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Abstract

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

Introduction

Bacillus thuringiensis, an insect pathogen genetically close to the human lethal pathogen *B. anthracis* and to the opportunistic food-borne pathogen *B. cereus*, produces biofilm pellicles floating on the culture medium (Auger *et al.*, 2006, Wijman *et al.*, 2007) or sticking to submerged solid surfaces (Houry *et al.*, 2012). The biofilm matrix of these species includes DNA, polysaccharides and proteins (Vilain *et al.*, 2009, Houry *et al.*, 2012). In the closely related species *Bacillus subtilis*, three proteins are required to build the biofilm. TasA forms amyloid fibers (Romero *et al.*, 2011), TapA is required for TasA polymerization and anchoring to cell surfaces (Romero *et al.*, 2014), and BslA coats the biofilm (Hobley *et al.*, 2013). The *B. subtilis tasA* and *tapA* genes are included in the *tapA-sipW-tasA* operon, in which *sipW* codes for a signal peptidase required for the secretion of TasA and TapA (Stover & Driks, 1999b, Stover & Driks, 1999a). Transcription of *tapA-sipW-tasA* is repressed by SinR and promoted by the anti-SinR protein SinI (Kearns *et al.*, 2005). In *B. thuringiensis*, *B. cereus* and *B. anthracis*, two orthologues of the *B. subtilis tasA* are found. One of these orthologues - also named *tasA* - is found just downstream of the signal peptidase gene *sipW*, in the SinR-regulated bicistronic operon *sipW-tasA* (Caro-Astorga *et al.*, 2015, Pflughoeft *et al.*, 2011, Fagerlund *et al.*, 2014). Located downstream from *sipW-tasA* is the second orthologue of *tasA* named *calY*. The *calY* gene is expressed from its own promoter and is also controlled by SinR (Caro-Astorga *et al.*, 2015, Pflughoeft *et al.*, 2011, Fagerlund *et al.*, 2014). In *B. cereus*, both CalY and TasA polymerize to form fibers in the biofilm matrix, although TasA was more efficient than CalY for fiber production (Caro-Astorga *et al.*, 2015). Consequently, in this species, the deletion of either *tasA* or *calY* led to a decrease in the biofilm biomass produced in microtiter plate assays (Caro-Astorga *et al.*, 2015).

CalY was at first called camelysin, a name which stands for ‘casein-cleaving membrane metalloproteinase’. CalY has indeed previously been described as a cell-surface, membrane-

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

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

Results

CalY is not a protease

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

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

The expression profile of the whole Bt407 genome was compared in 24 h biofilm cultures relatively to early stationary phase planktonic cultures by microarray analysis. Three hundred and five genes displayed a ratio of expression (biofilm/planktonic) greater than 2 (Table S1). By far, the most overexpressed gene in biofilms was *calY*, with an expression ratio of 36, whereas the *tasA* expression ratio was 7. However, *sipW-tasA* transcription starts at the onset of stationary phase, one hour earlier than *calY* transcription, and reaches high levels when *calY* transcription is still low at this time of the growth curve (Fig. 1), which can explain the higher expression ratio obtained for *calY*. Overall, 12 genes, putatively involved in biofilm formation, were found to be differentially expressed in biofilms. Six of these genes (*sipW*, *tasA*, BTB_c13240, *calY*, *sinI*, *sinR*) are located in the overexpressed *sipW-sinR* locus. The six other genes differentially

expressed in biofilm include 4 transcriptional or post-transcriptional regulators (*ai2K*, *abrB,* BTB_c16240 and the diguanylate cylase/phosphodiesterase BTB_c54300), and two genes involved in the biosynthesis and secretion of kurstakin - a lipopeptide shown to be required for biofilm formation (Dubois *et al.*, 2012, Fagerlund *et al.*, 2014, Gelis-Jeanvoine *et al.*, 2016).

CalY is a biofilm matrix component

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

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

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

CalY overexpression leads to fiber bundles

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

CalY is a cell-surface adhesin

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

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

SipW inactivation promotes the cell-surface location of CalY

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

CalY polymerization requires an activating factor

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

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

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

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

Discussion

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

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

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

by SipW to reach the extracellular medium. The transient presence of CalY at the cell surface cannot be a consequence of a delay in *sipW* transcription, because *sipW* transcription starts before *calY* transcription (Fig. 1). Alternatively, SipW activity could require the presence of a cofactor which production would start in mid-stationary phase. This hypothesis is supported by a previous report suggesting the existence of a SipW activator in *B. subtilis* (Tjalsma *et al.*, 2000).

The purified CalY used to raise antibodies did not polymerize spontaneously upon incubation. This result suggested that an activating factor, different from SipW (because the purified CalY was without signal peptide), was required for CalY polymerization. We investigated this possibility by mixing purified CalY with planktonic cultures fractions from the Bt407 Δ*calY* strain. We found that a cell-associated factor, most likely cell-surface bound, could promote fibers formation by the exogenous CalY. This activating factor was not present in cultures until mid-stationary phase, in agreement with the observation that planktonic cultures of the wild type strain produce CalY fibers only from this time of the stationary phase and later. The need of an activating factor for CalY polymerization is reminiscent of the requirement of TapA for TasA polymerisation in *B. subtilis* (Romero *et al.*, 2011). Yet, since there is no TapA homologue in *B. thuringiensis*, *B. cereus* or *B. anthracis*, the activation mechanism for CalY might be different from the activation of TasA by TapA in *B. subtilis*.

