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To cite this version:
Onya Opota, Nils C. Gauthier, Anne Doye, Colin Berry, Pierre Gounon, et al.. Bacillus sphaericus binary toxin elicits host cell autophagy as a response to intoxication. PLoS ONE, Public Library of Science, 2011, 6 (2), pp.e14682. 10.1371/journal.pone.0014682. hal-02646249

HAL Id: hal-02646249
https://hal.inrae.fr/hal-02646249
Submitted on 29 May 2020

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**Bacillus sphaericus** Binary Toxin Elicits Host Cell Autophagy as a Response to Intoxication

Onya Opota¹a,²*, Nils C. Gauthier²a,²b, Anne Doye², Colin Berry³, Pierre Gounon⁴, Emmanuel Lemichez², David Pauron¹*

1 Institut National de la Recherche Agronomique, UMR Interactions Biotiques et Santé Végétale, INRA 1301-CNRS 6243-Université de Nice Sophia Antipolis, Sophia Antipolis, France, 2 INSERM, U895, UNSA, Centre Méditerranéen de Médecine Moléculaire, C3M, Toxines Microbiennes dans la relation hôte pathogènes, Nice, France, 3 Cardiff School of Biosciences, Cardiff University, Cardiff, United Kingdom, 4 Centre Commun de Microscopie Electronique Appliquée, Faculté des Sciences, Université de Nice Sophia Antipolis, Nice, France

**Abstract**

*Bacillus sphaericus* strains that produce the binary toxin (Bin) are highly toxic to *Culex* and *Anopheles* mosquitoes, and have been used since the late 1980s as a biopesticide for the control of these vectors of infectious disease agents. The Bin toxin produced by these strains targets mosquito larval midgut epithelial cells where it binds to Cpm1 (*Culex pipiens* maltase 1) a digestive enzyme, and causes severe intracellular damage, including a dramatic cytoplasmic vacuolation. The intoxication of mammalian epithelial MDCK cells engineered to express Cpm1 mimics the cytopathologies observed in mosquito enterocytes following Bin ingestion: pore formation and vacuolation. In this study we demonstrate that Bin-induced vacuolation is a transient phenomenon that affects autolysosomes. In addition, we show that this vacuolation is associated with induction of autophagy in intoxicated cells. Furthermore, we report that after internalization, Bin reaches the recycling endosomes but is not localized either within the vacuolating autolysosomes or within any other degradative compartment. Our observations reveal that Bin elicits autophagy as the cell’s response to intoxication while protecting itself from degradation through trafficking towards the recycling pathways.

Citation: Opota O, Gauthier NC, Doye A, Berry C, Gounon P, et al. (2011) *Bacillus sphaericus* Binary Toxin Elicits Host Cell Autophagy as a Response to Intoxication. PLoS ONE 6(2): e14682. doi:10.1371/journal.pone.0014682

Editor: Diane Bashham, Iowa State University, United States of America

Received: February 21, 2010; Accepted: January 14, 2011; Published: February 14, 2011

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Funding: The work depicted in this paper has been granted by two French governmental agencies, the Institut National de la Recherche Agronomique (Inra) and the Institut National de la Santé et de la Recherche Médicale (INSERM). Moreover, this work represents a substantial part of Dr. Onya Opota’s PhD for which he received a fellowship from the French Ministère de la Education et de la Recherche. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: onya.opota@epfl.ch (OO); David.Pauron@sophia.inra.fr (DP)

Current address: Global Health Institute, School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

Current address: Department of Biological Sciences, Columbia University, New York, New York, United States of America

**Introduction**

*Bacillus sphaericus* is one of the few biopesticides available for controlling *Culex* and *Anopheles* mosquitoes, which are vectors of human diseases such as West Nile fever and malaria [1,2]. Bacillus sphaericus can produce different types of insecticidal proteins: the mosquitocidal toxins (Mtx) produced during vegetative growth and *Bacillus sphaericus* is one of the few biopesticides available for controlling *Culex* and *Anopheles* mosquitoes, which are vectors of human diseases such as West Nile fever and malaria [1,2]. Bacillus sphaericus can produce different types of insecticidal proteins: the mosquitocidal toxins (Mtx) produced during vegetative growth and the binary toxin (Bin) produced during sporulation. Bin-producing strains are by far the most toxic and kill mosquito larvae within twenty-four to forty-eight hours [3]. Bin is synthesized in a strain that produce the binary toxin (Bin) are highly toxic to *Culex* and *Anopheles* mosquitoes, and have been used since the late 1980s as a biopesticide for the control of these vectors of infectious disease agents. The Bin toxin produced by these strains targets mosquito larval midgut epithelial cells where it binds to Cpm1 (*Culex pipiens* maltase 1) a digestive enzyme, and causes severe intracellular damage, including a dramatic cytoplasmic vacuolation. The intoxication of mammalian epithelial MDCK cells engineered to express Cpm1 mimics the cytopathologies observed in mosquito enterocytes following Bin ingestion: pore formation and vacuolation. In this study we demonstrate that Bin-induced vacuolation is a transient phenomenon that affects autolysosomes. In addition, we show that this vacuolation is associated with induction of autophagy in intoxicated cells. Furthermore, we report that after internalization, Bin reaches the recycling endosomes but is not localized either within the vacuolating autolysosomes or within any other degradative compartment. Our observations reveal that Bin elicits autophagy as the cell’s response to intoxication while protecting itself from degradation through trafficking towards the recycling pathways.

