

# CalY is a major virulence factor and a biofilm matrix protein

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## 25 **Abstract**

26 The extracellular biofilm matrix often contains a network of amyloid fibers which, in the human  
27 opportunistic pathogen *Bacillus cereus*, includes the two homologous proteins TasA and CalY.  
28 We show here, in the closely related entomopathogenic species *B. thuringiensis*, that CalY also  
29 displays a second function. In the early stationary phase of planktonic cultures, CalY was located  
30 at the bacterial cell-surface, as shown by immunodetection. Deletion of *calY* revealed that this  
31 protein plays a major role in adhesion to HeLa epithelial cells and in the bacterial virulence  
32 against the insect *Galleria mellonella*, suggesting that CalY is a cell-surface adhesin. In mid-  
33 stationary phase and in biofilms, the location of CalY shifted from the cell surface to the  
34 extracellular medium, where it was found as fibers, and *calY* deletion led to biofilm impairment.  
35 The transcription study and the deletion of *sipW* suggested that CalY change of location is due to  
36 a delayed activity of the SipW signal peptidase. Using purified CalY, we found that the protein  
37 polymerization occurred only in the presence of cell-surface components. CalY is therefore a  
38 bifunctional protein, which switches from a cell-surface adhesin activity in early stationary  
39 phase, to the production of fibers in mid-stationary phase and in biofilms.

## 40 **Introduction**

41 *Bacillus thuringiensis*, an insect pathogen genetically close to the human lethal pathogen *B.*  
42 *anthracis* and to the opportunistic food-borne pathogen *B. cereus*, produces biofilm pellicles  
43 floating on the culture medium (Auger *et al.*, 2006, Wijman *et al.*, 2007) or sticking to  
44 submerged solid surfaces (Houry *et al.*, 2012). The biofilm matrix of these species includes  
45 DNA, polysaccharides and proteins (Vilain *et al.*, 2009, Houry *et al.*, 2012). In the closely  
46 related species *Bacillus subtilis*, three proteins are required to build the biofilm. TasA forms  
47 amyloid fibers (Romero *et al.*, 2011), TapA is required for TasA polymerization and anchoring  
48 to cell surfaces (Romero *et al.*, 2014), and BslA coats the biofilm (Hobley *et al.*, 2013). The *B.*  
49 *subtilis* *tasA* and *tapA* genes are included in the *tapA-sipW-tasA* operon, in which *sipW* codes for  
50 a signal peptidase required for the secretion of TasA and TapA (Stover & Driks, 1999b, Stover  
51 & Driks, 1999a). Transcription of *tapA-sipW-tasA* is repressed by SinR and promoted by the  
52 anti-SinR protein SinI (Kearns *et al.*, 2005). In *B. thuringiensis*, *B. cereus* and *B. anthracis*, two  
53 orthologues of the *B. subtilis* *tasA* are found. One of these orthologues - also named *tasA* - is  
54 found just downstream of the signal peptidase gene *sipW*, in the SinR-regulated bicistronic  
55 operon *sipW-tasA* (Caro-Astorga *et al.*, 2015, Pflughoeft *et al.*, 2011, Fagerlund *et al.*, 2014).  
56 Located downstream from *sipW-tasA* is the second orthologue of *tasA* named *calY*. The *calY*  
57 gene is expressed from its own promoter and is also controlled by SinR (Caro-Astorga *et al.*,  
58 2015, Pflughoeft *et al.*, 2011, Fagerlund *et al.*, 2014). In *B. cereus*, both CalY and TasA  
59 polymerize to form fibers in the biofilm matrix, although TasA was more efficient than CalY for  
60 fiber production (Caro-Astorga *et al.*, 2015). Consequently, in this species, the deletion of either  
61 *tasA* or *calY* led to a decrease in the biofilm biomass produced in microtiter plate assays (Caro-  
62 Astorga *et al.*, 2015).  
63 CalY was at first called camelysin, a name which stands for ‘casein-cleaving membrane  
64 metalloproteinase’. CalY has indeed previously been described as a cell-surface, membrane-

65 bound zinc metallopeptidase active against casein, plasminogen, actin, collagen or fibrinogen  
66 (Fricke *et al.*, 2001, Grass *et al.*, 2004). Because of the limited homology of the protein to other  
67 metalloproteases, a new family was created, the M73 peptidase family (MEROPS database  
68 accession number MER031615), which so far contains only CalY and TasA but has no identified  
69 zinc-binding or catalytic sites. Meanwhile, CalY is often cited in the literature as a protease  
70 involved in degradation of host tissues and in toxin activation (Nisnevitch *et al.*, 2006,  
71 Nisnevitch *et al.*, 2010, Bai *et al.*, 2002, Irshad *et al.*, 2018) but without experimental evidence  
72 for such activity. Furthermore, CalY has also been reported in *B. cereus* to be a cell-surface  
73 protein able to bind to fibronectin and to mucin (Sanchez *et al.*, 2009).

74 Both the putative proteolytic and binding activities of CalY suggest that it could take part in  
75 pathogenesis, while its ability to form fibers and the consequences of its deletion on biofilm  
76 formation shows that it is a component of the biofilm matrix. The *calY* gene is likely to have  
77 arisen from *tasA* by gene duplication, a process which can lead to the evolution of moonlighting  
78 proteins (Espinosa-Cantu *et al.*, 2015). We therefore hypothesized that CalY could be a  
79 bifunctional protein, involved both in the biofilm matrix construction and in adhesion to host  
80 tissues. We found that CalY is indeed a major virulence factor and a key component of the  
81 biofilm in *B. thuringiensis*, moving from one function to the other according to the subcellular  
82 location and to the culture growth state.

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## 87 **Results**

### 88 **CalY is not a protease**

89 The proteolytic activity previously described for CalY was obtained with a sample purified from  
90 *B. cereus* cell-surface extracts, and with azocasein as a substrate (Fricke *et al.*, 1995). Here, we  
91 assayed on the same substrate, azocasein, a recombinant CalY (rCalY) purified from *E. coli*. As  
92 shown in Table 1, azocasein was not hydrolysed by rCalY purified from two preparations but  
93 was strongly degraded by the two metalloproteases thermolysin and InhA2, used here as positive  
94 controls. In addition, a proteolytic activity has never been reported for *B. subtilis* TasA, and there  
95 is only one histidine in the CalY amino acids sequence whereas most zinc metalloproteases  
96 display two histidines in their catalytic or metal binding site (Cerdeira-Costa & Gomis-Ruth, 2014).

97

### 98 **Transcription analysis reveals that *calY* is the most overexpressed** 99 **gene in biofilms relatively to early stationary phase planktonic** 100 **cultures**

101 The expression profile of the whole Bt407 genome was compared in 24 h biofilm cultures  
102 relatively to early stationary phase planktonic cultures by microarray analysis. Three hundred  
103 and five genes displayed a ratio of expression (biofilm/planktonic) greater than 2 (Table S1). By  
104 far, the most overexpressed gene in biofilms was *calY*, with an expression ratio of 36, whereas  
105 the *tasA* expression ratio was 7. However, *sipW-tasA* transcription starts at the onset of stationary  
106 phase, one hour earlier than *calY* transcription, and reaches high levels when *calY* transcription is  
107 still low at this time of the growth curve (Fig. 1), which can explain the higher expression ratio  
108 obtained for *calY*. Overall, 12 genes, putatively involved in biofilm formation, were found to be  
109 differentially expressed in biofilms. Six of these genes (*sipW*, *tasA*, BTB\_c13240, *calY*, *sinI*,  
110 *sinR*) are located in the overexpressed *sipW-sinR* locus. The six other genes differentially

111 expressed in biofilm include 4 transcriptional or post-transcriptional regulators (*ai2K*, *abrB*,  
112 BTB\_c16240 and the diguanylate cyclase/phosphodiesterase BTB\_c54300), and two genes  
113 involved in the biosynthesis and secretion of kurstakin - a lipopeptide shown to be required for  
114 biofilm formation (Dubois *et al.*, 2012, Fagerlund *et al.*, 2014, Gelis-Jeanvoine *et al.*, 2016).

115

## 116 **CalY is a biofilm matrix component**

117 Deletion of *calY* led to a strong and significant decrease in the 48h-aged biofilms biomass, which  
118 could not be restored by complementation (Fig. 2A). We followed morphological changes in the  
119 formation of the pellicle in a 48 well microtiterplate in HCT medium over a 48h-time period for  
120 the wild-type strain, the *calY* mutant strain and the complemented *calY* mutant strain (Fig. 2B).  
121 Whereas the pellicle could be seen as soon as 18h in the wild-type strain, it was absent in the  
122 mutant strain and was present but incompletely formed in the complemented strain. After 24h of  
123 culture, the pellicle was thick in the wild-type strain, but remained very thin and displayed holes  
124 and tears in the mutant strain. In 48h-aged cultures, the wild type strain showed dense pellicles in  
125 which the structure was hidden by thick biofilm materials, while the mutant strain pellicle was  
126 thinner with a clear structure. At both 24h and 48h culture times, the complemented strain  
127 pellicle displayed an intermediate phenotype between the wild-type strain and the mutant strain  
128 (Fig. 2B), although the complemented strain did produce CalY (Fig. S2).

