

Necrotrophism Is a Quorum-Sensing-Regulated Lifestyle in *Bacillus thuringiensis*

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Abstract

How pathogenic bacteria infect and kill their host is currently widely investigated. In comparison, the fate of pathogens after the death of their host receives less attention. We studied *Bacillus thuringiensis* (*Bt*) infection of an insect host, and show that NprR, a quorum sensor, is active after death of the insect and allows *Bt* to survive in the cadavers as vegetative cells. Transcriptomic analysis revealed that NprR regulates at least 41 genes, including many encoding degradative enzymes or proteins involved in the synthesis of a nonribosomal peptide named kurstakin. These degradative enzymes are essential *in vitro* to degrade several substrates and are specifically expressed after host death suggesting that *Bt* has an active necrotrophic lifestyle in the cadaver. We show that kurstakin is essential for *Bt* survival during necrotrophic development. It is required for swarming mobility and biofilm formation, presumably through a pore forming activity. A *nprR* deficient mutant does not develop necrotrophically and does not sporulate efficiently in the cadaver. We report that necrotrophism is a highly regulated mechanism essential for the *Bt* infectious cycle, contributing to spore spreading.

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Introduction

Saprophytism, probably one of the most common lifestyle for micro-organisms, involves living in dead or decaying organic matter. For most pathogens, saprophytism is limited to necrotrophism (the use of the host cadaver). This step of the infection process is essential for the proliferation and horizontal transmission of these microorganisms (transfer of infection within a single generation) [1]. However, there have been very few studies addressing this major issue. The transition from a pathogenic to a necrotrophic lifestyle implies substantial metabolic changes for microorganisms [2]. The death of the host is a critical event which compels the micro-organisms to cope with a new series of challenges: competition with the commensal organisms and opportunistic incomers, stress, and nutrient deficiencies. Therefore, necrotrophism is likely to be highly regulated.

The insect pathogen *Bacillus thuringiensis* (*Bt*) is a suitable model for studying the time course of the infection process, including necrotrophism in the insect cadaver. *Bt* is an ubiquitous spore-forming bacterium belonging to the *Bacillus cereus* (*Bc*) group [3]. Its spores are found in a large variety of environments, such as soils, dead and living insects and plant phylloplane [4]. However, *Bt* probably does not grow in soil and reports of natural epizootic episodes are very rare [5,6]. Unlike soil bacteria, such as *Streptomyces spp* and *B. subtilis*, *Bc* group genomes contain a large

number of genes involved in nitrogen metabolism [3]. It is therefore likely that *Bt* multiplies in the host cadaver [1,6].

Bt carries plasmids encoding specific insecticidal toxins responsible for their insecticidal properties [7]. *Bt* spores and toxins are ingested by larvae, and the toxins bind to specific receptors on the midgut epithelial cells, inducing cell lysis and creating favorable conditions for the development of the bacteria [8]. The vegetative bacteria multiply in the insect hemocoel and cause septicemia [1,9]. *Bt* also harbors genes encoding exported virulence factors including enterotoxins, hemolysins, phospholipases and proteases [10]. The transcription of most of these virulence genes in bacteria growing in a rich medium is activated at the onset of stationary phase by the quorum-sensing system PlcR-PapR [11,12]. PlcR-regulated factors account for about 80% of the extracellular proteome of *Bt* during early stationary phase in rich medium [13]. In sharp contrast, the expression of the PlcR-regulated genes is repressed when the bacteria enter sporulation [14] and the stationary phase secretome of *Bt* and *B. anthracis* (*Ba*) growing in a sporulation medium is mainly composed of the metalloprotease NprA [15,16]. NprA (also designated NprB and Npr599 in *Ba*) cleaves tissue components such as fibronectin, laminin and collagen, thus displaying characteristics of pathogenic factors [17]. Transcription of *nprA* is activated during the late stationary phase by the regulator NprR [16]. NprR is a quorum sensor activated by its cognate signaling peptide, NprX. NprR-

Author Summary

Bacillus thuringiensis (*Bt*) is a well known entomopathogenic bacterium successfully used as a biopesticide for fifty years. The insecticidal properties of *Bt* are mainly due to specific toxins forming a crystal inclusion associated with the spore. After ingestion by susceptible insect larvae, toxins could induce favorable conditions for spore germination. The bacteria multiply in the insect and coordinate their behavior using signaling molecules involved in quorum sensing. The activation of the quorum sensor PlcR leads to the production of virulence factors allowing the bacteria to kill the insect host. Here we show that, in the cadaver, *Bt* shifts from a virulent to a necrotrophic lifestyle during which a second quorum sensor (NprR) becomes functional. NprR activates genes encoding degradative enzymes (proteases, lipases and chitinases) and a lipopeptide (kurstakin) involved in swarming and biofilm formation. The kurstakin is also essential for the survival of *Bt* after insect death. This suggests that NprR allows the bacteria to survive and eventually to sporulate in the host cadaver, thus improving their ability to disseminate in the environment. Altogether these results show that the pathogenic and necrotrophic lifestyles of *Bt* are tightly controlled by two quorum-sensing systems acting sequentially during the infection process.

NprX functions as a typical Gram-positive quorum-sensing system: the pro-signaling peptide NprX is exported from the cell, and after being processed to its active form is reimported, and binds to NprR allowing the recognition of its DNA target and the activation of *nprA* transcription [16].

The first stages of *Bt* infection are relatively well documented, but the fate of the bacteria after death of the host remains unclear. Here, we report evidence that the necrotrophic lifestyle of *Bt* is a specific and highly regulated process. The quorum-sensing system NprR-NprX controls at least 41 genes some of which are required for *Bt* to survive in the insect cadaver and to complete its development *in vivo* ending with the production of spores.

Results

NprR is activated after the host death

We tested whether NprR, the activator of *nprA* transcription [16], is involved in the pathogenicity of *Bt*. The LD₅₀s of the *Bt* 407 Cry⁻ (wt) strain and of the *nprR*-deficient (Δ RX) strain in the insect model *Galleria mellonella* were measured in two ways: by feeding larvae with spores mixed with the insecticidal toxin Cry1C and by injection of vegetative bacteria into the insect hemocoel (Table S1). The LD₅₀s of two strains did not differ significantly in either of the two conditions indicating that NprR was not required for pathogenicity. Consistently, an *nprA*-deficient strain was similarly found not to be affected in pathogenicity (not shown).