Electrophysiological analyses performed on cultered *Culex quinquefasciatus* cells and on large unilamellar phospholipids vesicles (LUVs) have shown the ability of Bin to induce channel formation [10,11]. By expressing Cpm1 in the mammalian epithelial cell line MDCK (Malden and Darby canine kidney) we have recently demonstrated the contribution of the Bin receptor Cpm1 to the formation of pores. While Bin had no effect on untransfected MDCK cells, it induced the formation of cationic channels in MDCK-Cpm1 [12]. When expressed in MDCK cells, Cpm1 fully retained its biochemical and functional characteristics such as GPI-anchoring to the apical side of polarized cells, enzymatic activity and high binding affinity to Bin. Moreover, we showed that Cpm1 is concentrated in lipid raft microdomains which may facilitate the oligomerization of the toxin/receptor complex and contribute to the pore formation process [12]. The synthesis of pore forming toxins is a strategy widely used by pathogenic bacteria to trigger their virulence [13]. Nevertheless, pore-
forming toxins display numerous modes of action ranging from the formation of lytic pores in the plasma membrane to the translocation of components displaying enzymatic activities or able to interfere with intracellular signalling pathways [9,14]. It has previously been shown that the lethal effect of Bin is not associated with epithelial cell lysis or epithelial disruption and, to date, the mechanism by which Bin kills mosquito larvae remains unsolved [3,15]. The midgut epithelial cells of mosquito larvae intoxicated with Bin display several cytopathologies affecting the microvilli, the mitochondria and the rough endoplasmic reticulum but the most dramatic feature of Bin intoxication is the appearance of abnormal, electron-clear vacuoles indicating an important cellular stress [15,16,17]. Remarkably, Bin-induced cytoplasmic vacuolation described in Culex mosquito midgut epithelial cells, was also found in Bin treated MDCK-Cpm1 cells [12]. In the present study, we take advantage of the MDCK-Cpm1 cell line to perform the first study investigating the Bin cellular mode of action. We demonstrate for the first time that Bin promotes the appearance of large vacuoles that present features of autolysosomes. Strikingly, we found that while this vacuolation is partially reversed over time, it continues to affect a significant subset of the cell population. A careful time-lapse videomicroscopic analysis reveals that in fact, dividing cells were preferentially affected by the vacuolation in an unreported phenomenon that we have called post-mitotic vacuolation. Furthermore, we found that induction of autophagy was elicited by Bin in intoxicated cells and that stimulation of autophagy prior to intoxication inhibited autolysosome vacuolation. Our findings suggest that the vacuolation concerned the initiation of the autophagy process. Furthermore, the study of the intracellular trafficking of fluorescent Bin derivatives revealed that after endocytosis, the toxin reached recycling endosomes but was not routed toward autophagosomes, autolysosomes or lysosomes. In addition, Bin trafficking reveals how a bacterial toxin can conserve its toxic potential by avoiding the target cell degradative pathway it elicits.

**Results**

**Bin induces the vacuolation of a compartment that possesses autolysosomal characteristics**

To determine the origin of the vacuolating structures induced by Bin in MDCK-Cpm1 cells we expressed organelle-specific markers fused with fluorescent proteins. While Bin-induced vacuoles could be distinguished with the acidotropic marker LysoTracker, we found that the early endosome marker Rab5 and the recycling endosome marker Rab4 were excluded from these vacuoles (Figs. 1A and B, Fig. S3 and video S6). In contrast, the late endosome marker Rab7 and the lysosome marker Lamp1 (Lysosome-associated membrane protein 1) were clearly associated with the membrane of the LysoTracker-positive vacuoles (Figs. 1C and D, Fig. S3 and video S5). Markers of the Golgi apparatus, the endoplasmic reticulum and the mitochondria were not found associated with the giant vacuoles decorated with Lamp1 and stained with LysoTracker (Figs. 1E to 1G). We also examined the distribution of the cytoskeleton network in vacuolated cells. Treating cells with Bin did not affect the microtubule architecture or the actin stress fiber network. Nevertheless, when examining cells under various focal planes we found actin filaments surrounding the membrane of Bin-induced vacuoles (Fig. S1).

Because of the convergence between the endocytic and the autophagic pathways [18,19], we investigated the possibility that Bin-induced vacuoles displayed both autophagic and late endocytic/lysosomal features. The microtubule-associated protein light chain 3 (LC3), is a mammalian homologue of Atg8 which is a constituent of the autophagic vesicles in yeast [20,21]. When autophagy is induced, LC3 is processed from a cytosolic form, LC3-I, to the LC3-II form that is associated with the autophagic vesicle membrane: elongating membranes and autolysosomes. In autophagic cells expressing GFP-LC3, these structures can be detected by fluorescence microscopy as dots (also named GFP-LC3 positive cells), while in non-autophagic cells GFP-LC3 presents a diffuse cytosolic pattern [20,22] (Fig 2A). While we could find Bin treated cells presenting an autophagic pattern, we could not detect any GFP-LC3 signal that could be related to an association with the membrane of the giant vacuoles (Fig 2B).

In order to initiate the autophagic catabolic step, autophagosomes fuse with lysosomes to generate autolysosomes where degradation ultimately occurs. Notably most of the GFP-LC3 is degraded, and its signal is lost. Adding NH4Cl to the medium reduces the luminal acidity of the vesicles so that the degradation of the GFP-LC3 marker is prevented and the detection of autolysosomes is increased [23,24,25]. A similar treatment allowed us to detect a GFP-LC3 signal associated with the membrane of Bin-induced vacuoles and inside these vacuoles (Fig. 2B). As a control, cells treated with NH4Cl alone did not display any vacuolation (Fig 2B). At the ultrastructural level, Bin-induced giant vacuoles looked like homogeneous electron-clear matrices limited by a single membrane characteristic of autolysosomes, in contrast to autophagosomes, which are enclosed by a double membrane (Fig. 2C). In addition, these vacuoles often contained partially degraded material and vesicular like structures that can be related to autophagic bodies [26] (Fig. 2C). Finally, neither homogeneous cytosol containing a low number of organelles nor nuclear fragmentation, hallmark signs of apoptosis or necrosis could be observed. Taken together these results support the hypothesis that Bin-induced vacuoles originate from an autolysosomal compartment.