129 The location of CalY in the biofilm matrix was determined by immunodetection using epi-  
130 fluorescence microscopy. The antibody, raised against rCalY, specifically recognizes CalY, but  
131 not TasA (see Experimental Procedures and Fig. S2 in supplementary materials). The matrix of  
132 the 48h-aged biofilm showed a dense network of CalY fibers in the wild type strain (Fig. 3). In  
133 contrast, no CalY fibers could be seen in the biofilm matrix prepared from the *calY* mutant  
134 strain. In the *calY* complemented strain, CalY was produced (Fig S2) but aggregated into rare

135 fiber bundles, thicker and longer than the fibers observed in the wild type strain (fig. 3), which is  
136 likely to be the cause of the partial defect in biofilm formation in the complemented strain.

137

### 138 **CalY overexpression leads to fiber bundles**

139 We wanted to determine the reason why CalY forms fiber bundles in the complemented strain.  
140 Sequencing of pCAL40, the multicopy plasmid used to express *calY* in the complemented strain,  
141 revealed no mutation in *calY*, and sequencing of the whole *sipW-sinR* region in the *calY* mutant  
142 showed 100% identity with the wild type strain, except for *calY* deletion (Fig S3). Therefore, the  
143 formation of fiber bundles by CalY in the complemented strain cannot be a consequence of a  
144 mutation in *calY* or in the region targeted by the recombination events. We then hypothesized  
145 that an overexpression of *calY* from the multicopy pCAL40 plasmid in the complemented strain  
146 could lead to an abnormal protein polymerization leading to the formation of fiber bundles and to  
147 a biofilm defect. To verify this hypothesis, we transformed the wild-type strain with pCAL40  
148 and determined the effect of this transformation on biofilm formation. The transformed strain  
149 produced less biofilm than the wild-type strain, and was not significantly different from the *calY*  
150 mutant and the *calY* complemented strains for this phenotype (Fig. 2A), supporting our  
151 hypothesis.

152

### 153 **CalY is a cell-surface adhesin**

154 The adhesion of the wild-type strain, the *calY* mutant and the complemented *calY* mutant grown  
155 in planktonic cultures and harvested in early stationary phase was assayed in polystyrene  
156 microtiter plates coated or non-coated with HeLa cells. Deletion of *calY* reduced drastically and  
157 significantly the adhesion to HeLa cells, down to the background level obtained in non-coated  
158 plates, where the three strains behave similarly (Fig. 4A), showing that CalY is a major adhesin

159 in *B. thuringiensis*. Complementation restored the adhesion capability of the *calY* strain, in  
160 contrast with the absence of complementation in the biofilm phenotype. This result suggested  
161 that CalY might be located at the cell surface in planktonic cultures. To check for this possibility,  
162 the subcellular location of CalY was determined by immunodetection in the wild-type strain, the  
163 *calY* strain and the complemented *calY* strain. At the end of the exponential phase, when *calY* is  
164 not transcribed (see Fig. 1), CalY could not be detected for any of the three strains (Fig. 4B). In  
165 early stationary phase, CalY could be seen on the bacteria cell-surface for the wild-type strain  
166 and for the complemented *calY* strain, but not for the *calY* mutant strain. In mid-stationary phase  
167 and later, CalY was no longer located on the cell-surface in the wild-type strain, but was seen as  
168 fibers surrounding the bacteria. CalY fibers were absent from the *calY* mutant strain cultures  
169 harvested in mid-stationary phase, and were present as fiber bundles in the complemented *calY*  
170 mutant strain cultures harvested at the same time (Fig. 4B).

171

## 172 **SipW inactivation promotes the cell-surface location of CalY**

173 Because no cell wall binding domain could be found in CalY sequence, we hypothesized that in  
174 the early stationary phase of growth, the protein could remain anchored to the bacterium  
175 cytoplasmic membrane by the hydrophobic region of its signal peptide. Cleavage of the signal  
176 peptide could be delayed if *sipW* transcription occurred later than *calY* transcription, but the  
177 reverse situation was observed (Fig. 1). An alternative hypothesis was that SipW could be in an  
178 inactive state in early stationary phase, and activated later. To determine if SipW inactivity could  
179 lead to a surface location of CalY, we deleted *sipW* and compared CalY location in the wild-type  
180 strain, the mutant strain and the complemented strain in cultures harvested in mid-stationary  
181 phase. In the wild type strain, CalY was present in the extracellular medium as fibers, but was  
182 found only at the cell surface in the *sipW* mutant strain, and complementation restored the  
183 extracellular location of the protein (Fig. 5).



184

## 185 **CalY polymerization requires an activating factor**

186 While CalY could form fibers in biofilm as well as in planktonic culture, rCalY (produced from  
187 *E. coli* without the signal peptide) did not spontaneously polymerize. These results suggested  
188 that a factor, either secreted or cell-surface bound, was required for CalY, polymerization. To  
189 check this hypothesis, we incubated rCalY with washed bacteria or with a cell-free supernatant  
190 obtained from a Bt407  $\Delta calY$  planktonic culture harvested in both early and mid-stationary  
191 phases, because CalY fibers were observed in a wild type strain culture harvested in mid- but not  
192 in early-stationary phase. The presence of CalY fibers in the two cultures fractions was then  
193 determined using the anti-CalY antibody. In cultures harvested in early stationary phase, no  
194 CalY fibers could be detected, either in the cell-free supernatant or in the washed bacteria (Fig.  
195 6). In contrast, bundles of antibodies-reacting fibers were seen in the cell fraction – but not in the  
196 cell-free supernatant fraction – obtained from cultures harvested in mid-stationary phase (Fig. 6  
197 and Fig. S4). These fibers bundles were present in the Bt407  $\Delta calY$  culture only when rCalY  
198 was added, and rCalY alone was unable to form fibers (Fig. 6). Therefore, a cell-associated  
199 factor is required for the extracellular CalY polymerization. This result also support the  
200 hypothesis that CalY in high concentrations polymerizes as fiber bundles.

201

## 202 **CalY is a major virulence factor in *B. thuringiensis***

203 CalY involvement in the *B. thuringiensis* pathogenesis against larvae of the lepidoptera *Galleria*  
204 *mellonella*, (the Greater wax moth) was assessed by force-feeding (ingestion) or by injection into  
205 the hemolymph of various doses of vegetative cells. Dose-response curves were built for the  
206 wild-type strain, the *calY* mutant strain and the complemented *calY* mutant strain, and LD50s  
207 were calculated from these dose-response curves. Deletion of *calY* resulted in a significant, 12-  
208 fold increase in the LD50 obtained by ingestion assays, whereas complementation of *calY* only

209 did not restore the wild-type strain virulence in these assays (Fig. 7). Similarly, injection assays  
210 resulted in a 13-fold increase of the LD50 when *calY* was deleted, but here, complementation  
211 fully restored the wild-type strain LD50 (Fig. 7).

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## 214 **Discussion**

215 Since its description by Fricke *et al.* in 1995 (Fricke *et al.*, 1995), CalY has been assigned a  
216 number of functions. This protein was first reported to be a cell-surface metallopeptidase. We  
217 could not detect such an activity on a purified, recombinant sample of the protein. We  
218 hypothesize that the metallopeptidase activity reported earlier for CalY is a consequence of the  
219 purification process from cell-surface extracts: on the one hand, metallopeptidases could have  
220 been co-purified with CalY; and on the other hand, our results show that CalY is present in high  
221 quantities as insoluble fibers in mid-stationary phase planktonic cultures, which are likely to be  
222 recovered during the cell-envelope preparation process. We therefore conclude that CalY is  
223 unlikely to be a metallopeptidase.

224 However, we confirm a recent finding showing that CalY is a component of the biofilm matrix,  
225 where it forms fibers (Caro-Astorga *et al.*, 2015). The authors suggested that CalY could play a  
226 prominent role in the matrix at the initiation of biofilm formation, and was likely to be replaced  
227 by TasA in mature biofilms. Using immunodetection methods and microarray analysis, we could  
228 observe that CalY is present as fibers in high quantity in 48h-aged biofilms, and that *calY* is the  
229 most overexpressed gene in mature biofilms compared to planktonic cultures in early stationary  
230 phase. Deletion of the gene encoding CalY leads to a decrease in the quantity of biofilm  
231 produced and in a reduction in the pellicle thickness.