The presence of CalY at the cell-surface, and the fact that it can bind mucin or fibronectin (Sanchez *et al.*, 2009), suggested that this protein could interact with host tissues. Our data show that CalY is needed for bacterial adhesion to HeLa cells. To our knowledge, CalY is the first *B. thuringiensis* or *B. cereus* cell-surface adhesin shown to be required for adhesion to HeLa epithelial cells. While the *B. cereus* cell-wall peptidase CwpFM was also reported to impact adhesion on HeLa cells, this effect was likely to be a side-effect of the autolysin activity of this protein (Tran *et al.*, 2010). In *B. anthracis*, two cell-wall anchored proteins, displaying LPXTG motifs, were found to bind collagen (Xu *et al.*, 2004), and orthologues of their genes are found

on the chromosome of *B. cereus* and of *B. thuringiensis*. However, the role of these collagen-binding proteins on the adhesion of bacteria to host cells or to host tissues was not investigated. BslA is a SLH protein (harboring a S-Layer Homology domain), which gene is located on the pathogenicity island of the *B. anthracis* virulence plasmid pXO1, and is therefore not present in *B. cereus* or *B. thuringiensis*. BslA is required for adhesion of *B. anthracis* vegetative cells on BJ1 fibroblasts (Kern & Schneewind, 2008). In addition, *bslA* deletion resulted in a dramatic increase in the lethal dose in an anthrax disease guinea pig model (Kern & Schneewind, 2010).

Deletion of *calY* also resulted in a strong increase in the lethal dose of *B. thuringiensis* in the insect infection model *Galleria mellonella*. The dose required to induce 50% mortality upon deletion of *calY* had to be increased in the same order of magnitude, respectively 13 times and 12 times, when bacteria were injected into the insect blood (hemocel) and when bacteria were force-fed. By this last contamination route, CalY is so far the only known virulence factor, together with the metallopeptidase InhA2 (Fedhila *et al.*, 2003), which plays a major role in the bacterial pathogenesis on its own. CalY activity both *per os* and by injection possibly reflects the dual function of this protein, acting as a biofilm matrix protein during the colonization of the gut following oral infection, and as an adhesin interacting with hemocytes, fatbody cells or other tissues following injection. CalY is therefore a major virulence factor of *B. thuringiensis*, and since it is highly conserved in *B. cereus* and *B. anthracis* (Fig. S5), it is likely that this protein is also involved in the pathogenicity of these species.

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

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

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

nature of these activators will certainly be a very promising aspect of future work dedicated to the elucidation of the mechanisms involved in CalY shift from one function to the other.

Experimental Procedures

Bacterial strains, plasmids and growth conditions

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

Genetic constructions

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

To disrupt *calY*, a 2709bp DNA fragment containing *calY* was amplified by PCR with oligonucleotides cal1 and cal4. The amplified DNA fragment was inserted into pGemT-easy

giving rise to pCAL10. pCAL10 was digested with *Hpa*I, and a spectinomycin-resistance cassette was inserted giving pCAL20. The *Bam*HI fragment from pCAL20 was ligated into pAT113 giving pCAL30 (Trieu-Cuot *et al.*, 1993). This recombinant suicide plasmid was transferred from *E. coli* to Bt407 by heterogamic conjugation (Pezard *et al.*, 1991, Trieu-Cuot *et al.*, 1987) giving rise to 407*calY::spc*. The markerless mutant Bt 407Δ*sipW* was obtained with the recombinant suicide plasmid pMAD*sipW*. This plasmid was built by inserting the BamHI/EcoRI 5'- and EcoRI/NcoI 3'-regions of *sipW*, amplified by PCR using primers sipWAmF/SipWAmR or sipWAvF/sipWAvR, in pMAD (Arnaud *et al.*, 2004) digested by BamHI and NcoI, and was transferred in Bt407 by electroporation (Lereclus *et al.*, 1989). The *calY* and *sipW* deletions were checked by PCR and by sequencing. To complement 407*calY::spc* with *calY* the PCR fragment containing *calY* and its promoter was amplified with Pcal1/calYa and inserted into pHT304 (Arantes & Lereclus, 1991) digested by *Hin*dIII/*Bam*HI to give pCAL40. To complement 407Δ*sipW* with *sipW* the PCR fragment containing *sipW* and its promoter was amplified with sipWcF/ sipWcR and inserted into pHT304 (Arantes & Lereclus, 1991) digested by *Hin*dIII/*Bam*HI to give pHT304*sipW*.

To follow *calY* promoter expression, a fusion with this promoter and the *lacZ* reporter was constructed. The *calY* promoter was amplified using Pcal1 and Pcal2 and cloned into pHT304-18Z (Agaisse & Lereclus, 1994). Likewise, the *sipW* promoter was cloned into pHT304-18Z using PsipWF and PsipWR primers to follow *sipW* transcription. The resulting plasmids were transferred into Bt407 by electroporation. β-galactosidase specific activity was measured as described previously, and are expressed in units of β-galactosidase per milligram of protein (Perchat *et al.*, 2011). Each assay was carried out at least three times using independent cultures.