**Bin-induced vacuoles form transiently**

In order to determine the dynamics of Bin-induced vacuolation of MDCK-Cpm1 cells we proceeded to time-lapse video microscopy. Vacuoles appeared within the first hour of intoxication in a subset of the cell population and rapidly increased in a time-dependent manner, finally affecting more than 50% of the cell population (Fig 3A and Video S1). Strikingly, this vacuolation was transient, as the number of vacuolated cells started to decrease after 6 h of intoxication. Interestingly, at late time points of intoxication the vacuolation still affected about 25% of the cell population (Fig 3A). By looking at individual cells we found that vacuolation could be reversed within a few hours. However, as shown in video S1 and S2 and figure 3B, we observed that the vacuolation reappeared in dividing cells and was dramatic after cytokinesis. We named this newly described phenomenon post-mitotic vacuolation. In fact, the number of cells vacuolating after mitosis increased as a function of time, and 24 h after intoxication almost all of the vacuoles were detected in post mitotic cells (Fig. 3A). These results suggest that Bin-induced vacuolation is a transitory phenomenon that is recurrent in newly-divided cells.

**Induction of autophagy contributes to reversion of Bin-induced vacuolation**

In Bin-treated MDCK-Cpm1 cells we could detect many autophagosomes that were either isolated or initiating fusion with the enlarged autolysosomes induced by Bin, which raised the hypothesis of an autophagy induction. A quantitative analysis confirmed that the number of autophagosomes was significantly higher in Bin-treated cells than in untreated cells grown in complete medium (Fig. 4A). Autophagy can also be monitored by scoring the fraction of GFP-LC3 positive cells [20,27]. Under starvation, the percentage of GFP-LC3 positive cells is dramatically enhanced as a result of the induction of autophagy (Fig. 4B).
In Bin treated cells, the percentage of GFP-LC3 positive cells was similarly enhanced (Fig. 4B). The increase in both the number of autophagosomes and the number of GFP-LC3 positive cells strongly suggested that autophagy is induced in MDCK-Cpm1 cells exposed to Bin. We next probed the effect of the induction of autophagy before Bin treatment. Figure 4C shows that when Bin was added to cells cultured in nutrient-free medium, vacuolation was inhibited. However, the percentage of vacuolating cells increased in a time-dependent manner and ultimately affected there again a subset of the cell population as in figure 4C. Videomicroscopy analysis indicated that this late vacuolation induced by Bin in nutrient-free medium (Fig. 4D and Video S3), was almost exclusively the post-mitotic phenomenon described in cells intoxicated in complete medium (Fig. 3B and Video S1). These results demonstrate that induction of autophagy prior to intoxication protects cells from vacuolation but does not inhibit the post-mitotic vacuolation. Because autophagy has been shown to be inhibited in dividing cells we hypothesised that this could explain the recurrence of post-mitotic vacuolation [28]. To challenge this hypothesis MDCK-Cpm1 cells were cultured in complete medium with Bin such that, after 3 h, approximately 50% of the cells were vacuolated (Figs. 4E and F). When the toxin was removed by washing with complete medium, the number of vacuolated cells continued to increase until it reached a maximum about 2 h after washing. Thereafter the vacuolation slowly decreased and was completely absent by 48 h after washing (unpublished data). In contrast, when the cells were washed with a nutrient-free medium, the vacuoles disappeared within 2 h (Figs. 4E and F). In order to confirm that this reversion was due to starvation alone, cells were grown again in toxin-free complete medium and the vacuolation rapidly reappeared (Figs. 4E and F). These results demonstrate that up-regulation of autophagy inhibited Bin-induced autolysosomal enlargement and in contrast, the consequence of a down-regulation of autophagy, similar to that which occurs in dividing cells, is the reappearance of the vacuolation.

Bin is internalized, but is not associated with the giant autolysosomes

We next looked for Bin internalization in MDCK-Cpm1 cells by directly labelling BinA with Alexa 488 (Al488) and BinB with Alexa 543 (Al543). After 10 min of intoxication we could detect...
**Figure 2. Bin-induced vacuoles originate from autolysosomes.** (A) MDCK-Cpm1 cells transfected with GFP-LC3 and grown in complete medium (CM) or in nutrient-free medium (EBSS). Diffuse GFP-LC3 pattern seen in CM-grown cells is representative of non-autophagic cells. In contrast, GFP-LC3 punctated pattern seen in EBSS-grown cells is characteristic of autophagic cells. (B) Localization of the autophagic marker GFP-LC3 in cells treated with Bin for 6 h. To allow the detection of GFP-LC3 associated with autolysosomes NH4Cl (5 mM, final concentration) was added to the culture medium during the last hour of intoxication. A similar treatment with NH4Cl in the absence of toxin had no toxic effect on the cells and did not induce any change in GFP-LC3 pattern. (C) Bin-treated cells were processed for EM analysis as described in Experimental procedures. Vacuoles induced by Bin are electron-clear matrices enclosed by a single membrane (black arrows). In contrast, autophagosomes are double membrane vesicles (white arrows). Bin-induced vacuoles often contained vesicle-like structure (arrow-head) and partially degraded material (large black arrows). Bars, 5 μm (A and B); 2 μm (C).

doi:10.1371/journal.pone.0014682.g002
BinB-Al543, as revealed by the colocalization of the two
proteins (GPI-APs) [29,30]. At 10 minutes post-infection BinB-
valuable marker of compartments enriched in GPI-anchored
receptor Cpm1 (Fig. 5B). After 30 min of intoxication,
the intracellular compartment reached by the
vaccuolation phenomenon (Fig. 7A). As shown in figure 7B,
and consistent with figure 6A, recycling endosomes stained with
GFP-Rab4 en route for partitioning into the daughter cells
were located in the same area [31,32]. Using time lapse microscopy,
we confirmed that while cells progressed through mitosis, the
internalized toxin was partitioned to the daughter cells and, when
post-mitotic vacuolation appeared, the toxin was not associated at
any time with the vacuoles but remained clustered in the free space
(Fig. 7C; Video S4) as reported above (Fig. 5C). Taken together,
these results established that the toxin is associated with recycling
endosomes but not with the degradative compartments.

