22b-*P22L-D20L* (Cm/Amp)	This study
***C. crescentus* Strains**		
127	*C. crescentus* CB15N (holdfast minus WT)	
5873	CB15N Δ*spmX*	[15]
8265	CB15N *spmX* :: *spmXCc-eGFP* (Kan)	YB127 electroporated with YB8271
8262	CB15N *spmXCc*-E19A "E11A"	YB127 electroporated with YB8252
8266	CB15N *spmX-E19A* :: *spmXCc-eGFP* (Kan) "E11A"	YB8262 electroporated with YB8271
8275	CB15N Δ*mur(1-150)-spmXCc*	YB127 electroporated with YB7395
1051	CB15N Δ*mur-spmXCc* :: *spmXCc-eGFP* (Kan)	YB8275 electroporated with YB8271
1052	CB15N Δ*spmX* :: *spmXCc-sfGFP* (Kan)	YB5873 electroporated with YB7409
1053	CB15N Δ*spmX* :: *spmXCc-E19A-sfGFP* (Kan) "E11A"	YB5873 electroporated with YB7410
1054	CB15N Δ*spmX* :: *P22Lyso*-Δ*mur-spmXCc-sfGFP* (Kan)	YB5873 electroporated with YB7417
1055	CB15N Δ*spmX* :: *P22Lyso-E11A*-Δ*mur-spmXcc-sfGFP* (Kan)	YB5873 electroporated with YB7418
1056	CB15N Δ*spmX* :: *MBs*-Δ*mur-spmXCc-sfGFP* (Kan)	YB5873 electroporated with YB1041
1057	CB15N Δ*spmX* :: *MPb*-Δ*mur-spmXCc-sfGFP* (Kan)	YB5873 electroporated with YB1042
***A. excentricus* Strains**		
258	*A. excentricus* AC48	[56]
6662	AC48 *spmX* :: *spmXAe-eGFP* (Spc/Str)	[17]
8238	AC48 Δ*spmX*	YB258 electroporated with YB8247
8260	AC48 *spmXAe-E19A* "E11A"	YB258 electroporated with YB8251
8261	AC48 *spmXAe-E19A* :: *spmXAe-eGFP* (Spc/Str) "E11A"	YB8260 electroporated with YB5686
8276	AC48 Δ*mur(1-150)spmXAe*	YB258 electroporated with YB7396
1058	AC48 Δ*mur-spmXAe* :: *spmXAe-eGFP* (Spc/Str)	YB8276 electroporated with

		YB7408
***A. biprosthecum* Strains**		
642	*A. biprosthecum* C19	[57]
1059	C19 *spmX* :: *spmXAb-eGFP* (Spc/Str)	[17]
8237	C19 Δ*spmX*	YB642 electroporated with YB8246
8257	C19 *spmXE19A* "E11A"	YB642 electroporated with YB8250
8258	C19 *spmXE19A* :: *spmXAb-eGFP* (Spc/Str) "E11A"	YB8257 electroporated with YB5681
8277	C19 Δ*mur(1-150)-spmXAb*	YB642 electroporated with YB7398
1060	C19 Δ*mur(1-150)-spmXAb* :: *spmXAb-eGFP* (Spc/Str)	YB8277 electroporated with YB7407

Materials and methods

Bacterial strains and growth conditions. All *C. crescentus*, *A. excentricus*, and *A. biprosthecum* strains used in this study were grown in liquid PYE medium. *C. crescentus* was grown at 30°C and *Asticcacaulis* species at 26°C. Strains were maintained on PYE plates supplemented with antibiotics as necessary (kanamycin 20 μgmL^{-1}, gentamycin 5μgmL^{-1}, and spectinomycin 100 μgmL^{-1}). For microscopy, *C. crescentus* and *A. excentricus* were inoculated from colonies, grown overnight, then diluted back 1:50 and grown for another 3-4 hours before being imaged in mid- to late-exponential phase. *A. biprosthecum* was inoculated from colonies and grown overnight to reach mid- to late-exponential phase for imaging. A detailed list of strains is included as **Table S4**. *E. coli* strains were grown as described in the Methods sections on purification and periplasmic expression.

Bioinformatics and gene trees. Sequences of the SpmX genes in **Table S1** and members of the GH24 family were retrieved by BLAST searches on the Integrated Microbial Genomes and Microbiomes (IMG/M) database [58] and the National Center for Biotechnology Information (NCBI) "nr" database. Multiple alignments were achieved with MUSCLE [59] and manually adjusted and visualized with Jalview [60]. Sequence conservation of SpmX residues was determined from the multiple sequence alignment of *spmX* alleles using ConSeq [61]. In order to improve visualization of conservation patterns, the ConSeq scores were averaged across a 20-residue sliding window. For estimating bacterial species phylogeny, assembled genome data were obtained from the genome database of the National Center for Biotechnology Information [62]. Amino acid sequences of 37 conserved housekeeping genes were automatically identified, aligned, and concatenated using Phylosift [63]. All phylogenetic reconstruction was performed using MrBayes v3.2.6 [64] to estimate consensus phylogenies and clade posterior probability support values. Sequence substitution was modeled according to a WAG substation model with gamma-distributed rate variation between sites. Trees were visualized and formatted using iTol [65]. The sequence cluster tree was built with NCBI's Conserved Domain Database tool (CDD). This tool uses reverse position-specific BLAST, a method that compares query sequences to databases of position-specific score matrices and obtains *E*-values, such as in PSI-BLAST