232 While CalY is produced in high quantities in biofilms, it is also highly expressed in planktonic  
233 cultures. Its transcription starts in early stationary phase and increases sharply in mid stationary  
234 phase. Upon entry into stationary phase, when *calY* transcription is still low, CalY is located only  
235 at the bacterium cell-surface. In contrast, later in the stationary phase, CalY is absent from the  
236 cell surface and is found as free extracellular insoluble fibers. We have shown here that, when  
237 *sipW* is deleted, CalY remains attached to the cell surface. Therefore, it is likely that CalY is first  
238 anchored to the cell membrane by the hydrophobic domain of its signal peptide and then cleaved

239 by SipW to reach the extracellular medium. The transient presence of CalY at the cell surface  
240 cannot be a consequence of a delay in *sipW* transcription, because *sipW* transcription starts  
241 before *calY* transcription (Fig. 1). Alternatively, SipW activity could require the presence of a  
242 cofactor which production would start in mid-stationary phase. This hypothesis is supported by a  
243 previous report suggesting the existence of a SipW activator in *B. subtilis* (Tjalsma *et al.*, 2000).  
244 The purified CalY used to raise antibodies did not polymerize spontaneously upon incubation.  
245 This result suggested that an activating factor, different from SipW (because the purified CalY  
246 was without signal peptide), was required for CalY polymerization. We investigated this  
247 possibility by mixing purified CalY with planktonic cultures fractions from the Bt407  $\Delta calY$   
248 strain. We found that a cell-associated factor, most likely cell-surface bound, could promote  
249 fibers formation by the exogenous CalY. This activating factor was not present in cultures until  
250 mid-stationary phase, in agreement with the observation that planktonic cultures of the wild type  
251 strain produce CalY fibers only from this time of the stationary phase and later. The need of an  
252 activating factor for CalY polymerization is reminiscent of the requirement of TapA for TasA  
253 polymerisation in *B. subtilis* (Romero *et al.*, 2011). Yet, since there is no TapA homologue in *B.*  
254 *thuringiensis*, *B. cereus* or *B. anthracis*, the activation mechanism for CalY might be different  
255 from the activation of TasA by TapA in *B. subtilis*.  
256 The presence of CalY at the cell-surface, and the fact that it can bind mucin or fibronectin  
257 (Sanchez *et al.*, 2009), suggested that this protein could interact with host tissues. Our data show  
258 that CalY is needed for bacterial adhesion to HeLa cells. To our knowledge, CalY is the first *B.*  
259 *thuringiensis* or *B. cereus* cell-surface adhesin shown to be required for adhesion to HeLa  
260 epithelial cells. While the *B. cereus* cell-wall peptidase CwpFM was also reported to impact  
261 adhesion on HeLa cells, this effect was likely to be a side-effect of the autolysin activity of this  
262 protein (Tran *et al.*, 2010). In *B. anthracis*, two cell-wall anchored proteins, displaying LPXTG  
263 motifs, were found to bind collagen (Xu *et al.*, 2004), and orthologues of their genes are found

264 on the chromosome of *B. cereus* and of *B. thuringiensis*. However, the role of these collagen-  
265 binding proteins on the adhesion of bacteria to host cells or to host tissues was not investigated.  
266 BslA is a SLH protein (harboring a S-Layer Homology domain), which gene is located on the  
267 pathogenicity island of the *B. anthracis* virulence plasmid pXO1, and is therefore not present in  
268 *B. cereus* or *B. thuringiensis*. BslA is required for adhesion of *B. anthracis* vegetative cells on  
269 BJ1 fibroblasts (Kern & Schneewind, 2008). In addition, *bslA* deletion resulted in a dramatic  
270 increase in the lethal dose in an anthrax disease guinea pig model (Kern & Schneewind, 2010).  
271 Deletion of *calY* also resulted in a strong increase in the lethal dose of *B. thuringiensis* in the  
272 insect infection model *Galleria mellonella*. The dose required to induce 50% mortality upon  
273 deletion of *calY* had to be increased in the same order of magnitude, respectively 13 times and 12  
274 times, when bacteria were injected into the insect blood (hemocel) and when bacteria were force-  
275 fed. By this last contamination route, CalY is so far the only known virulence factor, together  
276 with the metallopeptidase InhA2 (Fedhila *et al.*, 2003), which plays a major role in the bacterial  
277 pathogenesis on its own. CalY activity both *per os* and by injection possibly reflects the dual  
278 function of this protein, acting as a biofilm matrix protein during the colonization of the gut  
279 following oral infection, and as an adhesin interacting with hemocytes, fatbody cells or other  
280 tissues following injection. CalY is therefore a major virulence factor of *B. thuringiensis*, and  
281 since it is highly conserved in *B. cereus* and *B. anthracis* (Fig. S5), it is likely that this protein is  
282 also involved in the pathogenicity of these species.

283 High concentrations of CalY obtained by an exogenous addition of this protein to a bacterial  
284 culture resulted in the formation of thick bundles of fibers in small number instead of the dense  
285 network of thin fibers observed in the wild type strain without addition of CalY. The formation  
286 of these fiber bundles is unlikely to stabilize the biofilm. This hypothesis is supported by the fact  
287 that an overexpression of CalY, obtained by transformation of the wild type strain by pCAL40, a  
288 multicopy plasmid expressing *calY*, led to a decrease in biofilm formation. This can explain why

289 complementation by pCAL40 failed to restore biofilm formation in the  $\Delta calY$  strain, but  
290 succeeded to restore adhesion to epithelial cells – a phenotype for which we do not expect that  
291 CalY polymerization is required. Similarly, virulence in force-feeding assays (not  
292 complemented) might require the formation of a biofilm in the insect intestinal tract, while  
293 virulence in injection assays (complemented) could need only adhesion on host tissues.

294 Our results show that CalY is a bifunctional protein, expressed at a high level in stationary  
295 phase. In *Staphylococcus epidermidis* too, a bifunctional protein has been described, which can  
296 either work as an adhesin or promote biofilm formation (Buttner *et al.*, 2015). This protein, Aap,  
297 is a cell-surface protein which displays 3 domains. The C-terminal domain C anchors the protein  
298 to the bacterial cell surface. The N-terminal domain A is required for the binding of bacteria to  
299 epithelial cells. Removal of the A domain by proteolysis activates domain B, which can thereby  
300 form dimers and promote intercellular adhesion and biofilm formation. However, unlike CalY,  
301 Aap is not released in the extracellular medium and do not form long fibers. Another biofilm  
302 bifunctional protein, recently described, is the extracellular protein *B. subtilis* BslA, which plays  
303 a role both in the biofilm architecture and in the formation of a hydrophobic layer lining it  
304 (Arnaouteli *et al.*, 2017). Unlike these two proteins, CalY subcellular location changes during the  
305 culture course. Indeed, CalY can be located at the cell-surface, where it acts as an adhesin  
306 thereby promoting the binding of bacterial cells to host tissues. This function provides to CalY a  
307 major role in *B. thuringiensis* virulence. It can also be found free in the extracellular medium,  
308 where it polymerizes and form insoluble fibers. In this other function, CalY constitutes a major  
309 component of the biofilm matrix. The switch between the two functions, summarized in Fig. 8, is  
310 likely to be due to a change in the signal peptidase SipW activity, that itself most likely requires  
311 a cofactor or an activator. An inactive SipW would keep CalY in the cell membrane, while an  
312 active SipW would cleave CalY from the membrane and let it polymerize as fibers in the  
313 presence of another, at yet undetermined cell-surface activating factor. The determination of the

314 nature of these activators will certainly be a very promising aspect of future work dedicated to  
315 the elucidation of the mechanisms involved in CalY shift from one function to the other.  
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## 317 **Experimental Procedures**

### 318 **Bacterial strains, plasmids and growth conditions**

319 *Escherichia coli* TG1 (Sambrook *et al.*, 1989) was used as a host for derivatives of pUC19 (New  
320 England Laboratories), pQE30 (QIAGEN), pAT113 (Trieu-Cuot *et al.*, 1991) pHT304, pHT304-  
321 18Z (Arantes & Lereclus, 1991, Agaisse & Lereclus, 1994), pGemT-easy (Promega), and  
322 pUC1318Spc (Candela & Fouet, 2005). Strain M15 harboring pREP4 (QIAGEN) was used for  
323 the production of recombinant CalY. HB101 (pRK24) was used for mating experiments (Trieu-  
324 Cuot *et al.*, 1987). The *B. thuringiensis* strain (sequenced genome: GenBank accession number  
325 CP003889) used here was an acrySTALLIFEROUS derivative of strain 407 (Lereclus *et al.*, 1989),  
326 designated hereafter as Bt407. Planktonic cultures were grown in LB medium: the cultures were  
327 seeded at OD 0.01 and incubated at 30°C, agitation 175 rpm. Antibiotics were used as previously  
328 described (Bouillaut *et al.*, 2005).

329

### 330 **Genetic constructions**

331 Plasmid extraction, endonuclease digestion, ligation and agarose and polyacrylamide gel  
332 electrophoresis were carried out as described by Sambrook *et al.* (Sambrook *et al.*, 1989).  
333 Polymerase chain reaction (PCR) amplifications were carried out with rTaq according to the  
334 manufacturer (GE Healthcare). To produce His-tagged CalY, the *calY* open reading frame,  
335 without the leading sequence encoding CalY signal peptide, was amplified by PCR with the  
336 calYs and calYa oligonucleotides (Table 3). The amplified 534bp DNA fragment was inserted  
337 into pGemT-easy giving rise to pCAL5. The DNA fragment was then subcloned into pQE30  
338 (QIAGEN) using *Bam*HI/*Hind*III, giving pCAL7.  
339 To disrupt *calY*, a 2709bp DNA fragment containing *calY* was amplified by PCR with  
340 oligonucleotides cal1 and cal4. The amplified DNA fragment was inserted into pGemT-easy



341 giving rise to pCAL10. pCAL10 was digested with *HpaI*, and a spectinomycin-resistance  
342 cassette was inserted giving pCAL20. The *BamHI* fragment from pCAL20 was ligated into  
343 pAT113 giving pCAL30 (Trieu-Cuot *et al.*, 1993). This recombinant suicide plasmid was  
344 transferred from *E. coli* to Bt407 by heterogamic conjugation (Pezard *et al.*, 1991, Trieu-Cuot *et*  
345 *al.*, 1987) giving rise to 407*calY::spc*. The markerless mutant Bt 407 $\Delta$ *sipW* was obtained with  
346 the recombinant suicide plasmid pMAD*sipW*. This plasmid was built by inserting the  
347 *BamHI*/*EcoRI* 5'- and *EcoRI*/*NcoI* 3'-regions of *sipW*, amplified by PCR using primers  
348 *sipWAmF*/*SipWAmR* or *sipWAvF*/*sipWAvR*, in pMAD (Arnaud *et al.*, 2004) digested by  
349 *BamHI* and *NcoI*, and was transferred in Bt407 by electroporation (Lereclus *et al.*, 1989). The  
350 *calY* and *sipW* deletions were checked by PCR and by sequencing. To complement 407*calY::spc*  
351 with *calY* the PCR fragment containing *calY* and its promoter was amplified with *PcaI1*/*calYa*  
352 and inserted into pHT304 (Arantes & Lereclus, 1991) digested by *HindIII*/*BamHI* to give  
353 pCAL40. To complement 407 $\Delta$ *sipW* with *sipW* the PCR fragment containing *sipW* and its  
354 promoter was amplified with *sipWcF*/*sipWcR* and inserted into pHT304 (Arantes & Lereclus,  
355 1991) digested by *HindIII*/*BamHI* to give pHT304*sipW*.