Peptidase assay

In order to analyze a potential peptidase activity of CalY, azocasein (A2765, Sigma-Aldrich Inc., USA) was used as substrate. Thermolysin (peptidase family M4) from *Bacillus thermoproteolyticus* (P1512, Sigma-Aldrich Inc., USA) and purified InhA2 (peptidase family M6) were used as positive controls. InhA2 was purified from the supernatant of Bt407-ΔplcR [pHT3015Apha3inhA] (Fedhila *et al.*, 2003), a strain overexpressing InhA2. The supernatant from an early stationary LB culture was recovered, centrifuged and 0.22µm-filtrated. All proteins were precipitated by 85% ammonium sulphate and resuspended in Bis-Tris HCl 25mM, pH7.2 CaCl2 4mM. After desalting, InhA2 was purified by ion exchange chromatography (MonoQ5/50GL and HPLC) and eluted with 0-15% NaCl as a single 85 kDa protein. Azocasein was used as a 1% suspension in buffer bis-Tris, HCl pH 7.2, 25 mM, $CaCl_2$ 4mM, $ZnCl_2$ 0,1mM. Thermolysin, InhA2 and CalY were assayed at 10µg/ml. The reaction was stopped by the dilution of a 200 µl sample in 1 ml TCA 10%. The tube was centrifuged to pellet the undigested substrate and the supernatant was mixed with 750 µl NaOH 1 M before measuring the OD_{440}. The assays were repeated three times. The OD_{440} was corrected for blank (without peptidase) values. The peptidase activity was expressed as (nmoles azocasein degraded) / (min x mg peptidase).

Microarray analysis

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

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

Biofilm assays

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

Antibody production and immunodetection

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

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

CalY polymerization

Planktonic cultures of the Bt407 Δ*calY* mutant strain were harvested in early- and mid-stationary phases, and 100µl of the culture was centrifuged for 2 minutes at 7500 rpm at room temperature. The sedimented bacteria, washed twice with PBS and resuspended in 100µl of PBS, and the cell-free supernatant were each incubated with rCalY at a final concentration of 1µg/ml for 20 minutes at room temperature, centrifuged for 2 minutes at 7500 rpm at room temperature, resuspended in PBS, and treated for CalY determination as described in 'Antibody production and immunodetection'. Controls (rCalY alone, or sedimented bacteria from a 407 Δ*calY* mutant strain planktonic culture harvested in mid-stationary phase and incubated without rCalY) were incubated and treated as described above.

Insect virulence assays

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

Adhesion assays

Adhesion assays were performed as described previously (Ramarao & Lereclus, 2006). Bacteria harvested in early stationary phase were incubated for 15 minutes at 37°C in 24-well polystyrene microtiterplates in wells containing confluent Hela cells (supplied by the American Type Culture Collection, reference ATCC CCL-2) ($5x10^5$ per well) or no HeLa cells. $5x10^6$ bacteria were loaded per well. Non-attached bacteria were then removed by washing three times with PBS, and HeLa cells were detached by scraping. Serial dilutions were plated on LB plates to score adherent bacteria *versus* total bacteria. Three replications were performed using three

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

Acknowledgments

We are grateful to Agnès Fouet for pUC1318spc and to Patrick Trieu-Cuot for pAT113. We thank Sebastien Gelis-Jeanvoine for his help in genomic data handling. Thomas Candela was funded by the region Ile de France under the program DIM Astrea.

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599 characterization of two novel collagen binding MSCRAMMs of *Bacillus anthracis*. *J*
600 *Biol Chem* **279**: 51760-51768.
601

Tables

Protease :	Thermolysin M21663	InhA2 BTB_c06870	CalY BTB_c13250
replicates	3	3	3
mean	139	99	0.7
sem	25	11	0.3

Table 1: Metallopeptidase activity of CalY

The proteolytic activity of CalY was determined using azocasein as a substrate. Pure thermolysin and the metallopeptidase InhA2 were used as positive controls. InhA2 is produced by *B. thuringiensis* in stationary phase. Metallopeptidase activity is expressed in nmoles min^{-1} mg^{-1}. sem : standard error on the mean.

primer	sequence	use
calYs	GGATCCattggtggaggaacatttgcattctttagc	CalY overproduction
calYa	AAGCTTttatttttcttccccagcttcttggttagc	CalY overproduction & *calY* complementation
cal1	cgcGGATCCccgaaaacagttaatacgttaaaag	*calY* deletion
cal4	catgCCATGGgcgcatctgctaaacgttcttccgg	*calY* deletion
SipWAmF	cgGGATCCgaagcaattaggggcgaaagatag	*sipW* deletion
SipWAmR	gGAATTCgtctctctccctctccgttg	*sipW* deletion
SipWAvF	gGAATTCttcgtttggttatactttccgtttag	*sipW* deletion
SipWAvR	CATGCCATGGcgttccatactcacgctcaataaac	*sipW* deletion
sipWcF	gGAATTCgttacgccgtaatacaaaaggg	*sipW* complementation
sipWcR	aaCTGCAGctaaacggaaagtataaccaaacga	*sipW* complementation
Pcal1	cccAAGCTTcggaaggacaaaagaaagtagaag	P*calY-lacZ* fusion & *calY* complementation
Pcal2	tgcTCTAGAcacaatcaattccccctagc	P*calY-lacZ* fusion
PsipWF	aaCTGCAGgttacgccgtaatacaaaagg	P*sipW-lacZ* fusion
PsipWR	gcTCTAGAtcaccaccgctcgctttt	P*sipW-lacZ* fusion

Table 3: Primers used in this study

Upper-case letters show restriction sites

Figure captions

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 t_2 (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 t_0 which is the transition between the exponential phase and the stationary phase.