637 [21,22]. WebLogo3 was used to plot the amino acid distribution at each position of the GH motif
638 [53]. To create the alignments for logo generation, 94 T4 lysozyme-like sequences, 20
639 endolysin/autolysins from the CDD analysis, 60 SpmX muramidase-like sequences (BLAST
640 hits), and 66 SpmX muramidase sequences were simultaneously aligned to T4 lysozyme. Only
641 sequences with unambiguous alignment in the GH motif were included in this analysis.
642
643 **Recombinant DNA methods.** DNA amplification, Gibson cloning, and restriction digests were
644 performed according to the manufacturer. Restriction enzymes and Gibson cloning mix were
645 from New England Biolabs. Cloning steps were carried out in *E. coli* (alpha-select competent
646 cells, Bioline) and plasmids were purified using Zyppy Plasmid Kits (Zymo Research
647 Corporation). Sequencing was performed by the Indiana Molecular Biology Institute and
648 Eurofins MWG Operon Technologies with double stranded plasmid or PCR templates, which
649 were purified with a DNA Clean & Concentrator kits (Zymo Research Corporation).
650 Chromosomal DNA was purified using the Bactozol Bacterial DNA Isolation Kit (Molecular
651 Research Center). Plasmids were introduced into all *E. coli* strains using chemical transformation
652 according to the manufacturer's protocols. Plasmids were introduced into *C. crescentus*, *A.*
653 *excentricus*, and *A. biprosthecum* by electroporation based on previously published studies [66].
654 Allelic exchange in was achieved with pNPTS138, large genetic insertions with pMCS-2 [55],
655 and eGFP insertional fusions with pGFPC-1 and pGFPC-2 [55].
656
657 **Plasmid construction**.
658 *Expression plasmids* (pTB146SUMO, pET28a, pET22b): *spmX* gene fragments encoding amino
659 acids 2-150 of SpmX (SpmX-Mur) were amplified from genomic DNA and inserted into
660 linearized expression vectors using Gibson cloning (NEB) according to manufacturers protocols.
661 P22 lysozyme (P22Lyso) was amplified from a synthetic gene strand (Eurofins) for similar
662 construction with Gibson cloning. For pTB147SUMO, the vector was linearized with SapI and
663 XhoI to insert SpmX-Mur-*Ae*. For pET28a, the vector was linearized with NdeI and EcoRI to
664 insert SpmX-Mur-*Cc*, BamHI and XhoI to insert SpmX-Mur-*Ae*, SacI to insert SpmX-Mur-*Ab,*
665 and EcoRI to insert P22Lyso. In all pET28a plasmids, the constructs were cloned in frame with
666 the N-terminal His-tag and a stop codon to eliminate the C-terminal His-tag. For pET22b, the
667 vector was linearized with EcoRI and the C-terminal His-tag was preserved. Point mutants in
668 expression vectors were obtained by using standard quick-change procedures and primers with 3'
669 single stranded overhangs for increased efficiency.
670 *Integrating plasmids for allelic exchange* (pNPTS138): For allelic exchange, the desired
671 mutation was engineered into pNPTS138, bracketed by 1 kb up- and downstream of the
672 corresponding genetic region. Integrants were isolated by antibiotic selection and secondary
673 recombination events were selected by sucrose counter-selection using standard procedures. The
674 resulting clones were confirmed by PCR and sequencing isolated genomic DNA.
675 For genomic deletions of *spmX* in *Asticcacaulis*, pNPTS138 was linearized with EcoRI
676 and codons on either end of the gene were retained to avoid introducing frame-shifts in the
677 surrounding area. Therefore the final gene deletion in *A. excentricus* lacks residues 5-808 and in
678 *A. biprosthecum* lacks residues 5-815. For SpmXΔmur truncations, resides 2-150 were removed
679 in all three species. In all cases pNPTS138 was linearized with EcoRV, except for SpmX*Ab*-
680 E19A, where pNPTS138 was linearized with EcoRI. Point mutations E19A and N91R were
681 integrated into the Δ*spmX* background for ease of clone isolation and included full-length SpmX
682 flanked by 1 kb genetic context. pNPTS138 containing mutated SpmX were constructed using

683 Gibson cloning with fragments on either side of the intended mutation and overlapping primers
684 containing the mutation amplified from genomic DNA. In all cases pNPTS138 was linearized
685 with EcoRV, except for SpmX*Ab*-E19A, where pNPTS138 was linearized with EcoRI.
686 *Integrating plasmids for insertional eGFP fusions* (pGFPC): The last 600 bp of *spmX*
687 from *C. crescentus* was amplified from genomic DNA and cloned into pGFPC-2 using Gibson
688 cloning.
689 *Integrating plasmids for replacement at the* Δ*spmX locus* (pMCS-2). These constructs
690 were designed to allow insertion of various SpmX mutants fused to C-terminal sfGFP into the
691 Δ*spmX* locus in *C. crescentus*. For SpmX-sfGFP and SpmX-E19A-sfGFP, fragments containing
692 1 kb of genomic DNA upstream of *spmX* and *spmX* or *spmX-E19A* were amplified from existing
693 pNPTS138 constructs and fused to a fragment containing monomeric sfGFP amplified from
694 pSRKKm-Plac-sf*gfp* [67] using Gibson cloning. In the final construct, SpmX and sfGFP are
695 connected with the linker sequence GSAGSAAGSGEF [68]. Chimeras with P22 lysozyme
696 (P22Lyso) and its catalytic mutant were made by Gibson cloning together fragments containing
697 1kb of upstream genomic DNA, P22Lyso (with no stop codon), SpmXΔmur (residues 151-431)
698 and sfGFP with the same linker. P22Lyso and P22Lyso-E11A were amplified from pET28a
699 plasmids containing these genes. Chimeras with SpmX muramidase from *Brevundimonas*
700 *subvibrioides* (residues 1-140) and *Parvularcula bermudensis* (residues 1-168) were similarly
701 made with the muramidase fragments amplified from genomic DNA and synthetic gene strands
702 (Eurofins), respectively.
703
704 **Production of SpmX-Mur-*Ae* for crystallographic studies.** The muramidase domain of SpmX
705 from *A. excentricus* (SpmX-Mur-*Ae*, residues 2-150) was fused to a hexahistidine tag followed
706 by the SUMO cleavage site of the Ulp1 protease (His-SUMO tag) (**Table S3**) [69] and
707 overexpressed in *E. coli* BL21 (DE3) RIL cells. Cells were grown at 37 °C in 2 l of Terrific
708 Broth (BD Biosciences) supplemented with ampicillin (100 μg/ml) until the OD_{600nm} reached 0.8.
709 Production of the recombinant protein was induced by the addition of isopropyl β-D-1-
710 thiogalactopyranoside (IPTG) to 0.5 mM after the culture was cooled to 25°C. Cell growth was
711 continued overnight at 25°C, and cells were harvested by centrifugation. Cell pellets were
712 resuspended in 1/20th volume of buffer A (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 25 mM
713 imidazole, 10% (vol/vol) glycerol) containing the Complete^{TM} cocktail of protease inhibitors
714 (Roche). Cells were lysed by six passages through a cell disruptor (Constant Systems Limited) at
715 20 kPsi, and cell debris were pelleted by centrifugation at 40,000 × g for 30 min at 4 °C. The
716 centrifugation supernatant was loaded on a Ni-NTA agarose resin (Qiagen) equilibrated with
717 buffer A. After extensive washing with buffer A, His-SUMO-SpmX-Mur-*Ae* was eluted with a
718 linear 0-100% gradient of buffer B (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 500 mM
719 imidazole, 10% (vol/vol) glycerol) over 10 column volumes. Peak fractions were pooled, mixed
720 with a 1:100 dilution of a His-tagged Ulp1 (SUMO) protease preparation [70] and dialyzed
721 overnight at 4°C in buffer C (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10% (vol/vol) glycerol).
722 Cleavage reactions were passed through Ni-NTA resin to remove free His-SUMO tag and His-
723 Ulp1, and untagged protein was collected in the flow through. Flow-through fractions were
724 concentrated with Amicon Ultra Centrifugal filter units with a molecular weight cutoff of 10 kDa
725 (Millipore) and were injected onto an ENrich^{TM} SEC650 10x300 gel-filtration column (Biorad).
726 SpmX-Mur-*Ae* was eluted with buffer D (25 mM Tris-HCl (pH 8.0), 150 mM NaCl) and again
727 concentrated with Amicon Ultra Centrifugal filter units. Protein concentration was measured
728 using absorbance at 280 nm.