356 To follow *calY* promoter expression, a fusion with this promoter and the *lacZ* reporter was  
357 constructed. The *calY* promoter was amplified using *PcaI1* and *PcaI2* and cloned into pHT304-  
358 18Z (Agaisse & Lereclus, 1994). Likewise, the *sipW* promoter was cloned into pHT304-18Z  
359 using *PsipWF* and *PsipWR* primers to follow *sipW* transcription. The resulting plasmids were  
360 transferred into Bt407 by electroporation.  $\beta$ -galactosidase specific activity was measured as  
361 described previously, and are expressed in units of  $\beta$ -galactosidase per milligram of protein  
362 (Perchat *et al.*, 2011). Each assay was carried out at least three times using independent cultures.

363

## 364 Peptidase assay

365 In order to analyze a potential peptidase activity of CalY, azocasein (A2765, Sigma-Aldrich Inc.,  
366 USA) was used as substrate. Thermolysin (peptidase family M4) from *Bacillus*  
367 *thermoproteolyticus* (P1512, Sigma-Aldrich Inc., USA) and purified InhA2 (peptidase family  
368 M6) were used as positive controls. InhA2 was purified from the supernatant of Bt407- $\Delta$ plcR  
369 [pHT3015Apha3inhA] (Fedhila *et al.*, 2003), a strain overexpressing InhA2. The supernatant  
370 from an early stationary LB culture was recovered, centrifuged and 0.22 $\mu$ m-filtrated. All proteins  
371 were precipitated by 85% ammonium sulphate and resuspended in Bis-Tris HCl 25mM, pH7.2  
372 CaCl<sub>2</sub> 4mM. After desalting, InhA2 was purified by ion exchange chromatography  
373 (MonoQ5/50GL and HPLC) and eluted with 0-15% NaCl as a single 85 kDa protein. Azocasein  
374 was used as a 1% suspension in buffer bis-Tris, HCl pH 7.2, 25 mM, CaCl<sub>2</sub> 4mM, ZnCl<sub>2</sub> 0,1mM.  
375 Thermolysin, InhA2 and CalY were assayed at 10 $\mu$ g/ml. The reaction was stopped by the  
376 dilution of a 200  $\mu$ l sample in 1 ml TCA 10%. The tube was centrifuged to pellet the undigested  
377 substrate and the supernatant was mixed with 750  $\mu$ l NaOH 1 M before measuring the OD<sub>440</sub>.  
378 The assays were repeated three times. The OD<sub>440</sub> was corrected for blank (without peptidase)  
379 values. The peptidase activity was expressed as (nmoles azocasein degraded) / (min x mg  
380 peptidase).

381

## 382 **Microarray analysis**

383 Microarray analysis was performed as described previously (Fagerlund *et al.*, 2014). The Bt407  
384 strain was grown in bactopectone medium (10g l<sup>-1</sup> bactopectone, 5g l<sup>-1</sup> yeast extract, 10g l<sup>-1</sup>  
385 NaCl) at 30°C. For planktonic cultures, an overnight culture was diluted 1:100 in 50ml  
386 bactopectone medium, and harvested at the entry point into stationary phase (ie after 3 hours of  
387 growth at 250rpm). Biofilm were produced as follows: one gram of glass wool was dry-sterilized  
388 in a 500-ml erlenmeyer bottle. Overnight culture was diluted 1:200 in 100ml bactopectone  
389 medium, and grown at 50rpm (very slow shaking) in the flask containing glass wool. The glass

390 wool remained semi-immersed in the medium so that the biofilm could grow either on  
391 submerged parts of the glass wool or at the liquid/air interface. Cells were harvested after 24  
392 hours. To remove unattached cells, the glass wool was gently rinsed twice using 30°C fresh  
393 bactopectone medium. The attached cells were subsequently released from the glass wool by  
394 shaking in 60% ice-cold methanol. Six biological replicates of planktonic and biofilm RNA,  
395 respectively, were compared using the microarrays. Genes showing at least twofold differential  
396 expression ( $\log_2FC \geq 1.0$  or  $\log_2FC \leq -1.0$ ) between the biofilm and planktonic samples, and with a  
397 confidence level (adjusted P value)  $\leq 0.05$  were selected.

398

### 399 **Biofilm assays**

400 Biofilms were grown in HCT medium (Lecadet *et al.*, 1980), in glass tubes as described earlier  
401 (Fagerlund *et al.*, 2014) or in 48 well microtiterplates seeded at OD 0.01 and incubated at 30°C  
402 with no agitation. Biofilm biomass was assessed by measuring the OD<sub>600</sub> of disrupted biofilms  
403 recovered in 1ml PBS. Means were computed from 5 to 10 replicates obtained from 3  
404 independent cultures. Each pair of means was compared using the Tukey's range test, with an  $\alpha$ -  
405 level set at 0.05. Pellicles were observed and photographed with a Leica MZ FLIII binocular  
406 microscope and a Sony NEX-5 digital camera.

407

### 408 **Antibody production and immunodetection**

409 CalY was overexpressed using the pCal7 plasmid. His-tagged recombinant CalY (rCalY) was  
410 purified as described by QIAGEN on Ni-NTA coupled to Superflow resin. Anti-CalY serum was  
411 obtained from rabbits by three injections of 300  $\mu$ g of purified protein. Injections and serum  
412 preparations were performed by Covalab (<http://www.Covalab.com>). Anti-CalY serum was used  
413 at 1:10000 for Western blot detection and at 1:100 for immunodetection on culture samples.  
414 Antibody binding in Western blot was revealed with a goat anti-rabbit, peroxidase-conjugated

415 secondary antibody (Pierce antibodies, Thermo Fisher Scientific Inc., USA) and developed using  
416 the ECL Western blotting analysis system (Amersham ECL Detection Reagents, GE Healthcare  
417 Bio-Sciences Corp., USA). The selected antibodies were quite selective for CalY (Fig. S3).  
418 Immunodetection of CalY in culture samples for microscopy purposes was performed as follows.  
419 Biofilms recovered from glass tubes assays were homogenized in cold PBS by  
420 aspirating/pushing ten times through a 26-gauge needle. Planktonic cultures were centrifuged  
421 and the pellets were resuspended in cold PBS. Cultures in PBS were mixed with formaldehyde at  
422 a final concentration of 4%, washed twice with cold PBS and resuspended in PBS. Cultures were  
423 incubated for 10 minutes at room temperature with anti-CalY antibodies diluted 1:100, washed  
424 twice with cold PBS, resuspended in PBS and incubated with an AlexaFluor488-labelled goat  
425 anti-rabbit antibody (Molecular Probes, ThermoFisherScientific Inc., USA) diluted 1:100 at  
426 room temperature for 10 minutes. Cultures were then washed twice with cold PBS, resuspended  
427 in PBS, stained with FM4-64 (Molecular Probes, ThermoFisherScientific Inc., USA) diluted  
428 1:100 and observed on a Zeiss Z1-AxioObserver fluorescence microscope.

429

### 430 **CalY polymerization**

431 Planktonic cultures of the Bt407  $\Delta calY$  mutant strain were harvested in early- and mid-stationary  
432 phases, and 100 $\mu$ l of the culture was centrifuged for 2 minutes at 7500 rpm at room temperature.  
433 The sedimented bacteria, washed twice with PBS and resuspended in 100 $\mu$ l of PBS, and the cell-  
434 free supernatant were each incubated with rCalY at a final concentration of 1 $\mu$ g/ml for 20  
435 minutes at room temperature, centrifuged for 2 minutes at 7500 rpm at room temperature,  
436 resuspended in PBS, and treated for CalY determination as described in 'Antibody production  
437 and immunodetection'. Controls (rCalY alone, or sedimented bacteria from a 407  $\Delta calY$  mutant  
438 strain planktonic culture harvested in mid-stationary phase and incubated without rCalY) were  
439 incubated and treated as described above.