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 ($calY_c$), and the wild type strain overexpressing *calY* (wt_c). 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; $calY_c$: complemented *calY* mutant strain.

Figure 3: CalY location in biofilm.

The presence of CalY 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; *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).

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 ($calY_c$). 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).

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).

Figure 6: Polymerization of CalY.

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. rCalY was incubated with the cell-free supernatant (SNY) or with the washed bacteria resuspended in PBS (CY). Controls were rCalY incubated alone (Y), or washed bacteria incubated whitout rCalY (C). CalY fibers were revealed with a rabbit anti-CalY antibody and a goat anti-rabbit antibody tagged with Alexa488 (green). Phase: phase contrast.

Figure 7: CalY 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; $calY_c$: 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.

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 unkown, 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.

Supporting information

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

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

Fig. S2: CalY Western blot.

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

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

The *sipW-tasA* region from the wild type strain is shown in the mid-panel of the figure (shaded in yellow). This region was sequenced in the *calY* mutant strain, from the end of the BTB_c13210 gene to

the beginning of *inhA1* (4428 bp), using 5 PCR fragments amplified from both the direct and the reverse strands. The 10 generated fragments were aligned on the wild type strain sequence (available at https://www.ncbi.nlm.nih.gov/nucleotide/409171506) using the LASTZ tool of the Geneious software. In the lower panel, the black color shows the region covered by the assembled sequences. In the upper panel, the green color shows 100% identity between the amplified sequences and the wild type strain sequence. Mismatches were found only in *calY*, which was interrupted by a spectinomycin resistance cassette.

Fig. S4: Polymerisation of rCalY.

Examples of CalYr polymerisation in the presence of the cell fraction of different cultures of the 407 Δ*calY* strain collected in mid-stationary phase. Phase: phase contrast. Alexa488: immunodetection of CalY. Overlay: overlay of the phase and the immunodetection pictures.