**Discussion**

The molecular mechanism by which *B. sphaericus* binary toxin
Bin kills mosquito larvae, particularly from the genera *Culex*
and *Anopheles* is still undefined [3]. To date, the nature and the
sequence of the cellular events leading to larval death remain
unknown, cytoplasmic vacuolation being one of the major
cytotoxic responses of *Culex* mosquitoes to Bin intoxication
[15,16]. In the present study, by using a system of MDCK cells
expressing Bin receptor Cpm1 we show that Bin-induced giant
vacuoles originate from autolysosomes, the final and degradative
compartment of the autophagic pathway that result from the
fusion of autophagosomes with lysosomes. This vacuolisation was
not followed by cell death even at higher toxin concentrations. In
Bin-treated cells, neither the distribution of the microtubule
network nor the actin cytoskeleton that can induce the
accumulation of very large autophagic vacuoles [33] was affected.
Nevertheless, the detection of actin filaments surrounding the
vacuolating autolysosomes suggests that the actin cytoskeleton may
play a role in the invagination of the isolation membrane. Because
the size of autolysosomes is maintained by the equilibrium
between loading of autophagosomal content and the degradation
process, one hypothesis was that Bin impaired this balance.
Nevertheless, the material contained in the giant autolysosomes
was partially degraded, indicating that the catabolic activity of
autolysosomes remained effective. This does not exclude a delay
between the generation of autolysosomes and the degradation

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**Figure 3. Dynamics of Bin-induced vacuolation.** (A) Bin-treated
for 24 h) subconfluent MDCK-Cpm1 cells were submitted to time-lapse
phase contrast video microscopy, as exemplified in video S1. At the
indicated period of time the percentage of MDCK-Cpm1 cells displaying
at least one vacuole was determined. Mean values ± SD, n = 3. In the
same videos, the percentage of cells that present post-mitotic vacuolation
among the cells that display at least one vacuole, as illustrated in (B) was determined. Mean values ± SD, n = 3. (B) Bin-
treated cells displaying the post-mitotic vacuolation phenomenon,
images selected from video S2. The selection starts 15h after
intoxication, intervals in hours. Black and white arrows pinpoint two
dividing cells. Bars, 10 μm.
doi:10.1371/journal.pone.0014682.g003
Figure 4. Autophagy is enhanced in Bin treated cells and contributes to vacuolation reversion. (A) MDCK-Cpm1 cells were grown for 6 h in complete medium in the absence (CM) or the presence of Bin (CM + Bin), or in nutrient-free medium in the absence of the toxin (EBSS), and then processed for EM analysis. Pictures from randomly chosen EM sections were analyzed for the presence of autophagosomes as defined in figure 2. The results are expressed as the number of autophagosomes per cell. Mean values ± SD, n = 3. Different letters indicate statistically significant differences; P < 0.05. (B) MDCK-Cpm1 cells expressing GFP-LC3 were grown under the conditions described above. At the indicated time, the number of GFP-LC3 positive cells was scored. Mean values ± SD, n = 3. Different letters indicate statistically significant differences; P < 0.05. (C) Cells were grown in complete medium or in nutrient-free medium in the presence of Bin toxin. At the indicated period of time, the percentage of vacuolating cells was assessed. Mean values ± SD, n = 3. Different symbols indicate statistically significant differences; P < 0.05.
process in this compartment that has been shown to generate abnormal vacuoles [25]. Interestingly, we have shown that Bin-induced vacuolation is a transient phenomenon, which after complete reversion in most of the cell remains recurrent in dividing cells in a previously undescribed phenomenon that we named post-mitotic vacuolation. Autophagy induction by starvation protected cells from vacuolation, but strikingly even under this condition the post-mitotic vacuolation was observed. During cell division and even in the absence of nutrient, autophagy is down-regulated with the aim of protecting chromosomes in the absence of the nuclear membrane [28]. Our observations suggest that the interruption of inhibition by autophagy that occurs when cells divide is responsible of the recurrence of the post-mitotic vacuolation.

Autophagy plays an efficient role in cellular defense against bacterial invasion [34,35,36]. Nevertheless, several invasive bacteria have developed strategies to escape autophagy degradation either through modification of autophagosome compartments, as with *Legionella pneumophila*, or by subverting autophagy, as with *Shigella flexneri* in MDCK cells [37,38,39]. Recently, the non-invasive bacteria *Vibrio cholerae, Helicobacter pylori* and *Bacillus anthracis* have been shown to modulate autophagy through the secretion of the pore forming toxins VCC, VacA and Anthrax respectively [40,41,42,43]. An attractive hypothesis could be that the pore formation that can be detected by epithelial cells through the p38 mitogen-activated protein kinase (MAPK) activity [44], represents a signal danger that would elicit autophagy [13]. This hypothesis is supported by the requirement of VacA channel-formation activity to elicit autophagy in cells it intoxicates [41]. In agreement with this hypothesis, we found that the binding subunit BinB that is able by itself to disturb the membrane permeability [10,11], was also capable of inducing an autolysosome enlargement (unpublished data). In contrast to the vacuolation induced by the mixture of the two components of Bin, vacuolation induced by BinB alone was rapidly and completely reversed, perhaps due to the instability of BinB derived pores.

Autophagy induced by VCC and by VacA have been shown to target and degrade the toxin and as a consequence, contribute to the intoxicated cell’s survival [40,41]. We found that Bin and its GPI-anchored receptor Cpm1 are rapidly internalized in an early

**Figure 5. Internalized Bin is associated with its receptor but is not bound to vacuoles.** (A, C and E) MDCK-Cpm1 cells were incubated with BinA labeled with Alexa 488 (BinA-Al488) and BinB labelled with Alexa 543 (BinB-Al543) at 37°C for 10 min (A), 30 min (C) or 6 h (E) and processed for confocal microscopy analysis. At any time point, BinA-Al488 and BinB-Al543 were found colocalized in multiple intracellular vesicles. (B) Cells transfected with GPI-GFP and treated for 10 min with unlabelled BinA and BinB-Al543. Vesicles positive for BinB-Al543 were also stained by GPI-GFP. (D) Immunodetection of Cpm1 in MDCK-Cpm1 cells treated for 30 min with unlabelled BinA and BinB-Al543. Cpm1 signal and BinB-Al543 labeled vesicles were found colocalized. (E) BinA-Al488 and BinB-Al543-containing vesicles were found clustered between the vacuoles but not decorating the membrane of these vacuoles nor inside. Bars, 5 μm.