729
730 **Protein crystallization and structure determination.** High-throughput crystallization trials
731 were performed with a Cartesian PixSys 4200 crystallization robot (Genomic Solutions, U.K.).
732 Hanging drops containing 100 nl of protein (25 or 12.5 mg/ml) and 100 nl of reservoir solution
733 were set up in 96-well Crystal Quick plates (Greiner) and incubated at 20°C. Initial crystal hits
734 were refined manually by setting up hanging drops containing 1 µl of protein (25 or 12.5 mg/ml)
735 and 1 µl of reservoir solution in 24-well plates (Molecular Dimensions) incubated at 20°C.
736 Large needle-shaped crystals (dimensions of about 40 x 40 x 400 µm) were finally obtained for
737 SpmX-Mur-*Ae* in 0.1 M Tris-HCl pH 8.5, 12% PEG 3350, 0.2 M $MgCl_2$, at 20°C within 24–48
738 h. SpmX-Mur-*Ae* crystals were cryoprotected by transfer into 0.1 M Tris-HCl pH 8.5, 13% PEG
739 3350, 0.2 M $MgCl_2$, 10% glycerol, and then flash-frozen in liquid nitrogen. X-ray diffraction
740 data were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble, France) on
741 the ID30a1 (MASSIF-1) beamline [71,72].
742 Diffraction data were indexed and scaled using the XDS program suite [73]. SpmX-Mur-
743 *Ae* crystals belong to the trigonal space group $P3_221$, with unit cell dimensions of 100.44 x
744 100.44 x 96.62 Å and three molecules per asymmetric unit. Phase determination was carried out
745 by the molecular replacement method with PHASER [74], using as a search model the structure
746 of the phage P22 lysozyme (PDB entry 2ANX). The molecular replacement solution model was
747 rebuilt de novo using PHENIX [75] to prevent bias from the model.
748 The structure of SpmX-Mur-*Ae* was completed by cycles of manual building with COOT
749 [76] and addition of water molecules with ARP/wARP [77]. Several cycles of manual building
750 and refinement with REFMAC [78], as implemented in the CCP4 program suite, were performed
751 until R_{work} and R_{free} converged [79]. Stereochemical verification was performed with
752 PROCHECK [80]. The secondary structure assignment was verified with DSSP [81], with all
753 residues within most favorable or allowed regions of the Ramachandran plot.. Figures were
754 generated with PyMol (http://www.pymol.org). Coordinates of the final refined model were
755 deposited at the Protein Data Bank (PDB, http://www.rcsb.org) and were assigned PDB entry
756 code 6H9D. The data collection and refinement statistics are summarized in **Table S2**).
757
758 **Protein production for *in vitro* assays.** Fresh BL21(DE3) competent cells (Novagen) were
759 transformed with pET28a constructs containing various muramidase genes with N-terminal His-
760 tags (**Table S3**) and grown overnight in LB with 1% glucose and 50 µg/mL kanamycin.
761 Overnight cultures were diluted 100-fold in LB medium with 1% glucose and 50 µg/mL
762 kanamycin. Typically 500 mL cultures of cells were grown for 1.5-2 hours to an OD600 of 0.6-
763 0.7 and shifted to 20°C. When the OD600 reached 0.8−0.9, the cells were induced with 0.5 mM
764 IPTG. After growing for 4 h at 20 °C, cells were harvested and resuspended in 30 mL lysis
765 buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 20 mM imiadazole, 5 mM BME) with a EDTA-
766 free Protease Inhibitor Mini Tablet (Pierce) and phenylmethanesulfonyl fluoride (PMSF, 1 mM).
767 The 30 mL cell mixture was lysed on ice using a sonicating horn and spun down at 10,000g for
768 20-30 min. The clarified lysate was loaded onto a 5 ml HiTrap Chelating HP cartridge (GE
769 Healthcare) charged with Ni^{2+} and pre-equilibrated with lysis buffer. After loading, the column
770 was washed with lysis buffer followed by an elution via a 0-100% linear gradient of buffer B (25
771 mM HEPES pH 7.5, 100 mM NaCl, 500 mM imidazole, 2 mM BME). Muramidase-containing
772 fractions were pooled based on SDS-PAGE analysis and concentrated to 2.5 mL. Imidizole was
773 removed by passing the concentrated fraction over a PD10 desalting column (GE Healthcare)
774 equilibrated with 25 mM HEPES pH 7.5, 100 mM NaCl, 2 mM BME.

775
776 **Sacculi preparation and RBB labeling.** Sacculi were prepared from all species in the same
777 manner. For a typical 2L prep, cells were grown to an OD of 0.5-0.7 in their respective medium
778 (see bacterial strains and growth conditions) and harvested by centrifugation at 6,000*g* for 20
779 minutes. *C. crescentus* cells usually required multiple centrifugation steps to collect all the cells.
780 Cells were resuspended in 25 mL water (or PBS for *E. coli*) and added drop-wise into 50 mL of
781 boiling 7.5% SDS under stirring. The mixture was boiled for 30 minutes and then allowed to
782 cool to room temperature. Sacculi were then pelleted by ultracentrifugation at 100,000*g* for 30
783 minutes at room temperature. The resulting pellets were resuspended in 100 mL pure water, and
784 washed repeatedly until SDS was no longer detected in the supernatant. The pellet was
785 confirmed to be clear of SDS by mixing 0.2 mL of the supernatant with 1 uL 0.5% methylene
786 blue, 0.1 mL 0.7M $NaPO_4$ pH 7.2, and 0.6 mL chloroform and checking to make sure that, after
787 vortexing and allowing to settle, the solution had an upper blue phase and a lower clear phase
788 [82]. At this point, the pellets were resuspended in 10 mL PBS with 20 mM $MgSO_4$, 250 U/uL
789 Pierce Universal Nuclease (Thermo Fisher Scientific), and 10 mg/mL amylase (Sigma). The
790 mixture was incubated at 37°C for 1-4 hours. Afterwards, 10 mg/mL trypsin and 10 mM $CaCl_2$
791 was added and the mixture incubated overnight at 37°C. 800 uL of 15% SDS were then added to
792 the mixture and it was brought to a boil for about 10 minutes and allowed to cool to room
793 temperature. The sample was then pelleted (100,000*g*, 30 min, room temperature) and
794 resuspended in 4-5 wash steps until SDS was no longer detected. The final pellet was then
795 resuspended in 2 mL water and added to 0.8 mL 0.2M remazol brilliant blue (Sigma), 0.4 mL
796 5M NaOH, and additional water to 8 mL. The mixture was incubated, shaking, overnight at
797 37°C. After neutralizing the solution with 0.4 mL 5M HCl. The mixture was then pelleted
798 (21,000*g,* 20 minutes, room temperature), and resuspended in water until the supernatant became
799 clear.
800 To calibrate the concentration of RBB-labeled sacculi for dye-release assays and
801 peptidoglycan-binding assays, activity curves with Hen Egg White Lysozyme (Sigma) were
802 produced using different dilutions of the RBB-labeled sacculi. The RBB-labeled sacculi were
803 used at the dilution that resulted in an A595 of 0.5 when 5 μL of the RBB-labeled sacculi were
804 incubated with 4 uM HEWL.
805
806 **Peptioglycan-binding assays.** 5μL of calibrated RBB-labeled sacculi were incubated with 1 μM
807 of purified protein (protein constructs used are shown in Fig. S4) in PBS pH 7.4 to a final
808 volume of 50 μL for 30 minutes at 37°C and then pelleted (16,000*g*, 20 minutes). Fractions were
809 separated and the pellet resuspended in 50 μL PBS. 10 μL of each fraction was loaded onto Any
810 kD Mini-PROTEAN TGX Precast Protein Gels (BioRad) to visualize whether the protein
811 associated with the insoluble sacculi fraction. BSA (Sigma) was used at 1 μM as a negative
812 control.
813
814 **Remazol brilliant blue dye-release assays.** Methods were adapted from [32,83]. Assays were
815 carried out in 25-μL reactions using 25 mM HEPES pH 7.5, 100 mM NaCl and 5 μL of
816 calibrated RBB-labeled sacculi. Enzymes were added at various concentrations (see Figs. 5 and
817 S5) and incubated overnight at 37°C. Reactions were then centrifuged for 20 minutes at 16,000*g,*
818 and the supernatant carefully separated from the pellet. Final values in Fig. 5 and S5 are
819 normalized against absorbances measured for reactions with HEWL that were run in tandem for
820 every measurement to correct for differences in different sacculi preparations.