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

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

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

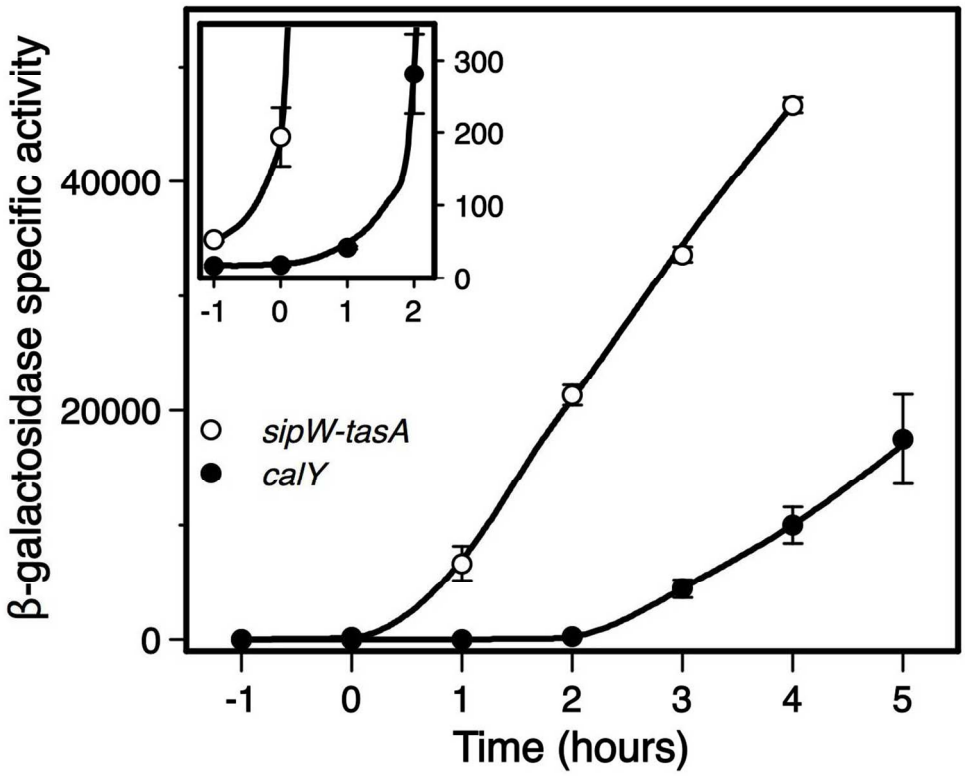

Figure 1: calY and sipW-tasA transcriptions.
Transcription of calY (white circles) or of sipW-tasA (black circles) was monitored in planktonic cultures in LB medium through the lacZ gene reporter. The inset shows a focus on the transition phase between t-1 (OD 1) and t2 (OD 7). Each circle is the mean 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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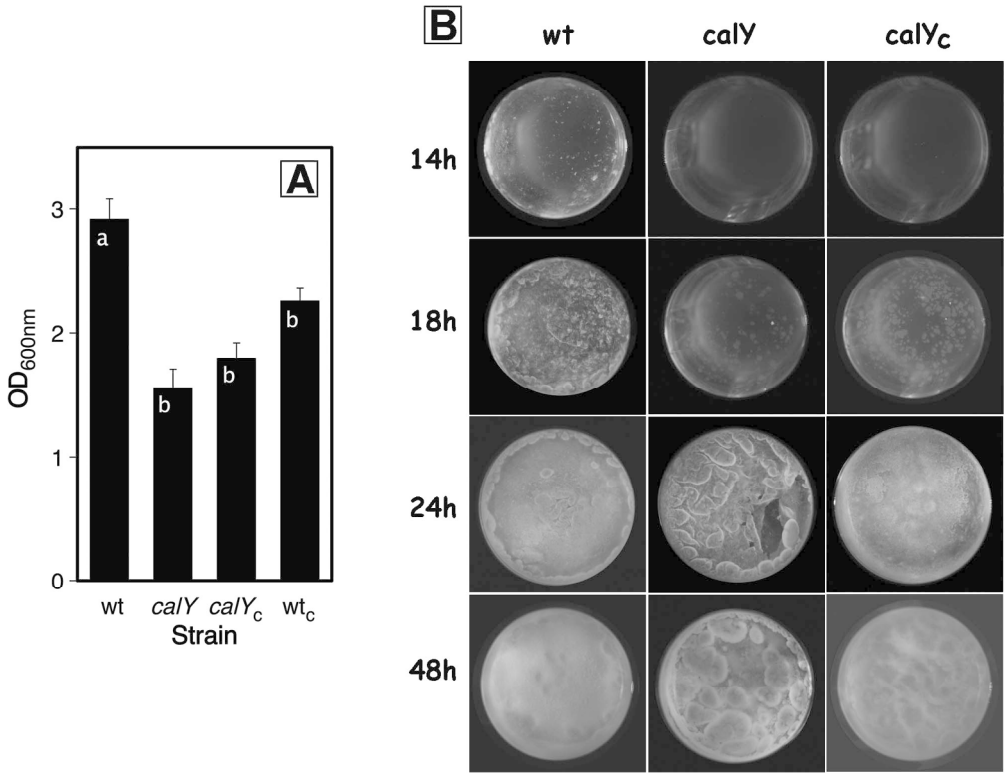

Figure 2: Role of CalY in biofilm formation.
A: the biomass of biofilms grown in glass tubes in HCT medium was determined for the wild-type strain (wt), the calY mutant strain (calY), the complemented calY mutant strain (calYc), and the wild type strain overexpressing calY (wtc). Each bar is the mean 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; calYc: complemented calY mutant strain.

170x134mm (300 x 300 DPI)

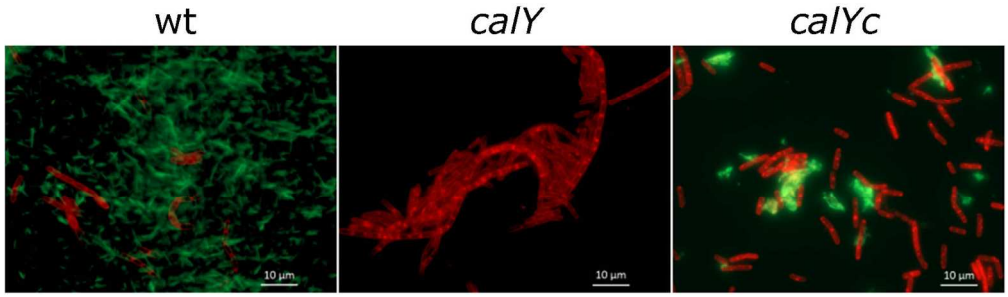

Figure 3: CalY location in biofilm.
The presence of CalY in 48h-aged biofilms was determined using an immunodetection method and fluorescence microscopy. Biofilms were grown in glass tubes in HCT medium. wt, wild-type strain; calY: calY mutant strain; calYc: complemented calY mutant strain. Anti-CalY antibody was revealed by a secondary antibody labelled with Alexa488 (green). Bacterial membranes were stained using FM4-64 (red).

150x43mm (300 x 300 DPI)