We investigated the involvement of NprR in the infection process by comparing, *in vivo*, the expression kinetics of *nprA* with that of the protease gene *mpbE*, reflecting the transcriptional activities of NprR and PlcR, respectively [16,18]. The reporter strains grew similarly in insect larvae and a constitutively expressed *P_{aphA3}-lacZ* fusion was used as the reference standard (Figure 1A and Figure S1A). Transcription of *mpbE* increased between 0 h and 24 h after injection and gradually decreased thereafter. In contrast, *nprA* transcription was low between 0 h and 24 h, increased between 24 h and 48 h and then decreased sharply

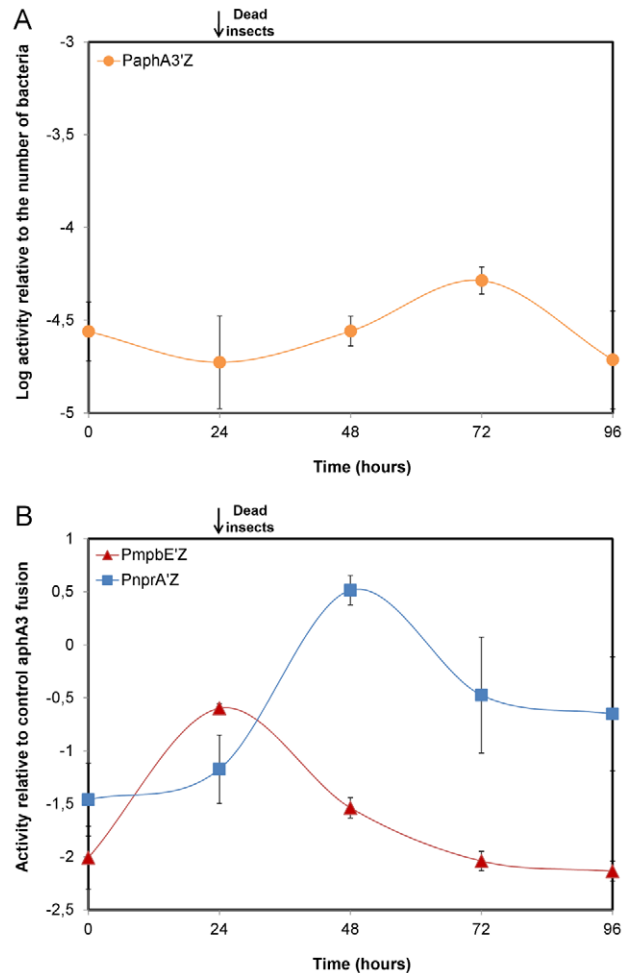


Figure 1. *mpbE* and *nprA* are sequentially activated during infection. (A) Correlation between the β -galactosidase activity obtained with the *P_{aphA3}-lacZ* fusion and the number of bacteria of the reporter strain 407 *paphA3'Z* in infected insects. The activity of the *aphA3* promoter remains constant over time in insects such that the *P_{aphA3}-lacZ* fusion can be used as an *in vivo* reference standard. (B) Expression of the *mpbE* and *nprA* genes *in vivo*. Each point is the log-transformed ratio of the β -galactosidase activity obtained with the 407 *pmpbE'Z* and 407 *pmpbE'Z* strains to that with the control strain 407 *paphA3'Z*. The black arrow indicates that assays were carried out with dead insects from 24 h post infection until the end of the experiment. Data are averages of at least three independent experiments (error bars are SEM from mean values). doi:10.1371/journal.ppat.1002629.g001

(Figure 1B). Thus, NprR is active later in the infection process than PlcR, and after the death of the host.

NprR allows *Bt* to survive in insect cadavers by a process independent of sporulation

To investigate the role of NprR during the late stage of infection, we compared the growth of the wt and Δ RX strains in insect larvae (Figure 2A). The total population of the two strains increased between 0 h and 24 h to reach about 1×10^8 cfu/mL. From 24 h to 96 h, the population of the wt strain remained stable, whereas the population of the Δ RX strain decreased sharply: 96 h post infection, the total population of the Δ RX strain was 6-log lower than that of the wt strain. Complementation of the Δ RX strain by pHT304-RX restored the wt phenotype. These

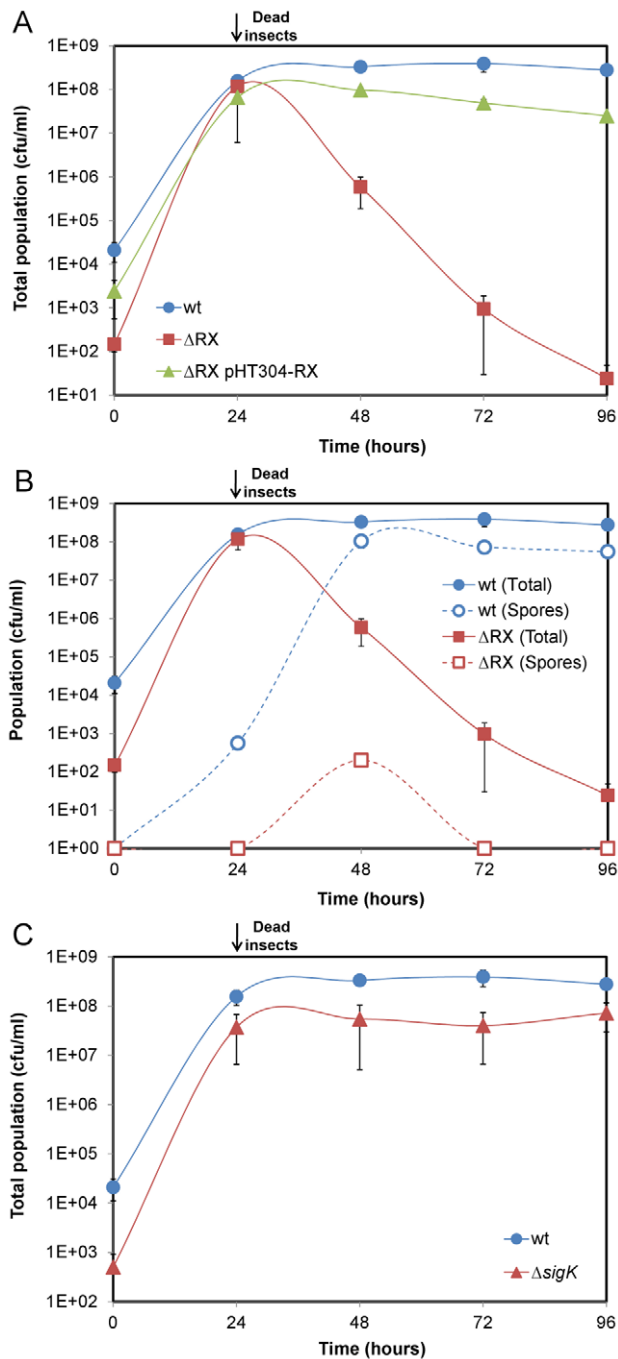


Figure 2. NprR allows *Bt* to survive in insect cadavers by a process independent of sporulation. (A) Comparison of the wt and Δ RX strain survival in the insect host. The survival defect of the mutant is genetically complemented by pHT304-RX. (B) Effect of the *nprR-nprX* deletion on sporulation. (C) Comparison of the wt and Δ *sigK* strain survival in the insect host. Data are averages of at least four independent experiments (error bars are SEM from mean values). doi:10.1371/journal.ppat.1002629.g002

findings indicate that NprR substantially improves the survival of *Bt* in insect cadavers.