821
822 **Fluorescence microscopy and image analysis**. Fluorescence imaging was done using an
823 inverted Nikon Ti-E microscope using a Plan Apo 60X 1.40 NA oil Ph3 DM objective with a
824 GFP/Cy3 filter cube and an Andor DU885 EM CCD camera. Images were captured using NIS
825 Elements (Nikon). Cells were mounted on 1% (w/v) agarose pads made with PYE (or PBS, in
826 the case of *E. coli*) for imaging. In general, the fluorescent channel of each image was
827 background subtracted and a Gausian Blur filter was applied using Fiji [84]. Quantification of
828 stalk morphotypes and stalks with multiple foci was done by hand using Fiji tools. Quantification
829 of fluorescence data was achieved using MicrobeJ [85]. Mean stalk intensity was measured in *A.*
830 *biprosthecum* cells by using Fiji to draw line ROIs that did not overlap with the focus at the base
831 of the stalk, measuring mean fluorescence along the ROI. Figures and statistics were performed
832 using GraphPad Prism version 8.00 for Mac, GraphPad Software, La Jolla California USA,
833 www.graphpad.com.
834
835 **Western blots.** Strains were grown to saturation (overnight for *C. crescentus* and *A. excentricus*,
836 usually 48 hours for *A. biprosthecum*). OD600 was determined and cells were collected at a
837 normalized density of OD600 = 1/1mL. 1 mL of each normalized culture was pelleted,
838 resuspended in 100 uL water, and prepared for analysis using standard procedures using SDS-
839 PAGE, transfer, and western blotting. 10 µL of each sample was loaded onto Any kD Mini-
840 PROTEAN TGX Precast Protein Gels (BioRad). The JL-8 monoclonal GFP antibody (Clontech)
841 was used as the primary antibody and Goat Anti-mouse HRP (Pierce) was used for the secondary
842 antibody. Transferred blots were visualized with SuperSignal West Dura Extended Duration
843 HRP substrate (ThermoFisher Scientific) using a Bio-Rad Chemidoc.
844
845 **Periplasmic expression in *E. coli*.** Fresh Lemo(DE3) competent cells (NEB) were transformed
846 with pET22b constructs containing various muramidase genes with N-terminal H-tags (**Table**
847 **S3**) and plated. Lemo21(DE3) carries a rhamnose-inducible copy of LysY that inhibits T7
848 polymerase and allows for tunable dampening of expression of toxic products. We could not
849 transform expression strains BL21(DE3) or Tuner(DE3) with the pET22b-P22Lyso construct,
850 but were able to isolate a few transformants carrying this construct using Lemo21(DE3) cells
851 under high rhamnose repression (2 mM). P22Lyso-D20L, and all the SpmX-Mur-*Cc* constructs,
852 efficiently transformed into all expression strains tested, and could be carried by Lemo21(DE3)
853 without rhamnose.
854 In the case of pET22b-P22Lyso, where cells lyse from leak, cell cultures were grown
855 directly from colonies in the presence of 5 mM rhamnose and monitored over time. Figure 4A
856 shows the same treatment for all tested constructs. For testing induction of CCM and P22Lyso-
857 D20L, colonies were grown overnight in LB with 100 ug/mL carbenicillin and 30 µg/mL
858 chloramphenicol. Overnight cultures were diluted 50-fold in LB medium with 100 ug/mL
859 carbenicillin and 30 µg/mL chloramphenicol. In experiments cases rhamnose was added at
860 specified concentrations. Typically 4 mL cultures of cells were grown for 1-1.5 hours to an
861 OD600 of 0.3-0.4, induced with 400 uM IPTG, and shifted to 20°C.
862 *Growth curves and live-dead staining*: Optical densities were measured over time and
863 cells were routinely checked for lysing by microscopy using standard procedures. Briefly, 1 uL
864 of a 1:1 mixture of solutions A and B from a LIVE/DEAD *Bac*Light Bacterial Viability Kit
865 (ThermoFisher Scientific) was directly added to 100 uL of cells diluted 1:10 in PBS. Cells were
866 visualized on 1% agar pads made with PBS using the methods described in microscopy.

867 *Periplasmic expression levels:* After growing for 4 h at 20 °C, OD600 was determined and cells
868 were collected at a normalized density of OD600 = 1/1mL. One mL of the normalized sample
869 was pelleted at 4000g for 15 min and the pellet resuspended in 250 uL 20% sucrose, 1 mM
870 EDTA, 30 mM TRIS pH 8 at room temperature. The sample was mixed gently by rotation at
871 room temperature for 10 minutes before being spun down at 13,000g for 10 minutes. The
872 supernatant was carefully removed and the pellet rapidly suspended in 250 uL ice cold pure
873 water. The sample was mixed gently by rotation at 4°C for 10 minutes before being spun down
874 at 13,000g at 4°C. The supernatant (periplasmic fraction) and pellet (cell fraction) were then
875 separated and prepared for analysis using standard procedures using SDS-PAGE, transfer, and
876 western blotting. Blots were incubated with His-Probe Antibody (H-3) sc-8136 HRP (Santa Cruz
877 Biotechnology) and visualized with SuperSignal West Dura Extended Duration HRP substrate
878 (ThermoFisher Scientific) using a Bio-Rad Chemidoc.
879
880 **Supplemental Figure Legends**
881
882 **Figure S1. Alignments of SpmX muramidases. Related to Figure 1.** Aligned protein
883 sequences of the muramidase domains from genes listed in **Table S1,** a subset of which were
884 used for the gene tree in **Fig. 1.** Key positions discussed in text are marked with a black dot and
885 annotated with T4L identities.
886
887 **Figure S2. Relationships of enzyme families within the lysozyme superfamily. Related to**
888 **Figures 1 and 2.** Sequence cluster tree diagram made using NCBI's Conserved Domain
889 Database tool (CDD) demonstrating the inferred relationship of various lysozyme families with
890 structural homology but no sequence similarity. This tool uses reverse position-specific BLAST,
891 a method that compares query sequences to databases of position-specific score matrices and
892 obtains *E*-values, such as in PSI-BLAST [21,22]. SpmX from *C. crescentus* is marked in red
893 type; bacteriophages discussed in the text are emphasized in bold, black type. The tree was
894 formatted using iTol [65].
895
896 **Figure S3. Binding assays of SpmX to peptidoglycan from various species. Related to**
897 **Figure 4.** Coomassie brilliant blue stained SDS–PAGE gel of supernatant (S) and pellet (P)
898 fractions from peptidoglycan binding assays. In brief, SpmX protein constructs from three
899 different species and of two different lengths were incubated in PBS with or without sacculi
900 (peptidoglycan) isolated from the three different species. Peptidoglycan is insoluble and can be
901 pelleted out of solution. As shown here, peptidoglycan from all three species pulled down the
902 SpmX protein constructs.
903
904 **Figure S4. *In vitro* activity and periplasmic expression/activity of SpmX and various**
905 **mutants. Related to Figure 4.** Active enzymes release peptidoglycan monomers covalently-
906 bound to RBB into the supernatant that are detected by absorbance at 595 nm. (**A**) RBB assays
907 with SpmX-Mur-*Cc*-E11A, the catalytic mutant. Due to poor expression and instability, SpmX-
908 Mur-E11A was not concentrated to higher than 12 μM and its activity is compared to WT SpmX
909 at 12 μm. Error bars are ± standard deviation from two replicates. (**B**) RBB assays with SpmX-
910 Mur-*Cc*-L20D, the mutant that restores the ancestral D20. **(C)** Final ODs of cultures expressing
911 various P22Lyso and SpmX-Mur-*Cc* constructs after four hours of induction. P22Lyso-D20L
912 showed signs of spheroplast formation and cell lysis but the other tested constructs did not.