doi:10.1371/journal.pone.0014682.g005
endocytic compartment that did not present classical markers of early endocytic vesicles such as Rab5, caveolin-1 or the transferrin receptor. Nevertheless this compartment was enriched in GPI-APs. Such compartments from which GPI-APs can be directly targeted to recycling endosomes have been described by Sabharanjak et al. and are named GPI-APs-enriched early endosomal compartments, GEECs [45]. After endocytosis Bin would exploit this endocytic route to avoid degradation as it was associated with recycling endosomes rather than with the degradative compartments. This localization provides additional facilities to a bacterial toxin: Shiga toxin is hypothesized to traffic through recycling endosomes before reaching the Golgi apparatus with the aim of exploiting a retrograde transport in the secretory pathway [46]. Thus Bin could trigger its lethal effect beyond the epithelial monolayer by exploiting a transcytotic pathway from the apical membrane to the basolateral membrane that could include trafficking through recycling endosomes. Such a hypothesis is further prompted by the fact that cytopathologies affecting nervous cells and skeletal muscles have been reported following Bacillus sphaericus ingestion [47]. Recently it has been demonstrated that another Bacillus sphaericus toxin pair, Cry48/Cry49 that contains the Bin-like Cry49 protein, also elicits cytoplasmic vacuolisation, characteristic of Bin intoxication [48]. Thus, the mechanism of vacuole formation elucidated above may be exploited by other toxins from this organism.

In this study, we provide new insights into the strategies used by non-invasive bacteria to overcome cellular defense responses, i.e. a stimulation of autophagy and ability to subvert this degradation processes by trafficking in the recycling pathway. While the survival of invasive bacteria depends on their ability to counteract autophagic degradation [37], the reason why non-invasive bacteria secrete toxins that would interfere with this process remains puzzling. This study on cell responses to Bin, and previous work with VCC or VacA, establish that autophagy forms part of

Figure 6. After internalization, Bin reaches recycling endosomes. MDCK-Cpm1 cells were transfected with markers of early and late endocytotic compartments, intoxicated with a mixture of unlabelled BinA and BinB-Al543 for 30 min and processed for confocal microscopy analysis. (A) BinB-Al543 fluorescent signal colocalized with GFP-Rab4, a typical marker of recycling compartments. (B) A significant colocalization was detected with TfR-myc that marks both endocytotic and recycling vesicles. (C - E) No colocalization was found with GFP-Rab7 and GFP-Lamp1 that marks late-endocytotic/lysosomal compartments and GFP-LC3 a marker of autophagic vesicles. Bars, 10 μm.
doi:10.1371/journal.pone.0014682.g006
the arsenal of responses to bacterial pore-forming intoxication. Future studies will help to identify the signal(s) and the cell sensor(s) that elicit(s) this pathway.

**Materials and Methods**

**Cell culture, transfection, and treatment**

Strain II MDCK cells expressing the full-length CPM1 cDNA sequence (MDCK-Cpm1 cells) were generated as previously described [12], and cultured in Eagle's minimum essential medium (MEM) supplemented with 5% fetal calf serum and 400 μg ml⁻¹ G-418 at 37°C in a humidified atmosphere containing 5% CO₂. Transfections were performed using Lipofectamine 2000 (Invitrogen) as a carrier according to the manufacturer’s instructions. All the assays were performed 24 hours after transfection. To induce autophagy by amino acid starvation, cells were washed three times with Earle’s balanced salts solution (EBSS, Gibco) and incubated in the same medium.

**Toxins**

BinA- and BinB-glutathione-S-transferase fusion proteins were expressed in E. coli as reported earlier [49]. The activated forms were purified by affinity chromatography on Akta-FPLC using GSTrap and Benzamidine-Sepharose columns (GE Healthcare, France). Purified BinA and BinB were stained with Alexa 488 and Alexa 543, respectively (Molecular Probes, France). All the

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**Figure 7. Fate of internalized toxin in dividing cells.**

(A) Immunodetection of β-tubulin and DNA staining were performed in MDCK-Cpm1 cells treated with unlabelled BinA and BinB-Al543 for 6 hours. (B) Cells were transfected with GFP-Rab4 then intoxicated as described above, fixed, and stained for DNA. Cells in the process of cell division were analyzed for the localization of the toxin. (C) Time lapse videomicroscopy analysis was performed on MDCK-Cpm1 cells treated with BinA-Al488 and BinB-Al543 for 24 h. The panel selected from video S4 shows a cell displaying post-mitotic vacuolation, the selection starts 16 hours after intoxication. While the cell was progressing through mitosis, internalized toxins were colocalized and partitioned in the daughter cells. Bars, 10 μm.

doi:10.1371/journal.pone.0014682.g007
Intoxications were performed with an equimolar mixture (50 nM final concentration) of BinA and BinB subunits present in the medium for the whole experiment. This concentration was suitable to induce MDCK-Cpm1 cell vacuolisation.

Plasmids, antibodies and fluorescent dyes

GFP-Rab 5 and GFP-Rab 7 fusion proteins, TIR-myc (a fusion of the transferrin receptor and the myc epitope), and GFP-Lamp1 (a fusion at the C-terminus of Lamp1 with GFP), were kindly provided by the INSERM U452, Nice, France. Rab4 fused with GFP was a gift from the INSERM U568, Nice, France. The constructs pEYFP-GolgI, pDsRed2-ER, and pDsRed2-Mitochondria were purchased from Clontech, France. GFP-LC3 (a fusion of the rat LC3 with GFP) was kindly provided by N. Mizushima (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). The decay accelerating factor (DAF) fused to GFP (GPI-GFP) was from A. Galmiche (Max Planck Institut für Infektionsbiologie, Berlin, Germany). Phalloidin–TRITC and the mAb anti-β-tubulin were purchased from Sigma-Aldrich, France. The mAb anti-myc (clone 9E10) was purchased from Babco (Richmond, CA). The polyclonal rat anti-Cpm1 antibody was generated as previously described [5]. The Alexa 480-labeled anti-mouse secondary antibody and FITC-labeled anti-rat secondary antibody were purchased from Molecular Probes. Acidic compartments were labeled by incubating the cells with 1 μM LysoTracker (Molecular Probes) in the culture medium for 5 min at 37°C. After incubation, cells were washed with PBS and immediately processed for immunofluorescence analysis. DNA was stained with the monomeric cyanine nucleic acid stains TO-PRO-3 (Invitrogen).