In sporulating microorganisms, sporulation is generally regarded as the key process ensuring survival in unfavorable conditions. We therefore investigated i) whether NprR was involved in the sporulation process of *Bt* in the insect cadaver, and ii) whether

sporulation is responsible for the survival of the bacteria in the insect cadaver. We compared the sporulation efficiencies of the wt and Δ RX strains in both LB and sporulation-specific medium (HCT) (Table S2). In HCT, the sporulation efficiencies of the two strains were similar. However, in LB medium, the total number of viable spores of the Δ RX strain was half that for the wt strain (8.30×10^7 vs 1.58×10^8), suggesting that NprR is involved in the sporulation of *Bt* in rich medium. Next, we monitored the counts of wt and Δ RX strain spores in insect larvae over 96 h (Figure 2B and Table S2). For the wt strain, heat-resistant spores were detected 24 h after injection and their number increased until 48 h. From 48 h to 96 h, the number of spores remained stable and represented one third of the total bacterial population. The large number of non sporulated bacteria 96 h after the death of the insect suggests that sporulation was not the main mechanism allowing *Bt* to survive. For the Δ RX strain, less than one percent of the bacterial population was heat-resistant spores throughout the infection process. The decrease in the number of heat-resistant spores from 48 h to 72 h is likely due to the germination of the spores. We suspected that the low number of spores is not a cause but a consequence of the inability of the Δ RX strain to survive in the insect cadaver. To test this idea, we tested the survival of a *sigK*-deficient strain (Figure 2C): SigK is a sigma factor involved in the transcription of late sporulation genes in the mother cell, and *sigK*-deficient strains are not able to form viable spores [19,20]. The total population of the *sigK* strain in the insect cadaver was similar to the total population of the wt strain, indicating that NprR ensures the survival of *Bt* by a process independent of sporulation.

NprR is a pleiotropic regulator involved in the necrotrophic development of *Bt* in the insect cadaver

The only gene described as being controlled by NprR was *nprA*. Therefore, we monitored the survival of a Δ *nprA* strain in infected larvae (Figure S2). The survival of the wt and Δ *nprA* strains was similar throughout the experiment, suggesting that other NprR-regulated genes are involved in bacterial survival. Microarray analysis was used to identify other NprR-regulated genes. Gene expression ratios between the wt and the Δ RX strains were determined 3 h after the onset of stationary phase (t3), when *nprA* transcription increases sharply [16]. For 107 genes, this expression ratio was greater than 2 ($p < 0.05$) (<http://www.ebi.ac.uk/arrayexpress/experiments/E-TABM-790>), suggesting that NprR has a direct or indirect effect on their transcription. Thirty-nine genes, with a relative expression ratio greater than 4, and a significance value (p) smaller than 0.01, were considered for subsequent analysis. The genes matching probes for BC2622, a macrolide glycosyltransferase, and BC3725, an exochitinase, were also investigated due to their functional similarity to the genes fulfilling these criteria. Quantitative RT-PCR confirmed that these 41 genes were at least four times up- or downregulated. Fusions to *lacZ* were constructed for nine of these genes and used to confirm that they are differentially regulated in the Δ RX mutant and wt strains (Figure S3). The expression kinetics of these genes were similar, with a sharp increase of expression after the onset of stationary phase. The final list of NprR-regulated genes is presented in Table S3. Of the 41 genes directly or indirectly regulated by NprR, 37 were down-regulated in the Δ RX strain, suggesting that NprR primarily acts as a transcriptional activator. The subcellular localizations of the products of these genes were assessed: 46% were cytoplasmic, 20% were associated with the membrane and 34% were extracellular or associated with the cell wall. The NprR-regulated genes can be distributed into four functional groups. The first group is composed of genes encoding proteins potentially involved in stress resistance: they include the

genes for cytochrome P450 (BC2613), cysteine dioxygenase (BC2617), and Transporter Drug/Metabolite exporter family members (BC1063). The second group is a four-gene locus encoding the oligopeptide permease system Opp required for the import of small peptides into the cell. The third group is a five-gene locus encoding a nonribosomal peptide synthesis (NRPS) system showing similarities with the systems involved in the synthesis of secreted factors like toxins and antibiotics. The last group codes for degradative enzymes (metalloproteases, esterases and chitinases) and for proteins which can bind organic material (chitin-binding protein and collagen adhesion protein). The role of NprR in the degradation of lipids, proteins and chitin was analyzed by growing the Δ RX and wt strains on specific culture media (Figure 3A). The lipolytic, proteolytic and chitinolytic activities of the Δ RX strain were significantly lower than those of the wt strain. We monitored the expression kinetics in infected insect larvae of two NprR-regulated genes encoding degradative enzymes (BC0429 and BC2167) (Figure 3B). The two genes were specifically expressed after the insect death, from 24 h to 96 h, suggesting that *Bt* displays a necrotrophic lifestyle in the insect cadaver.

A secreted factor regulated by NprR allows *Bt* to survive in insect cadavers

To identify the NprR-dependent survival factor we first tested whether this putative factor was secreted. Insect larvae were co-infected with two different ratios of wt and Δ RX strains: 90% of wt bacteria with 10% of Δ RX bacteria (90:10), and 10% of wt bacteria with 90% of Δ RX bacteria (10:90) (Figure 4A and Figure 4B). In insects infected with the ratio 10:90, the total population of the wt and the Δ RX strains decreased after 24 h. This may result from the Δ RX strain capturing NprX without being able to express NprR-regulated genes, but nevertheless removing the peptide from the environment. Consequently, the amount of signaling peptide was insufficient to activate NprR-regulated genes in the wt, resulting in clearance of both

populations. Co-infection with the ratio 90:10 led to the survival of the two subpopulations during the 96 h of the experiment. In this condition, the concentration of NprX in the host was presumably sufficient to maintain the expression of the NprR-regulated genes in the wt subpopulation, and this expression allowed the survival of the Δ RX population. Therefore, the wt strain may produce a secreted factor that enables the Δ RX strain to survive in the insect cadaver.