Compare to growth curves and microscopy shown in Fig. 4CD. **(D)** SpmX-Mur-*Cc* is expressed at similar levels in the periplasm as P22Lyso-D20L. Attempts at expressing SpmX-Mur-*Cc* more slowly in M9G medium at lower temperatures increased periplasmic levels but did not result in lysis. (**E**) RBB assays on *E. coli* sacculi isolated from Lemo21(DE3) using purified P22 lysozyme, P22 lysozyme D20L mutant, *C. crescentus* SpmX muramidase, and SpmX muramidase L20D mutant. Lines are drawn to help guide the eye toward basic trends. Normalization is to Hen Egg White Lysozyme (HEWL) assays (see methods).

Figure S5. Quantification of fluorescence and morphology data of SpmX mutant strains. Related to Figure 5. (A) Histograms of mean total cell fluorescence and integrated focal intensity of *C. crescentus* cells expressing fluorescently tagged WT (n = 1057 cells) and E11A (n = 792 cells) SpmX. Insets display the mean of these measurements with the standard deviation as error bars. Asterisks indicate $p < 0.001$ by Mann-Whitney U test. **(B)** Histograms (as in **A**) of mean total cell fluorescence and integrated focal intensity of *A. biprosthecum* cell expressing fluorescently tagged WT (n = 494 cells) and E11A (n = 462 cells) SpmX. Far right panel: boxplot of median and quartiles with whiskers indicating minimum and maximum values of mean fluorescence intensity of stalks in *A. biprosthecum* expressing WT or E11A (n = 10 stalks each strain). **(C)** Histogram (as in **A**) of mean total cell fluorescence and integrated focal intensity of *A. excentricus* expressing fluorescently tagged WT (n = 750 cells) and E11A (n = 693 cells) SpmX. Far right panel: quantification of the frequency of cells with multiple SpmX foci in the stalks of *A. excentricus* WT and E11A mutant. Chi-squared $p < 0.001$, n = 600 cells. **(D)** Quantification of the stalked morphotypes from *A. biprosthecum* WT and *spmX-E11A* mutant from cells without eGFP tags. Chi-squared $p < 0.001$, n = 500 cells. **(E)** Panels show bands from two independent blots using samples normalized by OD. Lanes have been cropped and rearranged to aid interpretation. In all cases, the primary antibody is directed against the C-terminal GFP fusion.