440

## 441 **Insect virulence assays**

442 The role of CalY in the pathogenicity of the bacterium was determined by comparing the lethal  
443 effect of the wild-type strain, the *calY* mutant strain and the complemented *calY* mutant strain in  
444 two assays (ingestion and injection) on *Galleria mellonella* (bred in our laboratory for over 10  
445 years), performed as previously described (Bouillaut *et al.*, 2005). Four (ingestion) or five  
446 (injection) concentrations of bacteria in vegetative phase were used, and for each concentration  
447 the experiment was repeated three times on a minimum of 20 larvae each time. Phosphate-  
448 buffered saline was used for negative controls. Infected larvae were kept at 37°C and mortality  
449 was recorded over 24 to 48 hours. The LD<sub>50</sub> values were based on mortality data obtained 48  
450 hours post injection and were determined by non-linear regression using JMP9 (SAS Institute  
451 Inc., USA). The model used for the regression was a derivative of the Hill equation  $\frac{x^n}{a^n+x^n}$ ,  
452 where  $x$  is the dose used and  $a$  and  $n$  are computed parameters. The parameters, determined with  
453 their 95% confidence intervals, represent the LD50 ( $a$ ) and the steepness of the curve ( $n$ ).

454

## 455 **Adhesion assays**

456 Adhesion assays were performed as described previously (Ramarao & Lereclus, 2006). Bacteria  
457 harvested in early stationary phase were incubated for 15 minutes at 37°C in 24-well polystyrene  
458 microtiterplates in wells containing confluent HeLa cells (supplied by the American Type Culture  
459 Collection, reference ATCC CCL-2) ( $5 \times 10^5$  per well) or no HeLa cells.  $5 \times 10^6$  bacteria were  
460 loaded per well. Non-attached bacteria were then removed by washing three times with PBS, and  
461 HeLa cells were detached by scraping. Serial dilutions were plated on LB plates to score  
462 adherent bacteria *versus* total bacteria. Three replications were performed using three

463 independent cultures. Each pair of means was compared using the Tukey's range test, with an  $\alpha$ -  
464 level set at 0.05.

465

466

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471

472

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601

For Peer Review

602 **Tables**603  
604

<b>Protease :</b>	<b>Thermolysin</b> M21663	<b>InhA2</b> BTB_c06870	<b>CaY</b> BTB_c13250
replicates	3	3	3
mean	139	99	0.7
sem	25	11	0.3

605  
606**Table 1: Metallopeptidase activity of CaY**

607 The proteolytic activity of CaY was determined using azocasein as a substrate. Pure  
608 thermolysin and the metallopeptidase InhA2 were used as positive controls. InhA2 is  
609 produced by *B. thuringiensis* in stationary phase. Metallopeptidase activity is expressed in  
610 nmoles min<sup>-1</sup> mg<sup>-1</sup>. sem : standard error on the mean.

611  
612

<b>primer</b>	<b>sequence</b>	<b>use</b>
calYs	GGATCCattggtggaggaacatttgcattcttttagc	CaY overproduction
calYa	AAGCTTttatttttcttccccagcttcttggttagc	CaY overproduction & calY complementation
cal1	cgcGGATCCcggaaaacagttaatacgttaaaag	calY deletion
cal4	catgCCATGGgcgcatctgctaaacgttcttccgg	calY deletion
SipWAmF	cgGGATCCgaagcaattagggcgaaagatag	sipW deletion
SipWAmR	gGAATTCgtctctctccctctcgttg	sipW deletion
SipWAvF	gGAATTCtctgtttgttatactttccgttag	sipW deletion
SipWAvR	CATGCCATGGcgttccatactcagctcaataaac	sipW deletion
sipWcF	gGAATTCgttacgccgtaatacaaaagg	sipW complementation
sipWcR	aaCTGCAGctaaacggaaagtataaccaaacga	sipW complementation
Pcal1	cccAAGCTTcggaggacaaaagaaagtagaag	PcalY-lacZ fusion & calY complementation
Pcal2	tgcTCTAGAcacaatcaattccccctagc	PcalY-lacZ fusion
PsipWF	aaCTGCAGgttacgccgtaatacaaaagg	PsipW-lacZ fusion
PsipWR	gcTCTAGAtcaccaccgctcgtttt	PsipW-lacZ fusion

627 **Table 3: Primers used in this study**

628 Upper-case letters show restriction sites

629

## 630 **Figure captions**

### 631 **Figure 1: *calY* and *sipW-tasA* transcriptions.**

632 Transcription of *calY* (white circles) or of *sipW-tasA* (black circles) was monitored in planktonic cultures  
633 in LB medium through the *lacZ* gene reporter. The inset shows a focus on the transition phase between  $t_1$   
634 (OD 1) and  $t_2$  (OD 7). Each circle is the mean of three replications and the error bars are the standard error  
635 of the mean. The x-axis time scale is relative to  $t_0$  which is the transition between the exponential phase  
636 and the stationary phase.

### 637 **Figure 2: Role of CalY in biofilm formation.**

638 A: the biomass of biofilms grown in glass tubes in HCT medium was determined for the wild-type strain  
639 (wt), the *calY* mutant strain (*calY*), the complemented *calY* mutant strain (*calY<sub>c</sub>*), and the wild type strain  
640 overexpressing *calY* (wt<sub>c</sub>). Each bar is the mean of 5 to 10 experiments, and error bars represent the  
641 standard error of the mean. Bars with different letters (a or b) represent results that are significantly  
642 different ( $P < 0.05$ ), as determined by the Tukey's range test.

643 B: biofilms grown in 48-wells polystyrene microtiter plates (well diameter: 10mm) in HCT medium were  
644 photographed at different culture times with a binocular microscope. wt: wild-type strain; *calY*: *calY*  
645 mutant strain; *calY<sub>c</sub>*: complemented *calY* mutant strain.

### 646 **Figure 3: CalY location in biofilm.**

647 The presence of CalY in 48h-aged biofilms was determined using an immunodetection method and  
648 fluorescence microscopy. Biofilms were grown in glass tubes in HCT medium. wt, wild-type strain;  
649 *calY*: *calY* mutant strain; *calY<sub>c</sub>*: complemented *calY* mutant strain. Anti-CalY antibody was revealed by a  
650 secondary antibody labelled with Alexa488 (green). Bacterial membranes were stained using FM4-64  
651 (red).

### 652 **Figure 4: CalY role in adhesion to epithelial HeLa cells.**

653 A: adhesion to epithelial HeLa cells grown in 24-wells polystyrene microtiterplates (left), or to  
654 microtiterplates alone (right), was determined for the wild-type strain (wt), the *calY* mutant strain (*calY*),  
655 or the complemented *calY* mutant strain (*calY<sub>c</sub>*). Each bar is the mean of 3 experiments, and error bars

656 represent the standard error of the mean. Bars with different letters (a or b) represent significantly  
657 different results ( $P < 0.05$ ), as determined by the Tukey's range test.

658 B: the subcellular location of CalY in planktonic cultures was determined using an immunodetection  
659 method and fluorescence microscopy. Planktonic cultures were grown in LB medium and harvested at the  
660 end of the exponential phase (end), in early- and in mid-stationary phase. wt, wild-type strain; *calY* : *calY*  
661 mutant strain; *calYc* : complemented *calY* mutant strain. Anti-CalY antibody was revealed by a secondary  
662 antibody labelled with Alexa488 (green). Bacterial membranes were stained using FM4-64 (red).

### 663 **Figure 5: Role of SipW in CalY subcellular location.**

664 The role of SipW on the subcellular location of CalY was determined using an immunodetection method  
665 and fluorescence microscopy. Planktonic cultures were grown in LB medium and harvested in mid-  
666 stationary phase. wt, wild-type strain; *calY*: *calY* mutant strain; *calYc*: complemented *calY* mutant strain.  
667 Anti-CalY antibody was revealed by a secondary antibody labelled with Alexa488 (green). Bacterial  
668 membranes were stained using FM4-64 (red).

### 669 **Figure 6: Polymerization of CalY.**

670 Cell-free supernatant (S) or pelleted and PBS-washed bacteria (C) were prepared from planktonic cultures  
671 grown in LB medium and harvested in mid-stationary phase. rCalY was incubated with the cell-free  
672 supernatant (SNY) or with the washed bacteria resuspended in PBS (CY). Controls were rCalY incubated  
673 alone (Y), or washed bacteria incubated without rCalY (C). CalY fibers were revealed with a rabbit anti-  
674 CalY antibody and a goat anti-rabbit antibody tagged with Alexa488 (green). Phase: phase contrast.

### 675 **Figure 7: CalY role in the bacterium virulence.**

676 Different concentrations of vegetative cells were injected or force-fed to larvae of the lepidopteran  
677 species *Galleria mellonella*. Mortality was recorded 48h post-treatment, and LD50s values were  
678 computed. Bars show LD50s values, and error bars represent the 95% confidence interval. The Y-scale is  
679 in log-units. wt: wild-type strain; *calY*: *calY* mutant strain; *calYc*: complemented *calY* mutant strain. Bars  
680 with different letters (a or b) represent significantly different results ( $P < 0.05$ ) different, as determined by  
681 the Tukey's range test.

### 682 **Figure 8: Schematic representation of CalY functions.**

683 Left panel: in early stationary phase, in planktonic culture, both SipW and CalY are expressed and located  
684 on the cell-surface. SipW does not display its peptidase activity and CalY remains anchored in the cell  
685 membrane by its signal peptide. CalY works as an adhesin which binds to epithelial cells, and strongly  
686 contributes to the bacterial virulence.

687 Right panel: later in the stationary phase or in biofilms, SipW is activated by an unknown, hypothetical  
688 factor and cleaves CalY signal peptide. The free, extracellular CalY is activated by an undetermined, cell-  
689 surface activating factor (not shown here), and polymerizes to produce amyloid fibers promoting biofilm  
690 formation.