Immunofluorescence studies, microscopy techniques, and video imaging

Immunofluorescence studies were performed as previously described [12,50]. After treatment MDCK cells were washed twice with PBS and fixed in PBS containing 2% (w/v) paraformaldehyde. For the immunofluorescence studies, cells were treated with PBS/0.1% TritonX-100, then blocked in PBS containing 2% (w/v) fish skin gelatine. Confocal images were obtained with an Axiovert 200 M microscope equipped with a confocal laser module LSM 510 META, using a 40x oil magnification lens (Carl Zeiss MicroImaging, Inc.). The images were combined and merged using Photoshop (Adobe software). All the confocal pictures represent single focal sections. Videomicroscopy analyses were performed on an Axiovert 200 microscope equipped with a shutter-controlled illumination (Carl Zeiss Micro-Imaging, Inc.) and a cooled digital charge-coupled device camera (Ropper Scientific). Images were processed using Metamorph 2.0 image analysis software (Invitrogen). For electron microscopy analysis, cells were fixed with 1.6% glutaraldehyde in 0.1M Phosphate buffer pH 7.4 at 22–24°C; then at 4°C for at least 18 hours. Samples were rinsed in the same buffer, then post-fixed with 1% osmium tetroxide and 1% potassium ferrocyanide for 1h at 22–24°C to enhance the staining of cytoplasmic membranes (de Bruijn, 1973). Cells were rinsed with distilled water and embedded in epoxy resin. Embedded samples were then conventionally processed for transmission electron microscopy (CM12; Philips).

Statistical analysis

The effects of Bin intoxication or autophagy induced by starvation on both the number of autophagosomes per cell (Fig. 4A) and the percentage of GFP-LC3 positive cells (Fig. 4B) were evaluated with a one-way analysis of variance, and the Student’s t-test with Bonferroni corrections was used for multiple comparisons between treatments. In order to evaluate the effect of the culture medium and the time of intoxication on the percentage of vacuolated cells (Fig. 4C), we used the Student’s t-test. All analyses were performed on transformed data: square root transformation was used for the number of autophagosomes per cell and arcsine of square root transformation was used for percentages.

Supporting Information

Figure S1 Figure S1 shows (A) microtubule network and (B) actin cytoskeleton analysis in Bin-treated cells. Cells were intoxicated with Bin for 6 h and processed for immunofluorescence analysis. Microtubules were visualized using anti-beta-tubulin mAb detected with a secondary antibody labeled with Alexa 488, and the actin cytoskeleton was visualized using TRITC-conjugated phalloidin. On middle confocal sections vacuoles (arrows) were found surrounded by actin. Bars, 10 μm.

Found at: doi:10.1371/journal.pone.0014682.s001 (0.47 MB TIF)

Figure S2 MDCK-Cpm1 cells were transfected with markers of early and late endocytotic compartments. Twenty-four hours after transfection, cells were intoxicated with an equimolar mixture of unlabelled BinA and BinB-Al543 (50 nM) for 10 min at 37°C. GFP-Rab5 (A) and Cav1-GFP (B) served as markers for early endocytotic vesicles, GFP-Rab4 (C) is a typical marker of recycling compartments while TIR-myc (D) marks both endocytotic and recycling vesicles. At early time point of endocytosis we did not find any colocalization with the toxin and these markers.

Found at: doi:10.1371/journal.pone.0014682.s002 (0.32 MB TIF)

Figure S3 Figure S3 shows MDCK-Cpm1 cells transfected with GFP-Rab7, GFP-Lamp1, GFP-Rab5 and GFP-Rab4. Twenty-four hours after transfection, cells were intoxicated with Bin specify which Bin eg BinA, BinB, BinA-Al98, BinB-Al543 and vacuolating cells were observed using a confocal microscope. The single focal sections and the total cell reconstitution show the association of GFP-Rab7 and GFP-Lamp1 with the vacuolating compartment. In contrast, GFP-Rab5 and GFP-Rab4 were excluded from the vacuoles.

Found at: doi:10.1371/journal.pone.0014682.s003 (0.08 MB JPG)

Figure S4 The upper panel shows MDCK-Cpm1 cells intoxicated with an equimolar mixture of unlabelled BinA-4H48 and BinB-Al543 (50 nM). After 6h of intoxication, the cells were processed for confocal microscopy. The single focal section and the total cell reconstitution show the colocalisation of the two subunits. The lower panel shows MDCK-Cpm1 cells transfected with the marker of late endocytotic compartments, GFP-Rab7. Twenty-four hours after transfection, cells were intoxicated with an equimolar mixture of unlabelled BinA and BinB-Al543 (50 nM). After 6h of intoxication, the cells were processed for confocal microscopy. The single focal section and the total cell reconstitution show that the membrane of the vacuoles are decorated by GFP-Rab7, and the toxin is clustered in the remaining space between the vacuoles but is not associated with the membrane of these vacuoles and is not found inside the vacuoles.

Found at: doi:10.1371/journal.pone.0014682.s004 (0.06 MB JPG)

Video S1 MDCK-Cpm1 cells treated with an equimolar mixture of BinA and BinB (50 nM) in complete medium at 37°C; the video starts 45 min after intoxication and ends 24 h after intoxication.