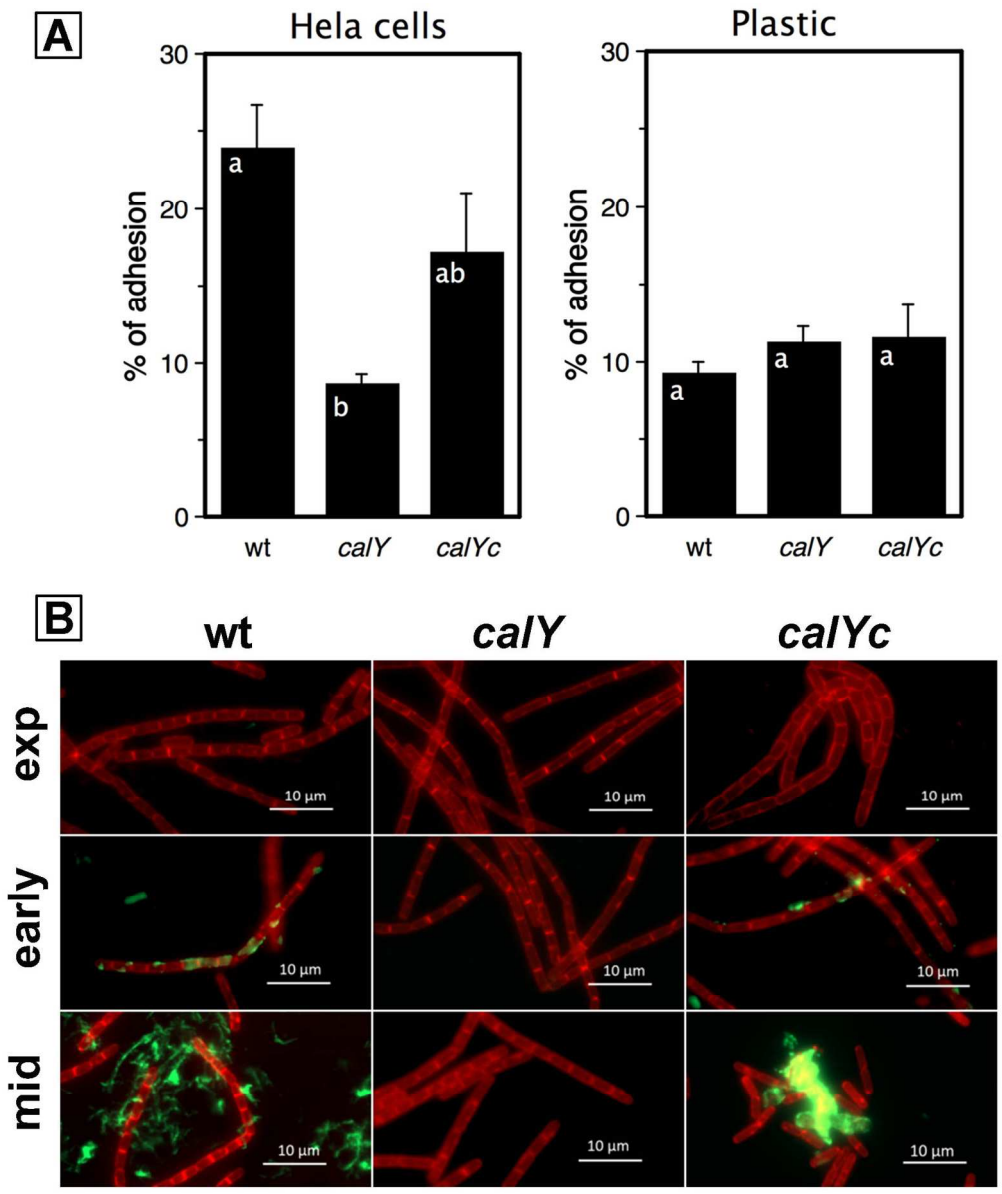

Figure 4: CalY role in adhesion to epithelial HeLa cells.
A: adhesion to epithelial HeLa cells grown in 24-wells polystyrene microtiterplates (left), or to microtiterplates alone (right), was determined for the wild-type strain (wt), the calY mutant strain (calY), or the complemented calY mutant strain (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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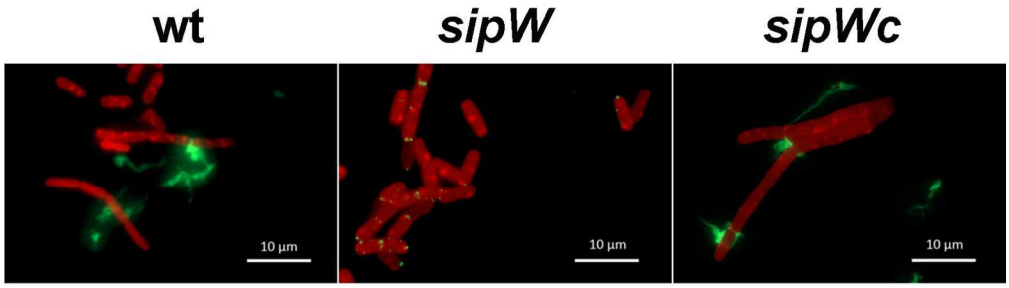


Figure 5: Role of SipW in CalY subcellular location.
The role of SipW on the subcellular location of CalY was determined using an immunodetection method and fluorescence microscopy. Planktonic cultures were grown in LB medium and harvested in mid-stationary phase. wt, wild-type strain; calY: calY mutant 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).

150x43mm (300 x 300 DPI)