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693

## 694 **Supporting information**

### 695 **Table S1: Microarray analysis of biofilm vs planktonic cultures.**

696 Planktonic cultures in early stationary phase and 24h-aged biofilms were harvested and compared for  
697 genes expression by microarray analysis. Genes showing at least twofold differential expression between  
698 the biofilm and planktonic samples, and with a confidence level (adjusted P value)  $\leq 0.05$  were selected.

699

### 700 **Fig. S2: CalY Western blot.**

701 Extracts of Bt407 (wt), *calY* mutant strain (calY) and complemented *calY* mutant strain (calYc) were  
702 analyzed by Western blot. The anti-CalY antibody was revealed by a HRP-conjugated goat anti-rabbit  
703 antibody and by the ECL detection system. CalYr was loaded on the gel as a positive control.

704

### 705 **Fig. S3: Sequencing of the *sipW-tasA* region in the *calY* strain.**

706 The *sipW-tasA* region from the wild type strain is shown in the mid-panel of the figure (shaded in  
707 yellow). This region was sequenced in the *calY* mutant strain, from the end of the BTB\_c13210 gene to

708 the beginning of *inhAI* (4428 bp), using 5 PCR fragments amplified from both the direct and the reverse  
709 strands. The 10 generated fragments were aligned on the wild type strain sequence (available at  
710 <https://www.ncbi.nlm.nih.gov/nucleotide/409171506>) using the LASTZ tool of the Geneious software. In  
711 the lower panel, the black color shows the region covered by the assembled sequences. In the upper panel,  
712 the green color shows 100% identity between the amplified sequences and the wild type strain sequence.  
713 Mismatches were found only in *calY*, which was interrupted by a spectinomycin resistance cassette.

714

715 **Fig. S4: Polymerisation of rCalY.**

716 Examples of CalYr polymerisation in the presence of the cell fraction of different cultures of the 407  
717  $\Delta calY$  strain collected in mid-stationary phase. Phase: phase contrast. Alexa488: immunodetection of  
718 CalY. Overlay: overlay of the phase and the immunodetection pictures.

719

720 **Fig. S5: CalY conservation in *B. cereus*, *B. anthracis* and *B. thuringiensis*.**

721 A- The CalY predicted sequences from the *Bacillus cereus* ATCC14579 strain, the *Bacillus anthracis*  
722 Ames ancestor strain and the *Bacillus thuringiensis* 407 strain were aligned using ClustalW, and  
723 displayed 94% sequence identity.

724 B- The TasA and CalY predicted sequences from *B. subtilis*, *B. cereus*, *B. anthracis* and *B. thuringiensis*  
725 strains were aligned and the genetic distances were plotted using the tree builder option from Geneious  
726 (Biomatters Ltd, New Zealand). CalY sequences are highly conserved and clusterize apart from TasA  
727 sequences.

728

729

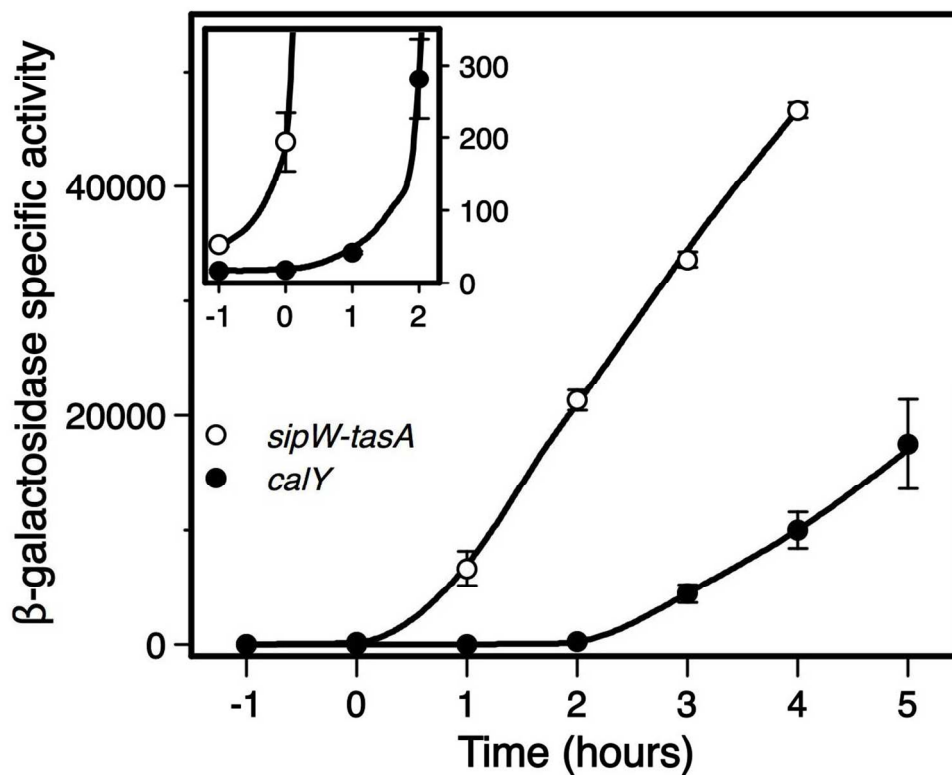


Figure 1: *calY* and *sipW-tasA* transcriptions.

Transcription of *calY* (white circles) or of *sipW-tasA* (black circles) was monitored in planktonic cultures in LB medium through the *lacZ* gene reporter. The inset shows a focus on the transition phase between t-1 (OD 1) and t2 (OD 7). Each circle is the mean of three replications and the error bars are the standard error of the mean. The x-axis time scale is relative to t0 which is the transition between the exponential phase and the stationary phase.

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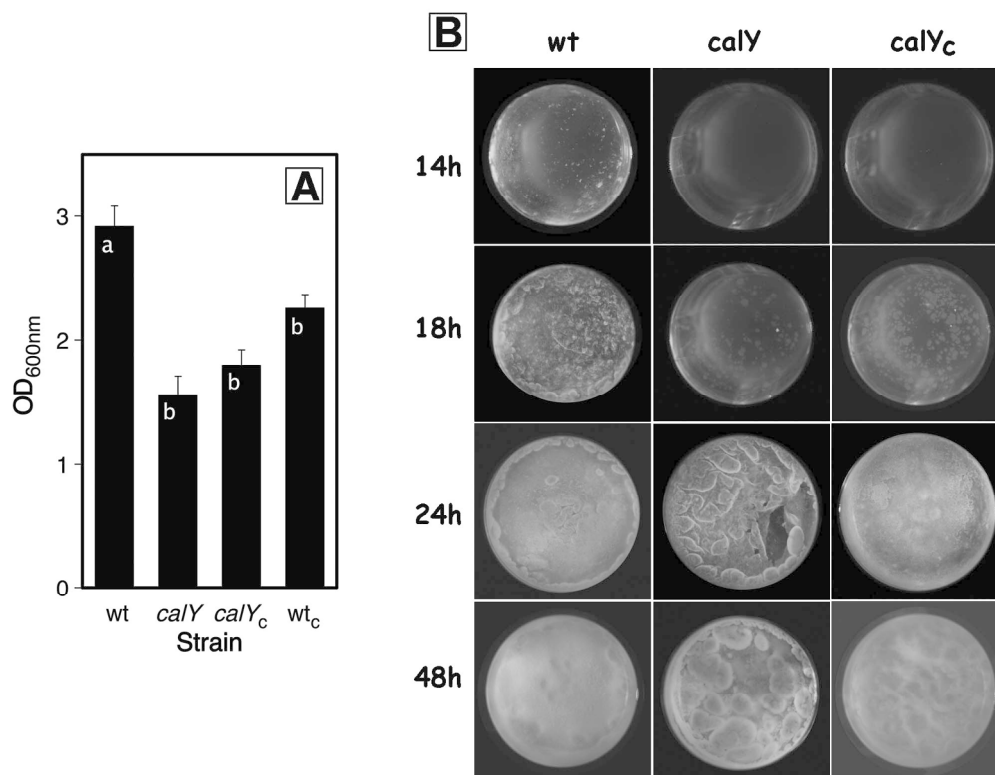


Figure 2: Role of CalY in biofilm formation.

A: the biomass of biofilms grown in glass tubes in HCT medium was determined for the wild-type strain (wt), the calY mutant strain (calY), the complemented calY mutant strain (calY<sub>c</sub>), and the wild type strain overexpressing calY (wt<sub>c</sub>). Each bar is the mean of 5 to 10 experiments, and error bars represent the standard error of the mean. Bars with different letters (a or b) represent results that are significantly different ( $P < 0.05$ ), as determined by the Tukey's range test.

B: biofilms grown in 48-wells polystyrene microtiter plates (well diameter: 10mm) in HCT medium were photographed at different culture times with a binocular microscope. wt: wild-type strain; calY: calY mutant strain; calY<sub>c</sub>: complemented calY mutant strain.

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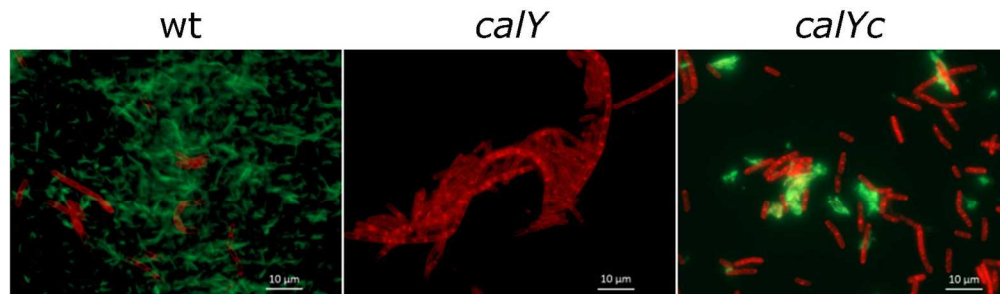


Figure 3: CaY location in biofilm.