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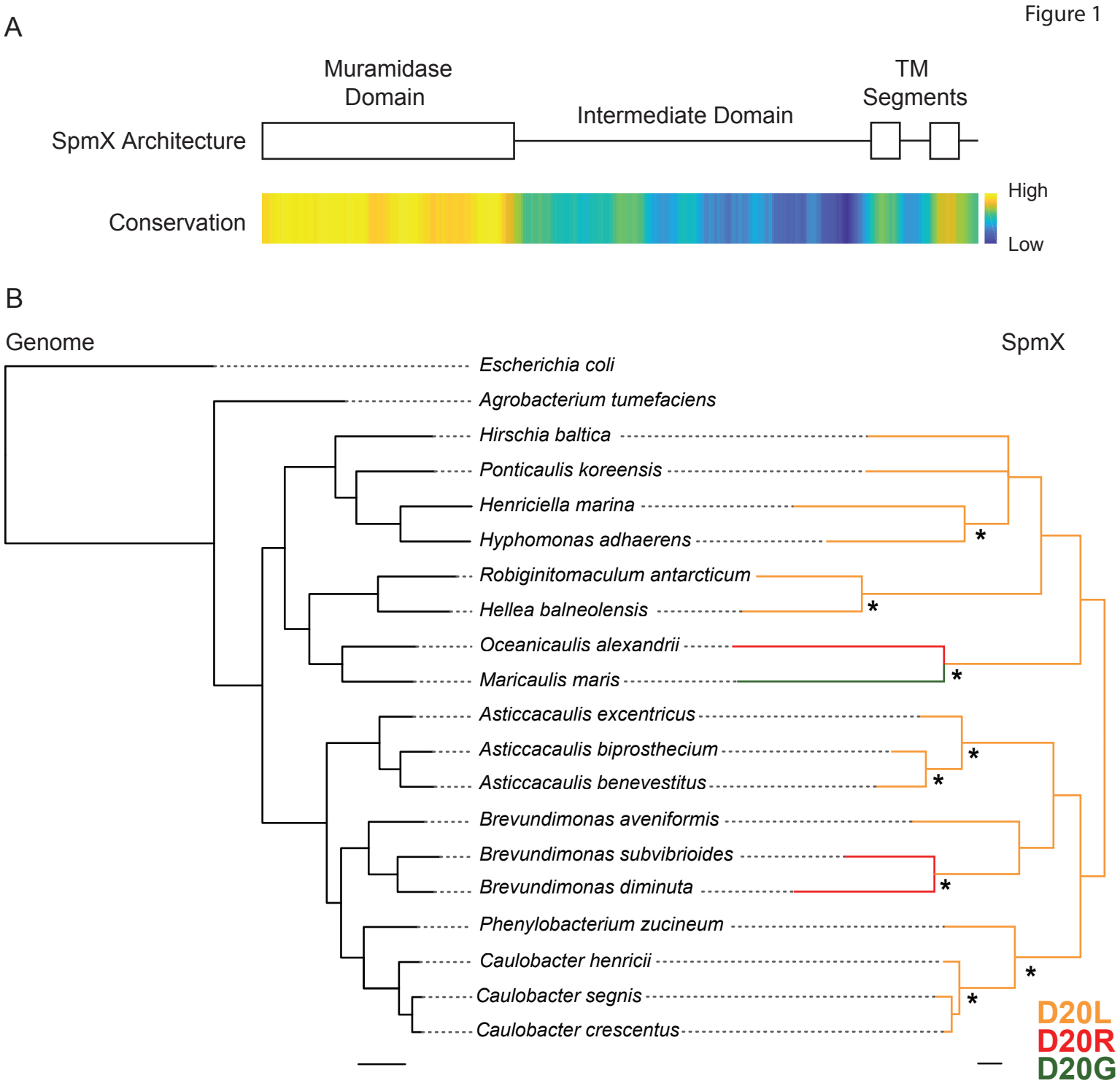
Figure 1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Conservation
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Ponticaulis koreensis
Henriciella marina
Hyphomonas adhaerens
Robiginitomaculum antarcticum
Hellea balneolensis
Oceanicaulis alexandrii
Maricaulis maris
Asticcacaulis excentricus
Asticcacaulis biprosthecium
Asticcacaulis benevestitus
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Brevundimonas subvibrioides
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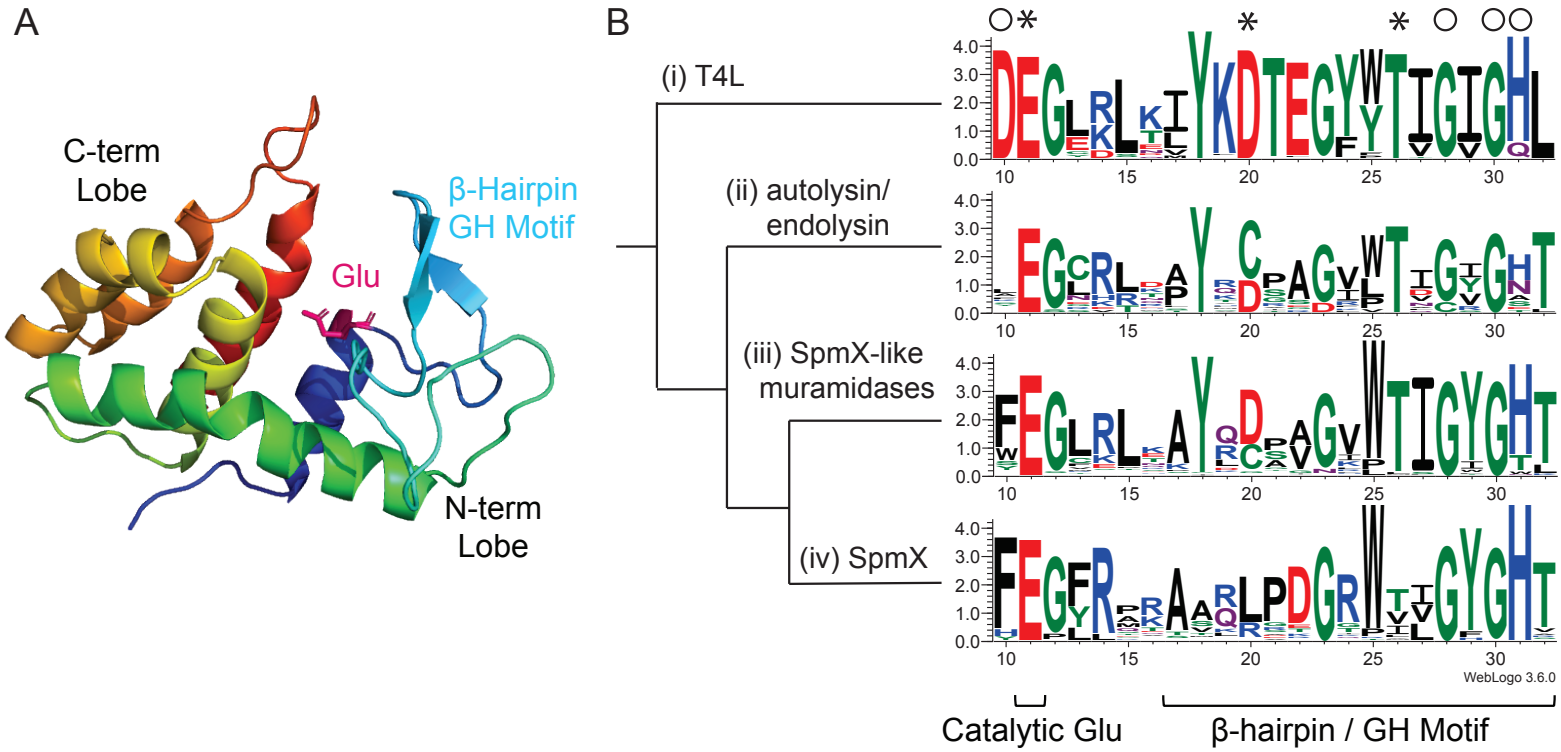
D20L
D20R
D20G

Figure 2


A
C-term
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N-term
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muramidases
(iv) SpmX
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Catalytic Glu
β-hairpin / GH Motif