The survival factor is a lipopeptide named kurstakin

NprR-dependent extracellular factors are degradative enzymes and the factor synthesized by the NRPS system. NprA, the major degradative enzyme produced during late stationary phase, is not required for bacterial survival in insect cadaver (Figure S2). The NRPS locus consists of seven open reading frames annotated BC2450 to BC2456 in the genome of the strain *Bc* ATCC 14579 used for designing the microarrays [3]. *In silico* analysis of all available sequenced *Bt* and *Bc* genomes, including that of strain *Bt* 407 used in this study, reveals that in all cases, this locus includes only four genes (<http://www.ncbi.nlm.nih.gov/bioproject/29717>). Several studies suggest that these four genes (designated *krsA, B, C, E*; Figure 5A) are involved in the production of the lipopeptide kurstakin [21,22,23]. *KrsE* is a presumed efflux protein and *KrsA, B, C* are the peptide synthetase subunits. The genes *krsA, krsB* and *krsC* were deleted from a wt strain and the survival of the mutant (Δ *krsABC*) in insects was monitored for 96 h (Figure 5B). The total population of the Δ *krsABC* strain declined from 2.10^7 cfu/ml at 24 h falling to 1.10^2 cfu/ml at 96 h. To test whether this effect was specifically dependent on the *krsABC* genes, we introduced a constitutive promoter upstream from these genes in the Δ RX strain. This NprR-independent expression of *krsABC* partially and significantly restored the survival of the Δ RX strain in the insect cadaver. These observations implicate the *krsABC* genes in the necrotrophic properties of *Bt*. We used MALDI-ToF-MS analysis to determine whether the *krsABC* genes are responsible for the production of kurstakin. Peaks characteristic

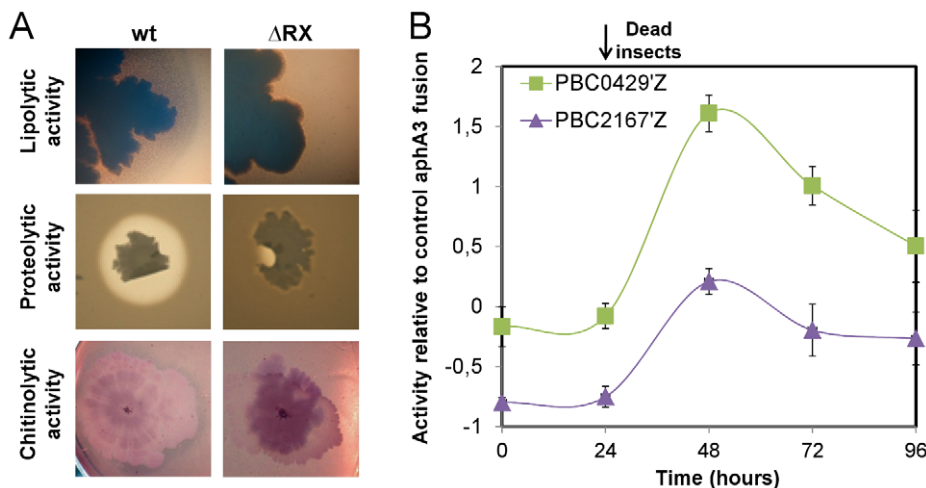


Figure 3. NprR is involved in the necrotrophic development of *Bt* in the insect cadaver. (A) Growth of the wt and Δ RX strains on specific culture media. The lipolytic activity was assayed on TS medium supplemented with Tween 80. Wild-type strain colonies were surrounded by a precipitate of oleic acid (a Tween 80 degradation product) whereas no precipitate was detected around the mutant strain colonies. The proteolytic and chitinolytic activities were assayed on HCT medium supplemented with cow's milk and on chitin medium, respectively. In both conditions, the wt strain was surrounded by a degradation ring whereas no such rings were detected for the mutant. (B) The genes BC0429 and BC2167 were specifically expressed after the insect death. Each point is the log-transformed ratio of the β -galactosidase activity obtained with the 407 pBC0429'Z and 407 pBC2167'Z strains to that with the control strain 407 *paphA3*'Z. The growth kinetics of both reporter strains are presented in Fig. S1B. Data are averages of at least three independent experiments (error bars are SEM from mean values). doi:10.1371/journal.ppat.1002629.g003

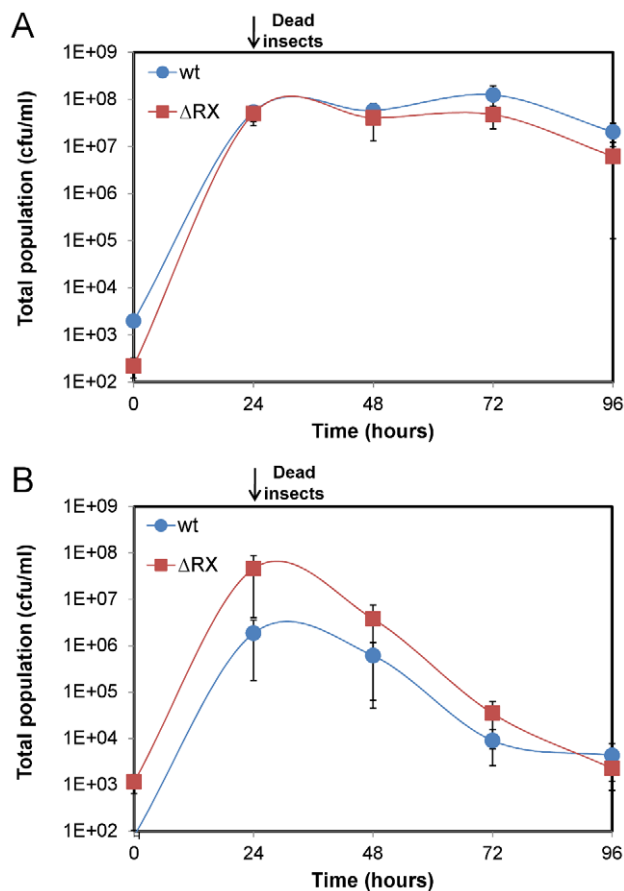


Figure 4. A NprR-regulated secreted factor allows *Bt* to survive in the insect host. Insect larvae were co-infected with both wt and Δ RX strains mixed at two different ratios: (A) 90% wt bacteria with 10% Δ RX bacteria (90:10) and (B) 10% wt bacteria with 90% Δ RX bacteria (10:90). Data are averages of at least four independent experiments (error bars are SEM from mean values). doi:10.1371/journal.ppat.1002629.g004

of kurstakin were found for whole cells of the wt strain and not for those of the Δ *krsABC* mutant (Figure 5C). This confirms that the *krsABC* genes are involved in kurstakin synthesis.

Kurstakin is involved in swarming and biofilm formation through a pore-forming activity

We compared properties of the wt and Δ *krsABC* strains on swimming plates (LB Agar 0.3%) and on swarming plates (LB Agar 0.7% and EPS Agar 0.7%) (Figure 6A). The wt and the Δ *krsABC* strains grown on LB 0.3% agar covered the plates, indicating that both strains were swimming proficient. However, unlike the wt, the Δ *krsABC* strain was unable to swarm or to form dendrites indicative of swarming mobility [24,25]. Lipopeptides are known to enhance biofilm formation [26] and it has been shown that the *Bt* 407 strain forms a thick biofilm at the air / liquid interface in glass tubes [27]. We tested the ability of the two strains to form biofilm in glass tubes (Figure 6A). The wt strain produced a significant ring at the air / liquid interface, whereas biofilm formation was abolished for the Δ *krsABC* strain. Kurstakin is therefore necessary for swarming and biofilm formation.