Found at: doi:10.1371/journal.pone.0014682.s005 (2.50 MB MOV)
Video S2  Selection of video 1 showing two cells that progress through division and present vacuolation just after mitosis; the selection starts 15 h after intoxication and ends 19 h after intoxication.  
Found at: doi:10.1371/journal.pone.0014682.s006 (1.19 MB MOV)

Video S3  The video shows two MDCK-Cpm1 cells that present vacuolation after mitosis when intoxicated in nutrient-free medium at 37°C; the video starts 20 h after intoxication.  
Found at: doi:10.1371/journal.pone.0014682.s007 (0.78 MB MOV)

Video S4  The video shows MDCK-Cpm1 cells treated with an equimolar concentration of Bina-A488 and BinB-Al543 (50 nM) at 37°C that present post-mitotic vacuolation. For all videos, frames were collected every 15 min and are displayed at 3 frames/sec.  
Found at: doi:10.1371/journal.pone.0014682.s008 (0.16 MB MOV)

Video S5  Animations of the total cell reconstitution of GFP-Rab7 expressing MDCK-Cpm1 cells shown in figure S3.  
Found at: doi:10.1371/journal.pone.0014682.s009 (1.23 MB MOV)

Video S6  Total cell reconstitution of GFP-Rab5 exclusion of Bin induced giant vacuoles shown in fig. S5.  
Found at: doi:10.1371/journal.pone.0014682.s100 (0.16 MB MOV)