Figure 3

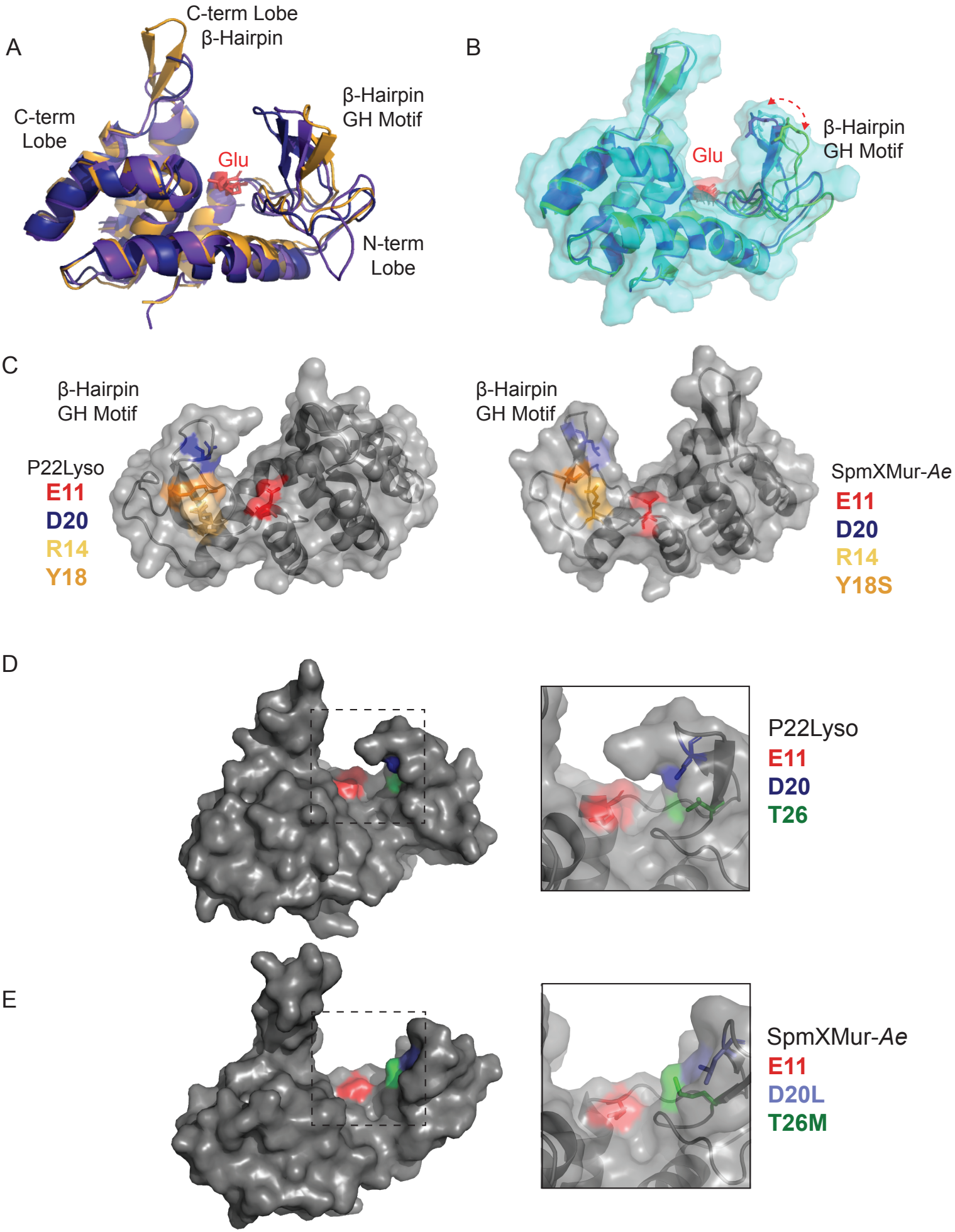
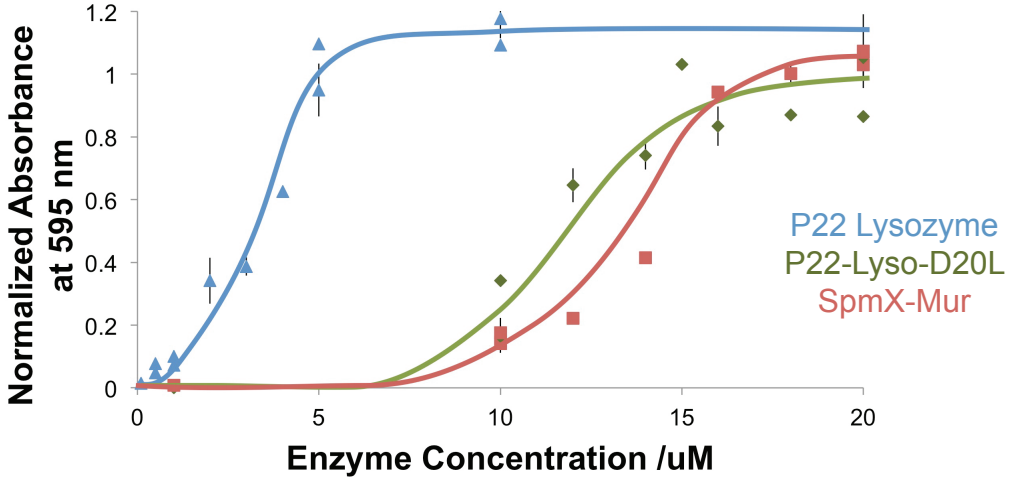
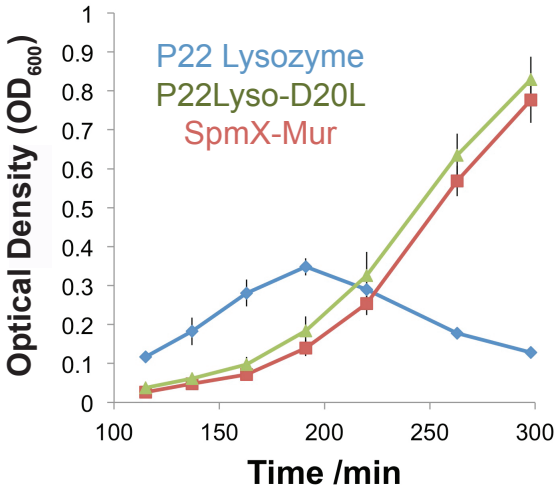

A
C-term Lobe
β-Hairpin
C-term
Lobe
β-Hairpin
GH Motif
Glu
N-term
Lobe
B
Glu
β-Hairpin
GH Motif
C
β-Hairpin
GH Motif
P22Lyso
E11
D20
R14
Y18
β-Hairpin
GH Motif
SpmXMur-Ae
E11
D20
R14
Y18S
D
P22Lyso
E11
D20
T26
E
SpmXMur-Ae
E11
D20L
T26M