Lopez and coll. have shown that swarming mobility in *B. subtilis* is triggered by surfactin, a lipopeptide, which acts as a pore-forming molecule causing potassium leakage across the cytoplasmic

membrane [26]. We tested the swarming mobility of the Δ *krsABC* strain on swarming plates with nystatin (a pore-forming molecule) and with nystatin plus K_2HPO_4 (Figure 6B). Nystatin restored the swarming mobility of the Δ *krsABC* strain and the addition of K_2HPO_4 reversed this phenotype. These results suggest that kurstakin is a pore forming molecule causing a potassium leakage across the cytoplasmic membrane of *Bt*.

Discussion

PtcR is the main virulence regulator in *Bt* and *Bc* [10,12] and is required for the early steps of the infection process [9,28]. We show here that another quorum sensor, NprR, is active after host death and is necessary for *Bt* to survive in the insect cadaver. NprR is a pleiotropic regulator directly or indirectly affecting the expression of at least 41 genes during the stationary phase. About 30% of the NprR-regulated genes encode extracellular or cell wall-associated proteins involved in the degradation of proteins, lipids and chitin. We report that *nprA* and two other NprR-regulated genes encoding degradative enzymes were expressed after death of the host. Therefore, it is likely that these enzymes allow *Bt* to use the content of the host, indicating that *Bt* displays a necrotrophic lifestyle in the insect cadaver. This nutrient acquisition may support the developmental program of *Bt* until sporulation. The degradative enzymes may also have other functions. Insect cuticles are made of chitin filaments arranged within a protein matrix which constitutes a physical barrier to the outside environment. Degradative enzymes may degrade this barrier and facilitate spore and toxin release into the environment. Degradative enzymes may also participate in cell protection against competitors. For example, the endochitinase ChiCW was reported as having antifungal properties [29], and InhA3 (BC2984) is a member of the Immune Inhibitor A metalloprotease family, which plays a key role in the resistance to the host immune defenses by degrading antimicrobial peptides [30,31,32]. In addition, two substrate-binding proteins (BC2827 and the BC3526) may increase the efficiency of these enzymes.

A large locus of three NprR-regulated genes (*krsABC*) codes for a NRPS system involved in the synthesis of a secreted lipopeptide called kurstakin. At least three suggestions could explain the important and surprising function of kurstakin:

- 1) We show that this lipopeptide is essential for *Bt* to survive in the cadaver. Lipopeptides have biosurfactant activity, and we show that kurstakin is necessary for swarming mobility and biofilm formation. Thus, the kurstakin may possibly allow *Bt* to spread across the cadaver, facilitating access to new substrates.
- 2) Lipopeptides are potent antimicrobials, and kurstakin has an antifungal activity [21,33,34]. Kurstakin may thus act as an antimicrobial molecule and prevent colonization and growth by competing microorganisms.
- 3) Lopez and coll. [26,35] demonstrated that surfactin is a pore-forming molecule causing a potassium leakage across the membrane of *B. subtilis*. This molecule acts as a signal triggering multicellularity: one subpopulation of bacterial cells produces surfactin and another responds to it by producing extracellular matrix. Here, we show that kurstakin may similarly induce potassium leakage across the cytoplasmic membrane of *Bt*. In addition, bioassays in *G. mellonella* indicate that at least two bacterial subpopulations coexist after host death: one subpopulation enters into sporulation, while the other remains in a vegetative form (Figure 2B) and expresses the NprR-regulated genes (Figure 3B). By analogy,