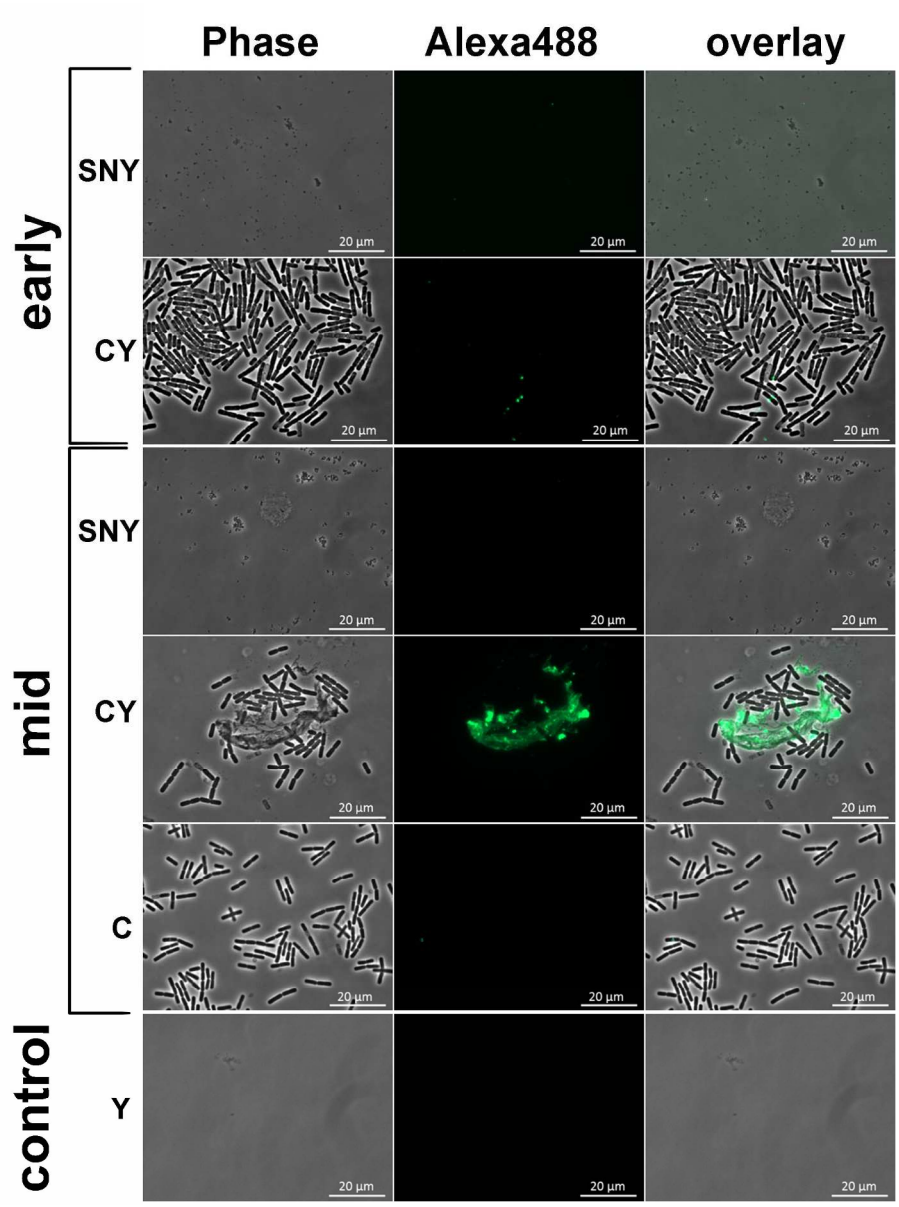


Figure 6: Polymerization of CalY.
Cell-free supernatant (S) or pelleted and PBS-washed bacteria (C) were prepared from planktonic cultures grown in LB medium and harvested in mid-stationary phase. rCalY was incubated with the cell-free supernatant (SNY) or with the washed bacteria resuspended in PBS (CY). Controls were rCalY incubated alone (Y), or washed bacteria incubated whitout rCalY (C). CalY fibers were revealed with a rabbit anti-CalY antibody and a goat anti-rabbit antibody tagged with Alexa488 (green). Phase: phase contrast.

178x239mm (300 x 300 DPI)

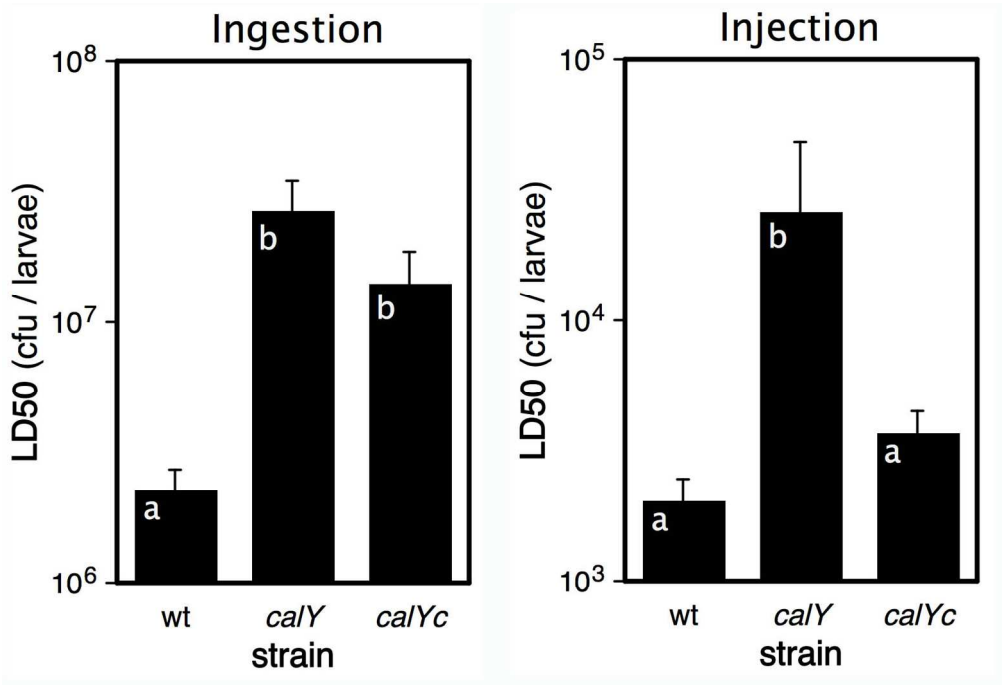


Figure 7: CalY role in the bacterium virulence.
Different concentrations of vegetative cells were injected or force-fed to larvae of the lepidopteran species Galleria mellonella. Mortality was recorded 48h post-treatment, and LD50s values were computed. Bars show LD50s values, and error bars represent 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.