The presence of CaY in 48h-aged biofilms was determined using an immunodetection method and fluorescence microscopy. Biofilms were grown in glass tubes in HCT medium. wt, wild-type strain; caY: caY mutant strain; caYc: complemented caY mutant strain. Anti-CaY antibody was revealed by a secondary antibody labelled with Alexa488 (green). Bacterial membranes were stained using FM4-64 (red).

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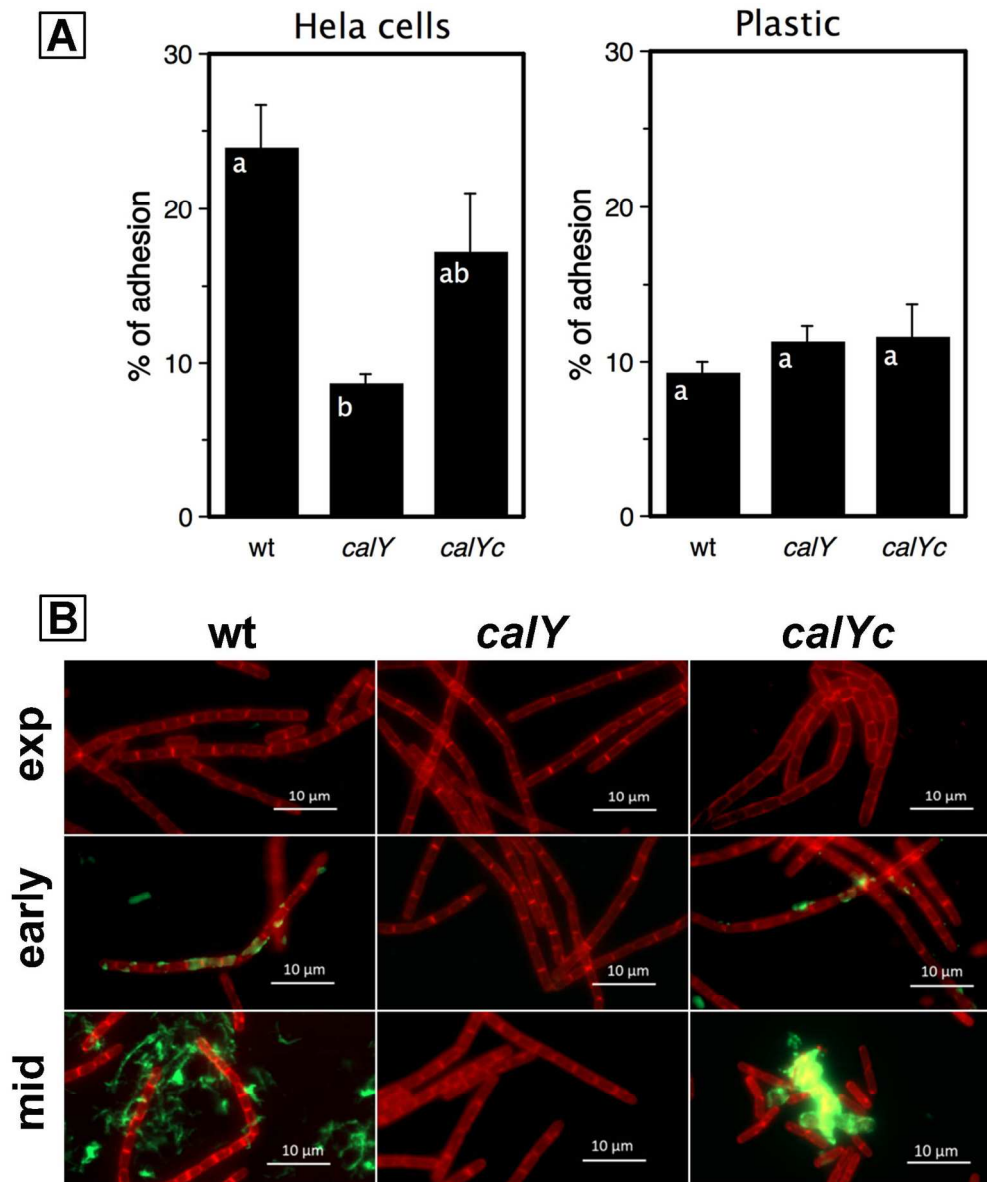


Figure 4: CaLY role in adhesion to epithelial HeLa cells.

A: adhesion to epithelial HeLa cells grown in 24-wells polystyrene microtiterplates (left), or to microtiterplates alone (right), was determined for the wild-type strain (wt), the *calY* mutant strain (*calY*), or the complemented *calY* mutant strain (*calYc*). Each bar is the mean of 3 experiments, and error bars represent the standard error of the mean. Bars with different letters (a or b) represent significantly different results ( $P < 0.05$ ), as determined by the Tukey's range test.

B: the subcellular location of CaLY in planktonic cultures was determined using an immunodetection method and fluorescence microscopy. Planktonic cultures were grown in LB medium and harvested at the end of the exponential phase (end), in early- and in mid-stationary phase. wt, wild-type strain; *calY* : *calY* mutant strain; *calYc* : complemented *calY* mutant strain. Anti-CaLY antibody was revealed by a secondary antibody labelled with Alexa488 (green). Bacterial membranes were stained using FM4-64 (red).

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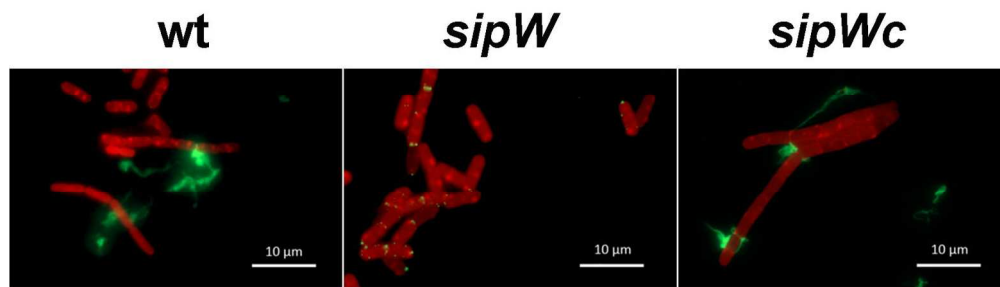


Figure 5: Role of SipW in CalY subcellular location.

The role of SipW on the subcellular location of CalY was determined using an immunodetection method and fluorescence microscopy. Planktonic cultures were grown in LB medium and harvested in mid-stationary phase. *wt*, wild-type strain; *calY*: *calY* mutant strain; *calYc*: complemented *calY* mutant strain. Anti-CalY antibody was revealed by a secondary antibody labelled with Alexa488 (green). Bacterial membranes were stained using FM4-64 (red).

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Peer Review

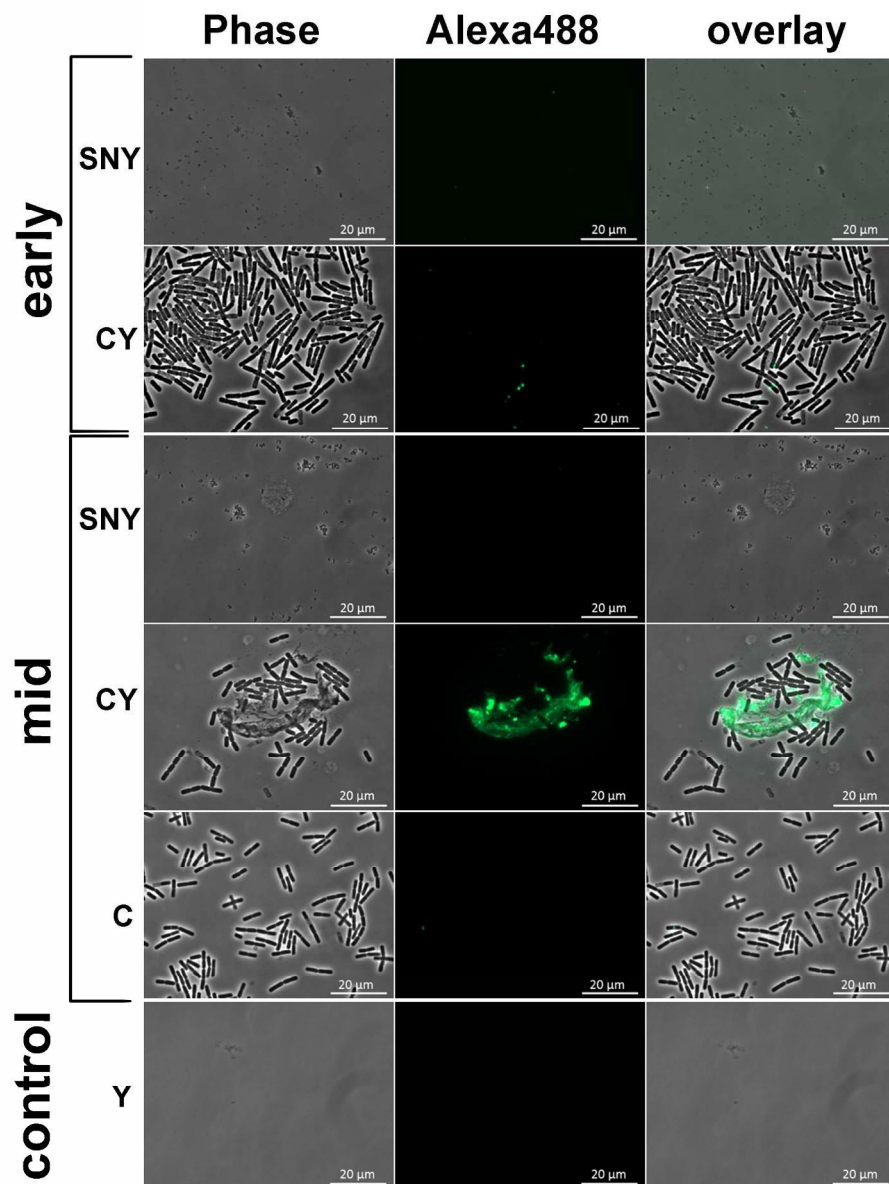


Figure 6: Polymerization of CaY.