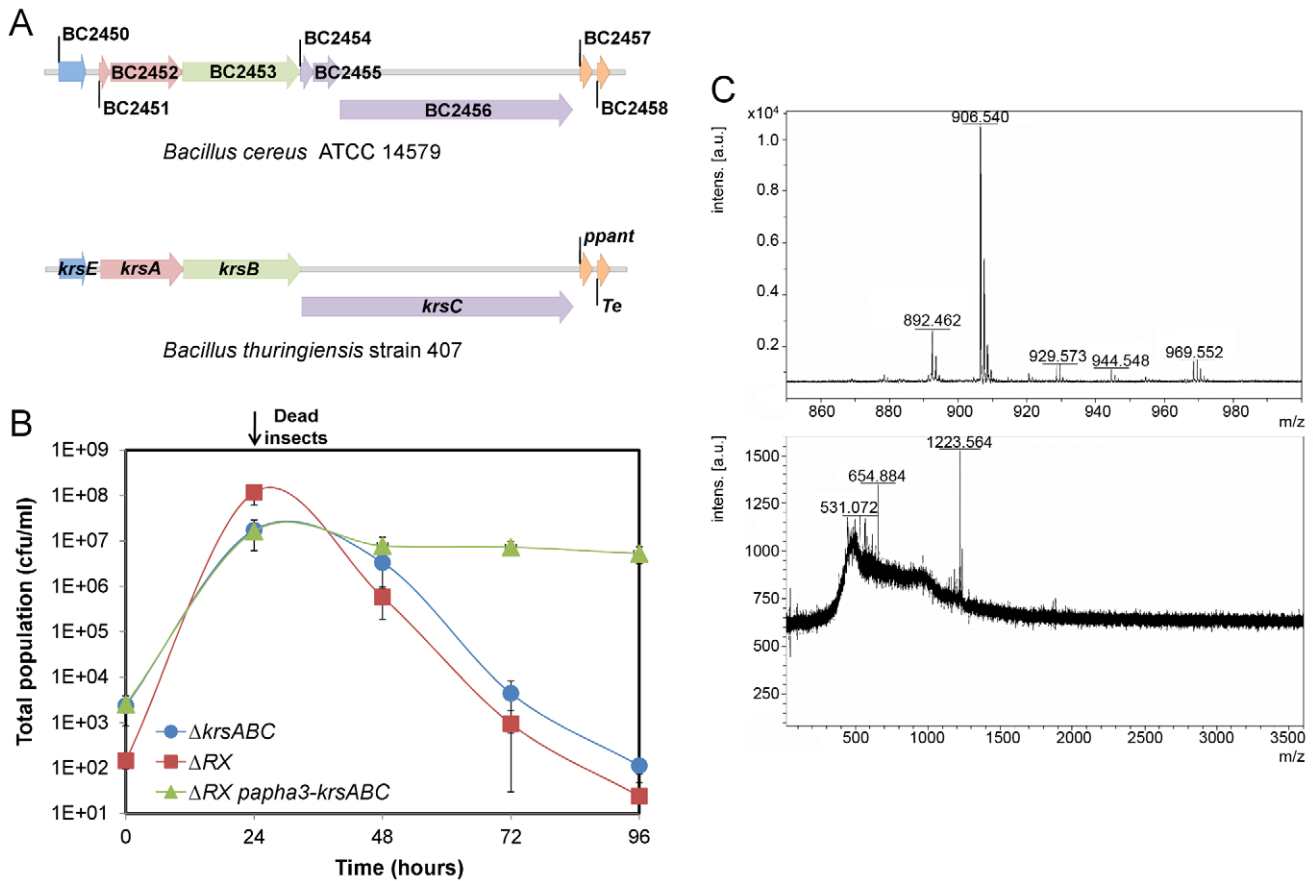


Figure 5. The *krsABC* genes are involved in the production of kurstakin, a lipopeptide essential for the necrotrophic lifestyle of *Bt*. (A) Genetic organization of the *krsEABC* locus in *Bc* ATCC14579 and *Bt* strain 407. *krsE* encodes a putative efflux protein. *krsA*, *krsB* and *krsC* encode non ribosomal peptide synthetases KrsA, KrsB and KrsC, respectively. Immediately downstream is a gene encoding a phosphopantheineyl transferase (*ppant*) and a gene encoding a protein with a thioesterase domain; both these genes are involved in kurstakin synthesis. (B) The total population of the $\Delta krsABC$ strain throughout the experiment was similar to the total population of the ΔRX strain. The constitutive expression of the *krsABC* genes in the ΔRX strain restored the survival of this mutant. (C) MALDI-ToF mass spectra of the wt (upper panel) and $\Delta krsABC$ (lower panel) strains. Four peaks corresponding to kurstakin were identified for the wt strain and none of these peaks were detected in the $\Delta krsABC$ strain. Molecular ions are apparent for kurstakins C12 [$M+H^+$] at 892 and C13 [$M+H^+$] at 906, [$M+Na^+$] at 929, and [$M+K^+$] at 944 identified by Price *et al.* (2007).

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kurstakin may also be a signaling molecule allowing *Bt* cells to differentiate into subpopulations. Bacterial heterogeneity could provide an advantage to the bacteria for the survival to sudden changes in the insect cadaver environment.

Some NprR-regulated genes encoding cytoplasmic or membrane-associated proteins may participate in the necrotrophic development of *Bt*. A putative efflux system (BC1063), two macrolide glycosyl transferases (BC2066 and BC2622) and a N-hydroxyarylamine O-acetyltransferase could be involved in resistance to antimicrobial molecules, and cytochrome P450 (BC2613) may be involved in resistance to reactive oxygen species. The membrane-associated proteins are mainly components of an oligopeptide permease system (Opp) involved in the uptake of PapR, the signaling peptide required for PlcR activation [36]. The operon encoding this Opp system is downregulated by NprR suggesting that NprR controls PlcR expression negatively through the Opp system. Interestingly, *nprA* expression was only slightly reduced in a *oppB* mutant strain suggesting that another oligopeptide permease system is involved for the uptake of NprX (Figure S4). Consequently, a down-regulation of the *opp* genes

could not have a significant effect on the expression of the NprR-regulated genes. Possibly, the extracellular concentration of NprX is a signal triggering the transition from a pathogenic to a necrotrophic lifestyle. During the early stage of infection, the PlcR regulon is expressed and the extracellular concentration of NprX might be low. Indeed, recent results indicate that *nprX* transcription starts in late stationary phase (TD, SP, DL, unpublished data). In view of these data, we hypothesize that after the host death, the extracellular concentration of NprX increases, leading to the expression of NprR-regulated genes and repression of the PlcR regulon *via* the Opp transporter. These various observations indicate that the necrotrophic lifestyle of *Bt* is a complex developmental stage, not limited to simple feeding on the host contents. They also imply that the transition from a pathogenic to a necrotrophic lifestyle is associated with significant metabolic changes.

It is becoming clear that the infectious cycle of *Bt* can be divided into four distinct and sequential phases starting with toxemia caused by the Cry proteins, followed by the action of PlcR in virulence, necrotrophism and the completion of the sporulation process involving NprR, and finally the dissemination of spores

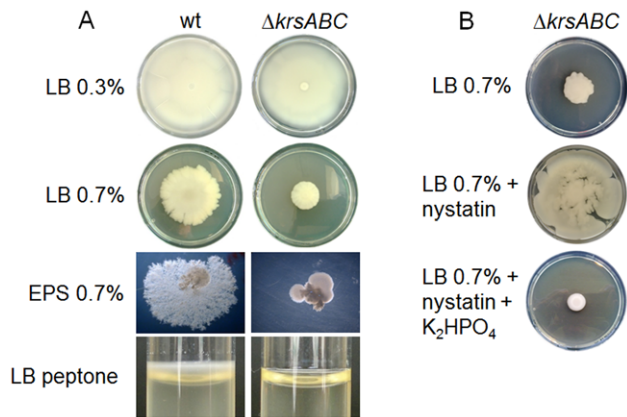


Figure 6. Kurstakin is required for swarming mobility and biofilm formation. (A) Mobility and morphology of the wt and $\Delta krsABC$ strains were compared on LB 0.3% agar, LB 0.7% agar and EPS 0.7% agar. Biofilm formation by the two strains was also assessed in glass tubes containing 2 ml of LB peptone. A $\Delta krsABC$ strain is unable to swarm or to form biofilm at the air/liquid interface. (B) The swarming mobility of the $\Delta krsABC$ strain is restored by the addition of nystatin, and this phenotype is reversed by the addition of K_2HPO_4 . doi:10.1371/journal.ppat.1002629.g006

(Figure 7). We describe a *nprR-nprX* mutant that does not develop necrotrophically and is unable to sporulate efficiently, demonstrating that the necrotrophic properties of *Bt* are essential for horizontal transmission. It is remarkable that the developmental process through the complete infection cycle in the insect host is coordinated by three regulator-signaling peptide cell-cell communication systems: PlcR-PapR, NprR-NprX and the Rap-Phr complexes which control the phosphorylation of Spo0A [37,38,39].

Members of the *Bc* group may be able to follow two different life cycles: an infectious cycle (described above) and an endosymbiotic cycle in which the bacteria live in a symbiotic relation with their invertebrate hosts [6,40]. Here, we report that *Bt* multiplies efficiently in the insect cadaver and has a genetic developmental program to live in this biotope. Consequently, we propose the existence of a strictly necrotrophic life cycle in which *Bt* colonizes a wide variety of dead insects, and uses the cadaver as a bioreactor to multiply and to produce spores and toxins.

In silico analysis reveals that *nprR* is found in all strains of the *Bc* group, except that in *Bc* ATCC14579 *nprR* is disrupted by a transposon [3]. The published genome sequences of *Ba* and *Bc* strains provide no evidence for any loss of genetic determinants, which might be crucial for saprophytic survival [41]. Therefore, the function of NprR is probably conserved in the *Bc* group, and it would be interesting to determine whether the necrotrophic development of *Bc* and *Ba* in their mammalian hosts requires the same quorum-sensing regulated network. It is also important to characterize kurstakin to determine how it promotes survival in insect cadavers. Moreover, the properties of this molecule may indicate possibilities for the development of phytosanitary products or adjuvants to improve both the ecological fitness and the efficacy of biopesticides.