144x98mm (300 x 300 DPI)

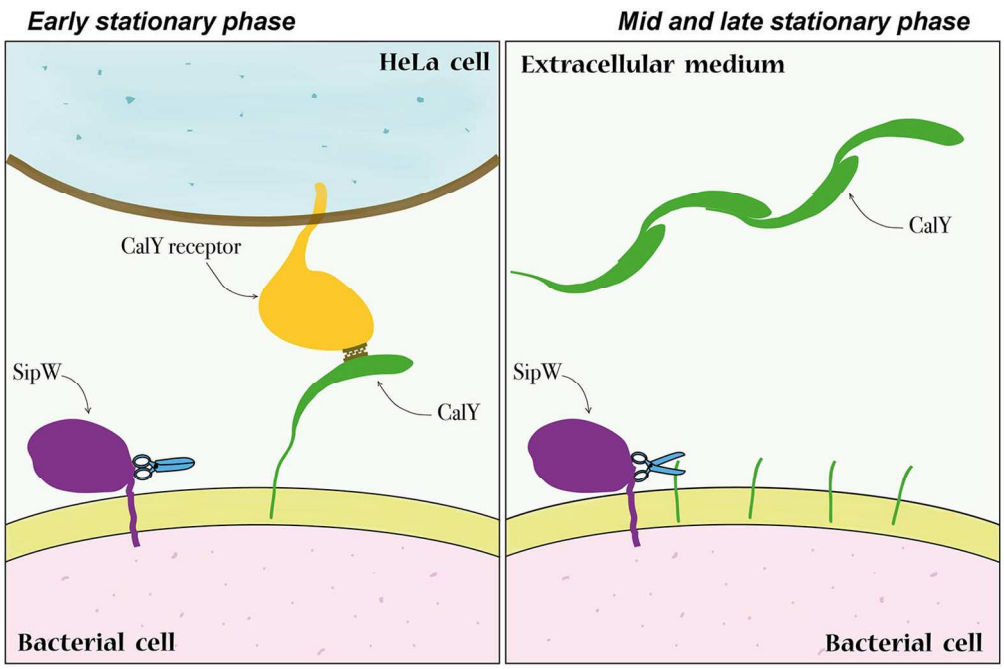
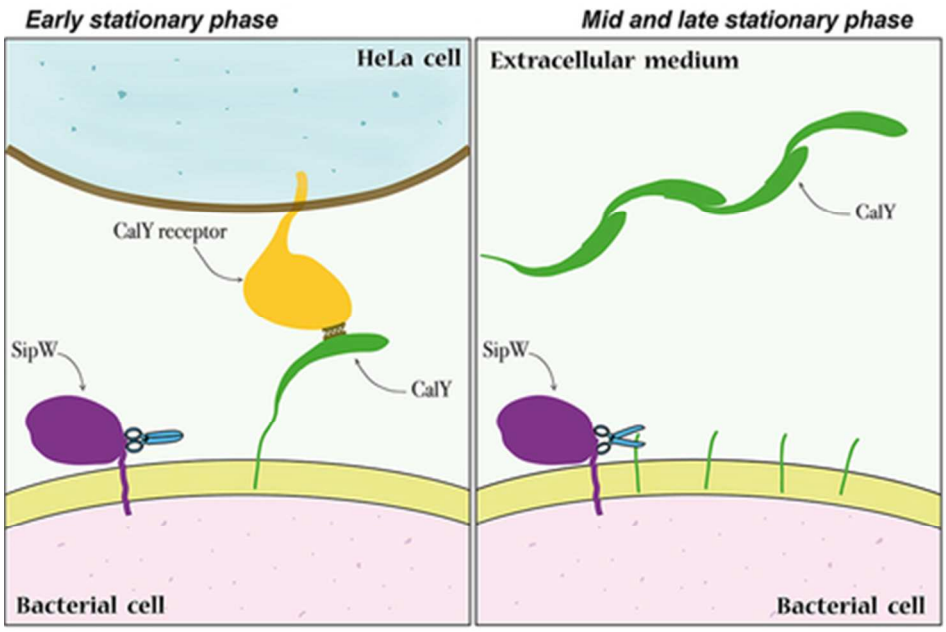


Figure 8: Schematic representation of CalY functions.
Left panel: in early stationary phase, in planktonic culture, both SipW and CalY are expressed and located on the cell-surface. SipW does not display its peptidase activity and CalY remains anchored in the cell membrane by its signal peptide. CalY 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 unkown, 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.

129x86mm (300 x 300 DPI)



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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