References

1. de Maagd RA, Bravo A, Berry C, Crickmore N, Schneef HE (2003) Structure, diversity, and evolution of protein toxins from spore-forming endotoxophilic bacteria. Annu Rev Genet 37: 409–433.
2. Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, et al. (1998) Bacillus thuringiensis and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62: 775–806.
3. Charles JF, Darboux I, Pauron D, Nielsen-LeRoux C (2005) Mosquitocidal Bacillus sphaericus toxin genes, genetics, mode of action, use, and resistance mechanisms. In: Gilbert KlStG LI, ed. Elsevier Pergamon. pp 207–231.
4. Darboux I, Nielsen-LeRoux C, Charles JF, Pauron D (2001) The receptor of Bacillus sphaericus binary toxin in Culex pipiens (Diptera: Culicidae) midgut: molecular cloning and expression. Insect Biochem Mol Biol 31: 981–990.
5. Darboux I, Pauchet Y, Castella C, Silva-Filha MH, Nielsen-LeRoux C, et al. (2002) Loss of the membrane anchor of the target receptor is a mechanism of bioinsecticidal resistance. Proc Natl Acad Sci U S A 99: 5380–5383.
6. Oporta O, Charles JF, Warot S, Pauron D, Darboux I (2007) Identification and characterization of the receptor for the Bacillus sphaericus binary toxin in the malaria vector mosquito, Anopheles gambiae. Comp Biochem Physiol B Biochem Mol Biol.
7. Silva-Filha MH, Nielsen-LeRoux C, Charles JF (1999) Identification of the receptor for Bacillus sphaericus crystal toxin in the brush border membrane of the mosquito Culex pipiens (Diptera: Culicidae). Insect Biochem Mol Biol 29: 711–721.
8. Charles JF, Nielsen-LeRoux C, Delecluse A (1996) Bacillus sphaericus toxins: molecular biology and mode of action. Annu Rev Entomol 41: 451–472.
9. Barth H, Aktories K, Popoff MR, Stiles BG (2004) Binary bacterial toxins: biochemistry, biology, and applications of common Clostridium and Bacillus proteins. Microbiol Mol Biol Rev 68: 373–402, table of contents.
10. Schultz JA, Potvin L, Gous F, Charles JF, Berry C, et al. (2001) Permeabilization of model lipid membranes by Bacillus sphaericus mosquito-cidal binary toxin and its individual components. J Membr Biol 184: 572–582.
11. Colman C, Davidson EW, Cooper K (1997) Electrophysiological effects of Bacillus sphaericus binary toxin on cultured mosquito cells. J Invertebr Pathol 69: 197–204.
12. Pauchet Y, Luton F, Castella C, Charles JF, Romey G, et al. (2005) Effects of a mosquitocidal toxin on a mammalian epithelial cell line expressing its target receptor. Cell Microbiol 7: 1335–1344.
13. Gonzalez MR, Rioschberger M, Pernot L, de Groot F, Freche B (2008) Bacterial pore-forming toxins: the (w)hole story? Cell Mol Life Sci 65: 493–507.
14. Soberon M, Fernandez LE, Perez C, Gill SS, Bravo A (2007) Mode of action of mosquitocidal Bacillus thuringiensis toxins. Toxicon 49: 597–600.
15. Charles JF (1987) Ultrastructural midgut events in Culicidae larvae fed with Bacillus sphaericus 2297 spore/crystal complex. Ann Inst Pasteur Microbiol 138: 471–484.
16. Silva-Filha MH, Peixoto CA (2003) Immunochemical localization of the Bacillus sphaericus binary toxin components in Culex quinquefasciatus (Diptera: Culicidae) larva midgut. Pesticide Biochem Physiol 77: 138–146.
17. Naraos ML, Cloppin-Kap K (1988) Effect of Bacillus sphaericus 1593 toxin on choline acetyl transferase and mitochondrial oxidative activities of the mosquito larva. Indian J Biochem Biophys 25: 253–256.
18. Lou W, Greuze HJ, Greelen MJ, Slot JW (1997) The autophagic and endocytic pathways converge at the nascent autophagic vacuole. J Cell Biol 136: 61–70.
19. Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature 451: 1069–1073.
20. da Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE (2003) Structure, diversity, and evolution of protein toxins from spore-forming endotoxophilic bacteria. Annu Rev Genet 37: 409–433.
21. Schwarz JA, Potvin L, Gous F, Charles JF, Berry C, et al. (2001) Permeabilization of model lipid membranes by Bacillus sphaericus mosquito-cidal binary toxin and its individual components. J Membr Biol 184: 572–582.
22. Liang XH, Jackson S, Seaman M, Brown K, Kemplekis B, et al. (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402: 672–676.
23. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, et al. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. Embo J 19: 5720–5728.
24. Tanida I, Minematsu-Ikeguchi N, Ueno T, Komimana E (2005) Lysozyme as a noninvasive intracellular pH indicator. Biophys J 74: 1591–1599.
25. Elkinen EL, Prescott AR, Cooper JE, Brachmann SM, Wang L, et al. (2002) Inhibition of autophagy in mitotic animal cells. Traffic 3: 478–487.
26. Gutierrez NC, Monzo P, Gonzalez T, Doye A, Oldani A, et al. (2007) Early endosomes associated with dynamin: Fxrrin structures are required for late trafficking of H. pylori VacA toxin. J Cell Biol 177: 343–354.
27. Mizushima N, Lossev M, Keddie S, Gauthier NC, Monzo P, Keddie S, et al. (2007) Inhibition of autophagy in mitotic animal cells. Traffic 3: 478–487.
28. Mizushima N, Lossev M, Keddie S, Gauthier NC, Monzo P, Keddie S, et al. (2007) Inhibition of autophagy in mitotic animal cells. Traffic 3: 478–487.
29. Mizushima N, Lossev M, Keddie S, Gauthier NC, Monzo P, Keddie S, et al. (2007) Inhibition of autophagy in mitotic animal cells. Traffic 3: 478–487.
30. Mizushima N, Lossev M, Keddie S, Gauthier NC, Monzo P, Keddie S, et al. (2007) Inhibition of autophagy in mitotic animal cells. Traffic 3: 478–487.
31. Bergeland T, Widerberg J, Bakke O, Nordeng TW (2001) Mitotic partitioning of chromosomes in C. elegans. Hum Genet 109: 565–573.
32. Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature 451: 1069–1073.
33. Da Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE (2003) Structure, diversity, and evolution of protein toxins from spore-forming endotoxophilic bacteria. Annu Rev Genet 37: 409–433.
34. Schwarz JA, Potvin L, Gous F, Charles JF, Berry C, et al. (2001) Permeabilization of model lipid membranes by Bacillus sphaericus mosquito-cidal binary toxin and its individual components. J Membr Biol 184: 572–582.
35. da Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE (2003) Structure, diversity, and evolution of protein toxins from spore-forming endotoxophilic bacteria. Annu Rev Genet 37: 409–433.
36. Da Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE (2003) Structure, diversity, and evolution of protein toxins from spore-forming endotoxophilic bacteria. Annu Rev Genet 37: 409–433.
37. Da Maagd RA, Bravo A, Berry C, Crickmore N, Schnepf HE (2003) Structure, diversity, and evolution of protein toxins from spore-forming endotoxophilic bacteria. Annu Rev Genet 37: 409–433.
32. Schweitzer JK, Burke EE, Goodson HV, D’Souza-Schorey C (2005) Endocytosis resumes during late mitosis and is required for cytokinesis. J Biol Chem 280: 41628–41633.
33. Munado DB, Colombo MI (2001) A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. J Cell Sci 114: 3619–3629.
34. Nakagawa I, Amano A, Mizushima N, Yamamoto A, Yamaguchi H, et al. (2004) Autophagy defends cells against invading group A Streptococcus. Science 306: 1037–1040.
35. Rich KA, Burket C, Webster P (2003) Cytoplasmic bacteria can be targets for autophagy. Cell Microbiol 5: 455–468.
36. Yano T, Mita S, Ohmori H, Oshima Y, Fujimoto Y, et al. (2008) Autophagic control of listeria through intracellular innate immune recognition in drosophila. Nat Immunol 9: 908–916.
37. Dorn BR, Dunn WA, Jr., Progulske-Fox A (2002) Bacterial interactions with the autophagic pathway. Cell Microbiol 4: 1–10.
38. Ogawa M, Yoshimori T, Suzuki T, Sagara H, Mizushima N, et al. (2005) Escape of intracellular Shigella from autophagy. Science 307: 727–731.
39. Schinzi A, Kasljar H, Leggio SA, Addicks K, Kronke M, et al. (2007) Staphylococcus aureus subvert autophagy for induction of caspase-independent host cell death. J Biol Chem 282: 2693–2706.
40. Gutierrez MG, Saka HA, Chinera I, Zoppino FC, Yoshimori T, et al. (2007) Protective role of autophagy against Vibrio cholerae cytolysin, a pore-forming toxin from V. cholerae. Proc Natl Acad Sci U S A 104: 1829–1834.
41. Terebiznik MR, Raju D, Vazquez CL, Torbricki K, Kulkarni R, et al. (2009) Effect of Helicobacter pylori’s vacuolating cytotoxin on the autophagy pathway in gastric epithelial cells. Autophagy 5: 370–379.
42. Tan YK, Vu HA, Kusuma CM, Wu A (2009) Implications of autophagy in anthrax pathogenicity. Autophagy 5: 734–735.
43. Ha SD, Ham B, Mogridge J, Safig P, Lin S, et al. (2010) Cathepsin B-mediated autophagy flux facilitates the anthrax toxin receptor 2-mediated delivery of anthrax lethal factor into the cytoplasm. J Biol Chem 285: 2120–2129.
44. Rater AJ, Hippe KR, Aguilar JL, Bender MH, Nelson AL, et al. (2006) Epithelial cells are sensitive detectors of bacterial pore-forming toxins. J Biol Chem 281: 12994–12998.
45. Sabharanjak S, Sharma P, Parton RG, Mayor S (2002) GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. Dev Cell 2: 411–423.
46. Mallard F, Antony C, Tenza D, Salamero J, Gouat B, et al. (1998) Direct pathway from early/recycling endosomes to the Golgi apparatus revealed through the study of shiga toxin B-fragment transport. J Cell Biol 143: 973–990.
47. Singh GJ, Gill SS (1988) An electron microscope study of the toxic action of Bacillus sphaericus in Culex quinquefasciatus larvae. J Invertebr Pathol 52: 237–247.
48. de Melo JV, Jones GW, Berry C, Vasconcelos RH, de Oliveira CM, et al. (2009) Cytopathological effects of Bacillus sphaericus Cry4Ba/Cry49Aa toxin on binary toxin-susceptible and -resistant Culex quinquefasciatus larvae. Appl Environ Microbiol 75: 4782–4789.
49. Oei C, Hindley J, Berry C (1992) Binding of purified Bacillus sphaericus binary toxin and its deletion derivatives to Culex quinquefasciatus gut: elucidation of functional binding domains. J Gen Microbiol 138(Pt 7): 1515–1526.
50. Gauthier NC, Ricci V, Gounon P, Dow C, Tauc M, et al. (2004) Glycosylphosphatidylinositol-anchored proteins and actin cytoskeleton modulate chloride transport by channels formed by the Helicobacter pylori vacuolating cytotoxin VacA in HeLa cells. J Biol Chem 279: 9481–9489.