Materials and Methods

Bacterial strains and growth conditions

The *Bt* strain 407 Cry⁻ is an acrySTALLIFEROUS strain cured of its *cry* plasmid [42]. This strain shares high phylogenetic similarity with *Bc* [43]. *Bacillus* 407 *oppB::tet*, *Bacillus* 407 *sigK::aphA3*, *Bacillus* 407 *nprRX::tet* (ΔRX), *Bacillus* 407 *nprA::lacZ* and *Bacillus* 407 *nprRX::tet*

nprA::lacZ mutant strains were described previously [16,20,36]. *Escherichia coli* K-12 strain TG1 was used as host for the construction of plasmids and cloning experiments. Plasmid DNA for *Bacillus* electroporation was prepared from the Dam⁻ Dcm⁻ *E. coli* strain ET12567 (Stratagene, La Jolla, CA, USA). *E. coli* and *Bt* cells were transformed by electroporation as described previously [42,44]. *E. coli* strains were grown at 37°C in Luria Broth (LB). *Bacillus* strains were grown at 30 or 37°C in LB or in HCT, a sporulation-specific medium [45]. The following concentrations of antibiotic were used for bacterial selection: 100 µg/ml ampicillin for *E. coli*; 200 µg/ml kanamycin, 10 µg/ml tetracycline, 200 µg/ml spectinomycin and 10 µg/ml erythromycin for *Bacillus*. Numbers of viable cells were counted as total colony-forming units (cfu) on LB plates. Numbers of spores were determined as heat-resistant (80°C for 12 min) cfu on LB plates.

In vivo experiments

Force-feeding and intrahemocelic injection experiments with *G. mellonella* were carried out as described previously [9]. LD₅₀ data were analyzed using the program StatPlus 2007 of Analysoft. *Bt* cells in living and dead insects were counted as follows. For each strain, each larva was injected with 2.10⁴ bacteria and kept at 30°C for 96 h; 24 h after injection, surviving insects were eliminated. At the injection time and every 24 h for the 96 h of the experiment, two larvae were crushed and homogenized in 10 ml of physiological water and dilutions were plated onto LB agar plates containing appropriate antibiotics. To follow the spore population, bacterial colony-forming units were determined before and after treatment of the insect homogenate for 12 min at 80°C. At least four independent replicates were performed for each time and for each strain tested. *In vivo* β-galactosidase activity was assayed from 2 ml aliquots of the insect homogenate as described previously [16]. At least three independent measurements were performed for each time and for each transcriptional fusion tested.

DNA manipulations

Chromosomal DNA was extracted from *Bt* cells using the Puregene Yeast/Bact. Kit B (QIAGEN, France). Plasmid DNA was extracted from *E. coli* using QIAprep spin columns (QIAGEN, France). Restriction enzymes (New England Biolabs, USA) and T4 DNA ligase (New England Biolabs, USA) were used in accordance with the manufacturer's recommendations. Oligonucleotide primers (Table S4) were synthesized by Sigma-Proligo (Paris, France). PCRs were performed in a Applied Biosystem 2720 Thermakycler (Applied Biosystem, USA). Amplified fragments were purified using the QIAquick PCR purification Kit (QIAGEN, France). Digested DNA fragments were separated on 1% (w/V) agarose gels after digestion and extracted from gels using the QIAquick gel extraction Kit (QIAGEN, France). Nucleotide sequences were determined by Beckman Coulter Genomics (Takeley, UK)

Plasmid constructions

The plasmid pRN5101 [46] was used for homologous recombination. The low-copy-number plasmid pHT304 was used for complementation experiments with wild-type *nprR-nprX* genes under their own promoters [16]. Transcriptional fusions were constructed in pHT304-18Z [47]. All the plasmids used in this study are described in Table S5.

Construction of the *B. thuringiensis* recombinant strains

The *krsABC* genes were disrupted by inserting a spectinomycin resistance gene into the coding sequence. The thermosensitive

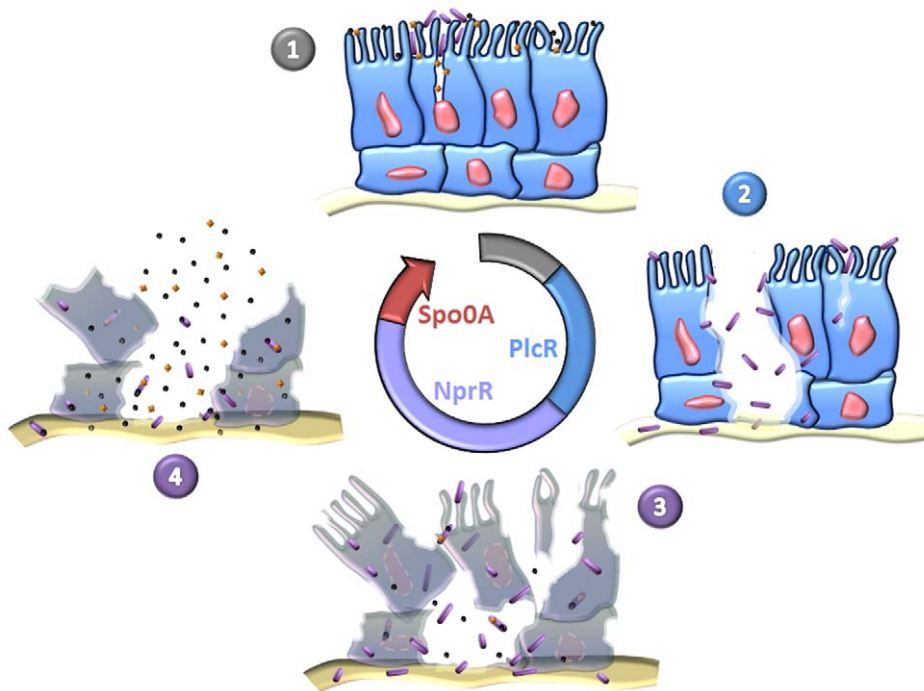


Figure 7. Sequential expression of master regulators during the infectious cycle of *Bt*. (1) Toxins and spores reach the intestinal epithelium. The toxins induce cell lysis and create favorable conditions for spore germination. The resulting vegetative bacteria adhere to cells weakened by toxins and can grow. (2) The bacteria produce PlcR-regulated virulence factors which attack the intestinal barrier. This event allows the bacteria to penetrate into deeper tissues, such that they can multiply in body fluids and cause host death. (3) The bacteria shift from a virulent lifestyle to a necrotrophic lifestyle and change their gene expression pattern. The pleiotropic regulator NprR activates the transcription of genes encoding proteins involved in food supply, stress resistance, protection against competitors and kurstakin synthesis. These factors increase bacterial fitness in the host cadaver, allowing the bacteria to produce Cry toxins (orange diamonds) and to pursue their developmental program until sporulation and production of resistant spores. (4) Spores and toxins disseminate in the environment through various abiotic (wind, rain) and biotic (parasites, predators) mechanisms.