Figure 4

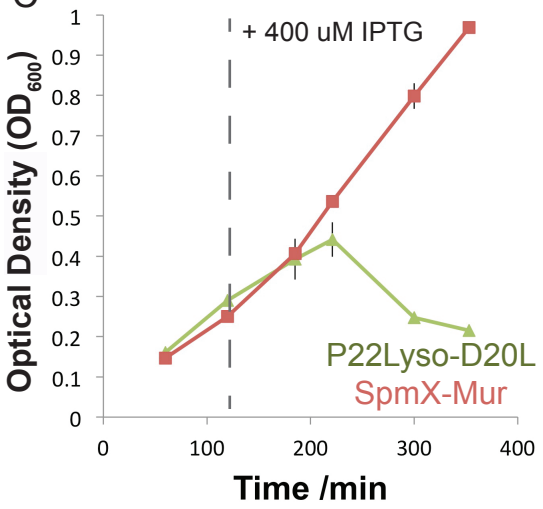
A



B



C



D

Live-Dead Stain:
SYTO9: live cells / PI: dead cells, free DNA

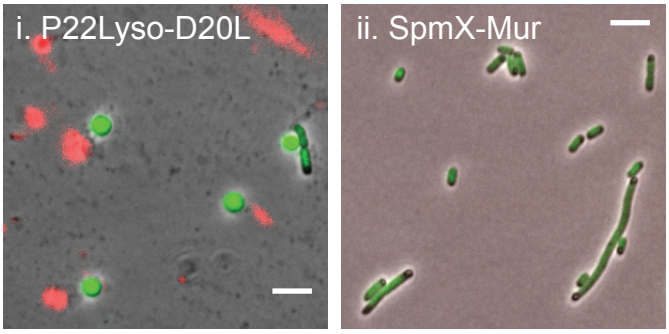


Figure 5

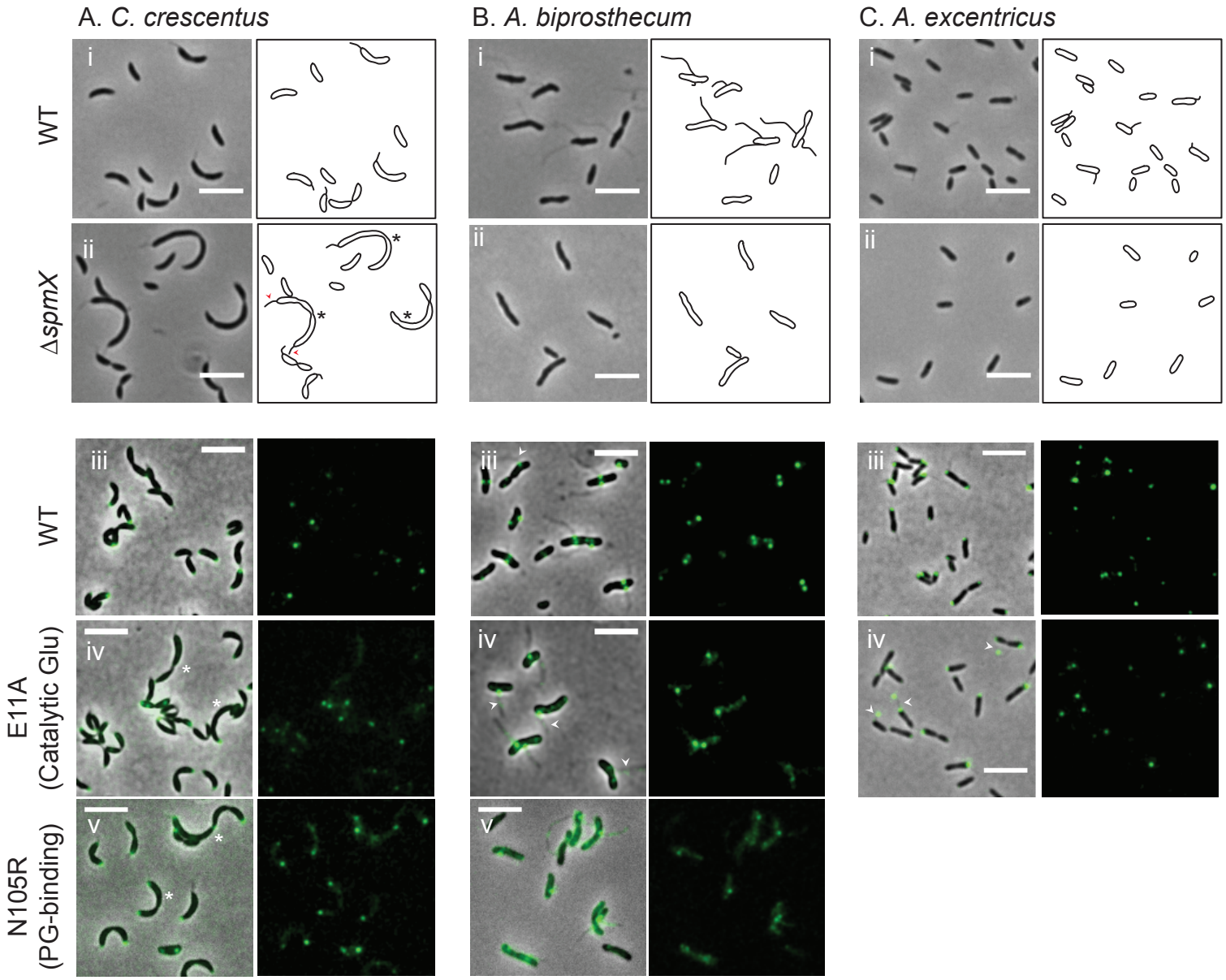
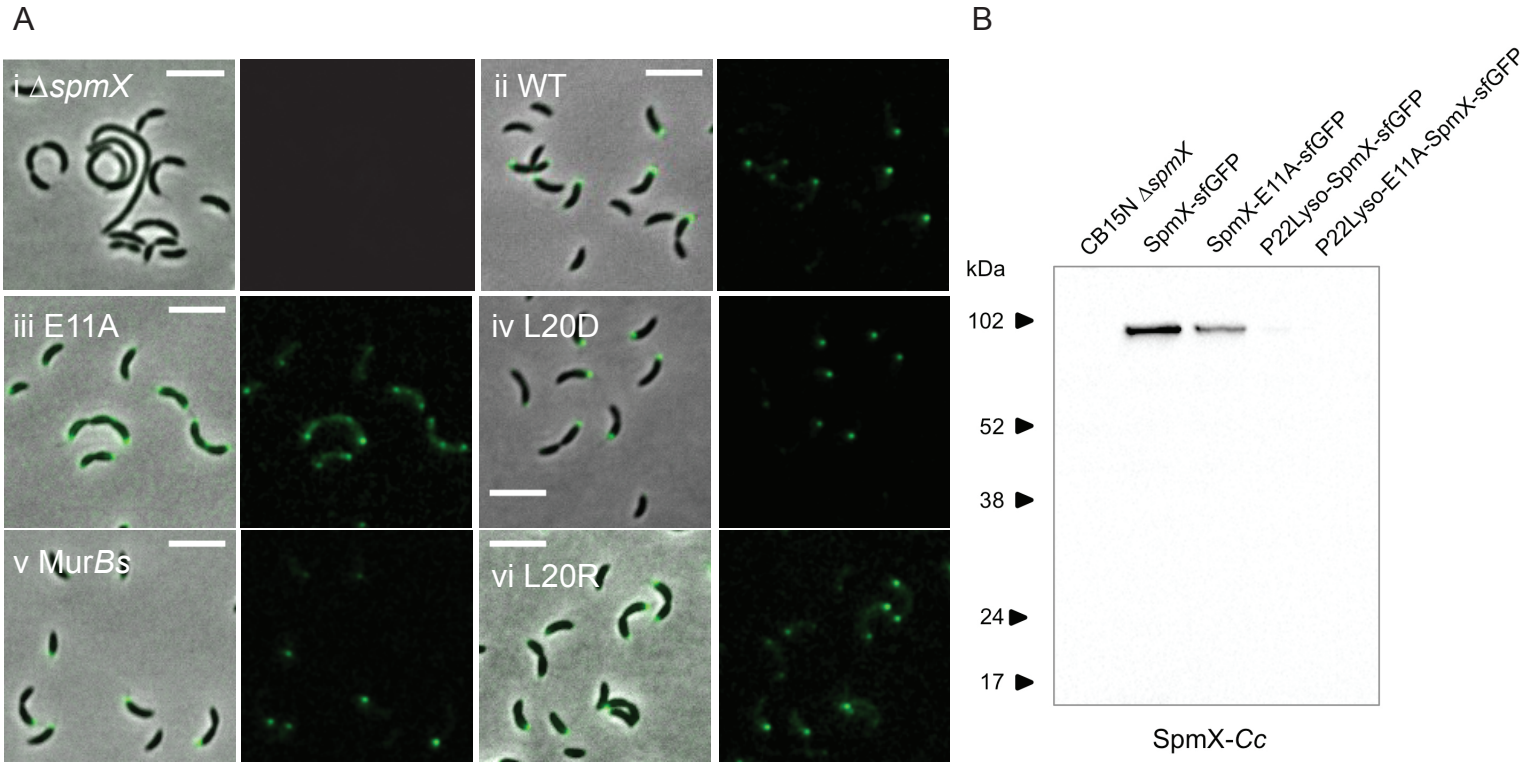

A. C. crescentus
B. A. biprosthecum
C. A. excentricus
WT
ΔspmX
WT
E11A
(Catalytic Glu)
N105R
(PG-binding)
i
ii
iii
iv
v

Figure 6


A
i Δ*spmX*
ii WT
iii E11A
iv L20D
v Mur*Bs*
vi L20R
B
CB15N Δ*spmX*
SpmX-sfGFP
SpmX-E11A-sfGFP
P22Lyso-SpmX-sfGFP
P22Lyso-E11A-SpmX-sfGFP
kDa
102
52
38
24
17
SpmX-*Cc*