Cell-free supernatant (S) or pelleted and PBS-washed bacteria (C) were prepared from planktonic cultures grown in LB medium and harvested in mid-stationary phase. rCaY was incubated with the cell-free supernatant (SNY) or with the washed bacteria resuspended in PBS (CY). Controls were rCaY incubated alone (Y), or washed bacteria incubated without rCaY (C). CaY fibers were revealed with a rabbit anti-CaY antibody and a goat anti-rabbit antibody tagged with Alexa488 (green). Phase: phase contrast.

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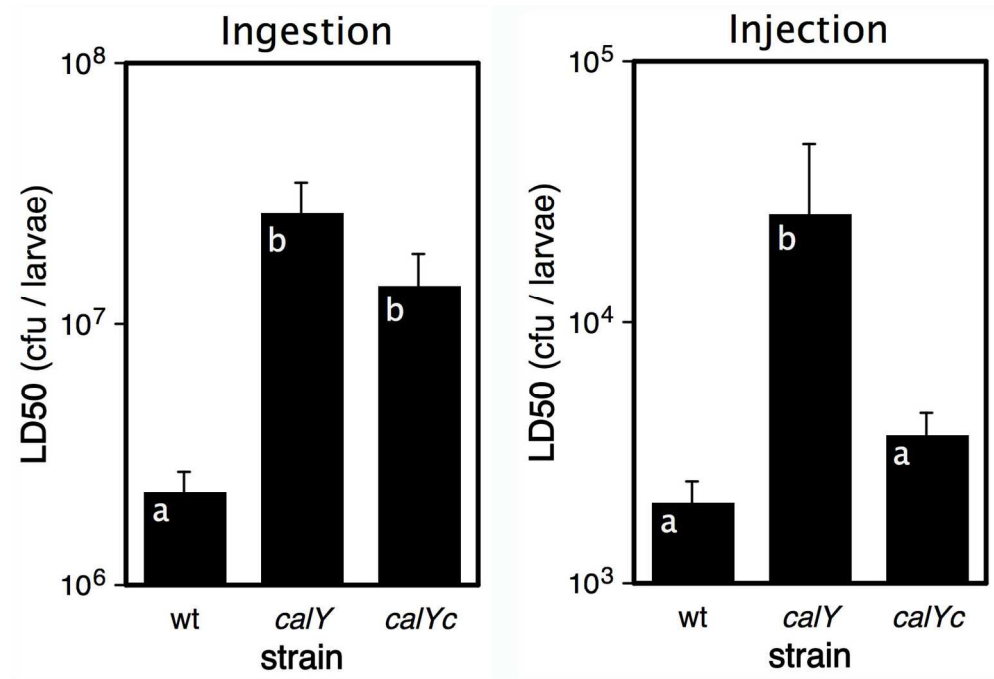


Figure 7: CaY role in the bacterium virulence.

Different concentrations of vegetative cells were injected or force-fed to larvae of the lepidopteran species *Galleria mellonella*. Mortality was recorded 48h post-treatment, and LD50s values were computed. Bars show LD50s values, and error bars represent the 95% confidence interval. The Y-scale is in log-units. wt: wild-type strain; calY: calY mutant strain; calYc: complemented calY mutant strain. Bars with different letters (a or b) represent significantly different results ( $P < 0.05$ ) different, as determined by the Tukey's range test.

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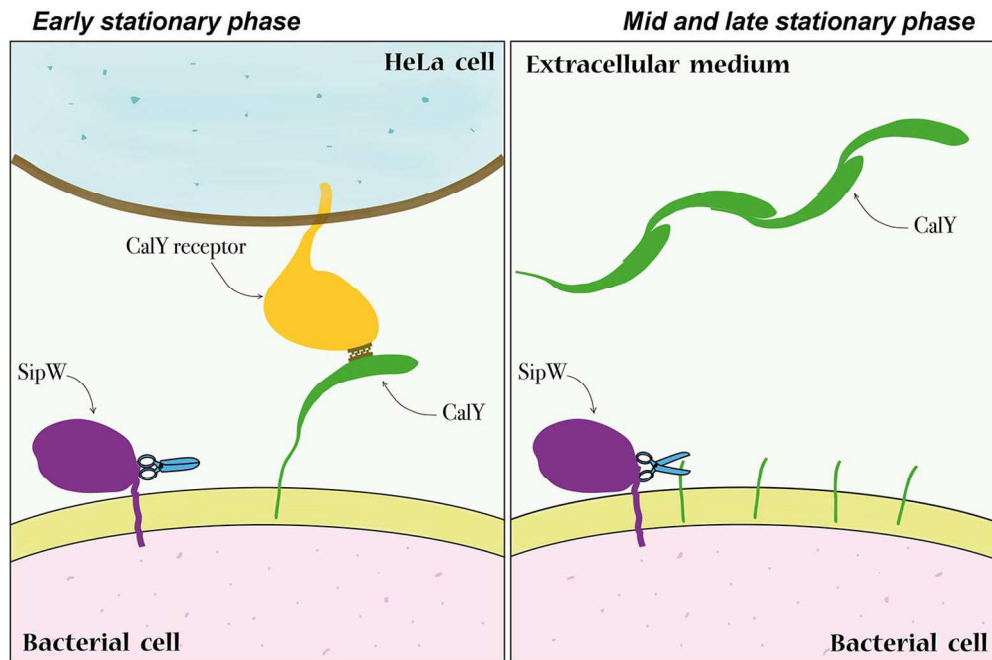
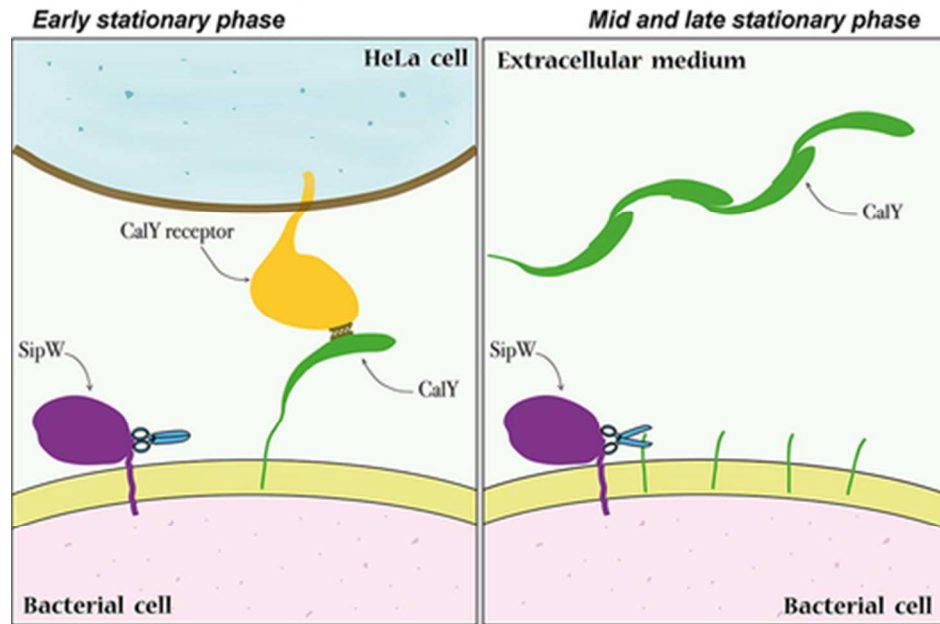


Figure 8: Schematic representation of CalY functions.

Left panel: in early stationary phase, in planktonic culture, both SipW and CalY are expressed and located on the cell-surface. SipW does not display its peptidase activity and CalY remains anchored in the cell membrane by its signal peptide. CalY works as an adhesin which binds to epithelial cells, and strongly contributes to the bacterial virulence.

Right panel: later in the stationary phase or in biofilms, SipW is activated by an unknown, hypothetical factor and cleaves CalY signal peptide. The free, extracellular CalY is activated by an undetermined, cell-surface activating factor (not shown here), and polymerizes to produce amyloid fibers promoting biofilm formation.

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CalY is a biofilm protein produced in high quantities. We found that in early stationary phase, this protein is located at the cell surface where it promotes the bacterium binding to epithelial cells. Later, in mid- or late-stationary phase, CalY is released in the extracellular medium by the signal peptidase SipW and polymerizes as fibers promoting biofilm formation.

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