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plasmid pRN5101 Ω *krsABC::spc* was used to disrupt the chromosomal wild-type copy of the *krsABC* genes in the *Bacillus 407* wt strain by homologous recombination as described previously [46]. The recombinant strain, designated *Bacillus 407* Δ *krsABC*, was resistant to spectinomycin and sensitive to erythromycin. The thermosensitive plasmid pRN5101 Ω P_{*krsABC*}::*aphA3* was used to replace the natural promoter region of the *krsABC* genes in the *Bacillus 407* Δ RX strain by *aphA3* and its constitutive promoter. In the resulting *Bacillus* recombinant strain, the *krsABC* genes were transcribed from the *aphA3* promoter; it was designated *Bacillus 407* Δ RX P_{*aphA3*}-*krsABC*, and was resistant to kanamycin and sensitive to erythromycin.

Phenotype analysis

The methods used to study the proteolytic activity, the chitinolytic activity and the lipolytic activity have been described previously [32,48]. Swimming and swarming were evaluated using LB 0.3% agar plates and LB 0.7% agar plates, respectively. Biofilm formation was assayed in LB medium and in glass tubes as described previously [27]. Dendrite formation was evaluated on EPS 0.7% agar. Strains were cultured in LB medium at 37°C until the beginning of stationary phase and 2.10⁶ bacteria were spotted onto the center of the agar plate. Plates were incubated at 37°C for 24 h to 96 h.

β -Galactosidase assay

For *in vitro* β -galactosidase activity measurements, *Bt* cells containing *lacZ* transcriptional fusions were cultured in LB medium at 37°C. *In vivo* β -galactosidase activity was assayed from

2 ml aliquots of insect homogenate (see *in vivo* experiments). β -Galactosidase activities were measured as described previously [49]. The specific activities are expressed in units of β -galactosidase per milligram of protein (Miller units).

Samples for microarrays and quantitative RT-PCR

Prewarmed 500 ml baffled erlenmeyer flasks with 50 ml LB medium were inoculated with 1 ml overnight cultures of *Bacillus 407* *nprA::lacZ* or *Bacillus 407* Δ RX *nprA::lacZ*, and incubated at 37°C and 250 rpm. Samples for microarray analysis were taken three (t3) hours after the onset of the stationary phase. Samples were harvested as described previously [12] mixed with RLT buffer from the RNeasy midi kit (Qiagen, France) and frozen at -70°C. After thawing samples at 37°C for 15 min, RNA isolation, cDNA synthesis, labeling and purification were performed as described [12].

Microarray comparisons

The microarray slides were printed, prehybridized and hybridized as described previously [12], except that hybridization was extended to 17 hours. The slides were scanned on an Axon 4000B scanner (Molecular Devices). Gridding, spot annotation and calculation of raw spot intensities was done with the GenePix Pro 6.1 software (Molecular Devices). The LIMMA package [50,51,52] on the R 2.7.1 platform [53] was used for filtering, normalization and further analysis. The raw data were filtered and weighted by quality [54], and the four technical replicates on each slide were averaged to increase robustness. P-values were computed using a false discovery rate of 0.05. The analysis was

based on hybridization to three slides, all employing biological replicates.

Quantitative RT-PCR

Gene expression was investigated in *Bacillus* 407 *nprA::lacZ* and *Bacillus* 407 Δ RX *nprA::lacZ*. Reverse transcription was performed according to the SuperScript III reverse transcriptase protocol from Invitrogen, but RNaseOUT was replaced with 0.1 μ l SUPERase-In (Ambion). A negative control without reverse transcriptase was included. In all samples, the reaction volume was adjusted to 20 μ l with DEPC-treated water (Ambion) before reverse transcription. The reaction product was diluted (1 μ l in 39 μ l) with water, and 8 μ l applied to each well (2 μ l for 5 s rRNA samples). Primers were added to a final concentration of 0.56 μ M. A volume of 9 μ l LightCycler 480 DNA SYBR Green I Master (Roche) was added, and the volume was adjusted to 18 μ l. Primers (available on request) were designed to give PCR products of around 100 bp. The reference genes, *gatB* (BC4306) and 5 s rRNA, were included on every plate. The samples were analyzed on a Roche Lightcycler 480 (Roche Diagnostics GmbH, Mannheim, Germany). Cycling conditions were 95°C for 5 minutes followed by 45 cycles at 95°C for 10 seconds, 58°C for 10 seconds, and 72°C for 8 seconds. Cp values were determined using 2nd derivative max, and are averages of two technical replicates. The results were calculated by the delta-delta C_t approximation. The log₂ expression ratios of *Bacillus* 407 Δ RX *nprA::lacZ* over *Bacillus* 407 *nprA::lacZ* in Table S3 are averages for three biological replicates.

Mass spectrometry analysis

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-ToF MS) was used to screen kurstakin production from whole bacterial cells on solid media. Cultures were performed on AK agar plates incubated at 30°C for 24 h or 48 h. A saturated solution of α -cyano-4-hydroxy-cinnamic acid was prepared in 1:2 (v/v) solution of CH₃CN and H₂O containing 0.1% TFA. Measurement was performed using UV laser MALDI-ToF spectrometer (Bruker UltraFlex TOF; Bruker Daltonics) equipped with a pulsed nitrogen laser ($\lambda = 337$ nm). The ions were extracted from the ionization source with an acceleration voltage of 20 kV. Samples were measured in the reflector mode, positive mode. The equivalent of about 1 μ l of cell material was picked from agar plates with an automatic pipette. The tip with the culture was deposited in an eppendorf tube. 20 μ l of matrix solution (saturated solution of α -cyano-4-hydroxycinnamic acid in a 1:2 v/v solution of CH₃CN/H₂O with 0.1% TFA) are added. The eppendorf with the tip and the matrix solution was vortexed for 30 seconds. 1 μ l of this sample solution was deposited on the MALDI target and let dry at room temperature. The spectrum was obtained with 5 \times 30 shots on the sample. Analyses were performed on two different samples.

Supporting Information

Figure S1 Growth kinetics of reporter strains. (A) Total population of the 407 *paphA3'*Z, 407 *pnprA'*Z and 407 *pmpbE'*Z strains in the insect larvae. (B) Total population of the 407 pBC0429'Z and 407 pBC2167'Z strains in the insect larvae. Data are averages of at least three independent experiments (error bars are SEM from mean values). (TIF)

Figure S2 *nprA* is not required for the necrotrophic lifestyle of *Bt*. The total population of the Δ *nprA* strain was similar to that of the wt strain throughout the experiment

indicating that *nprA* is not required for *Bt* to survive in the insect host. Data are averages of at least four independent experiments (error bars are SEM from mean values). (TIF)

Figure S3 Differential expression of nine genes was confirmed with *lacZ* fusions. Fusions of the promoter region of the genes tested to a *lacZ* reporter on the plasmid pHT304-18Z were introduced into the wt (circles) and the Δ RX strains (squares), and expression was measured. Time on the x-axis is given relative to the transition to stationary phase (t₀). β -Galactosidase activity in Miller units (MU) is plotted on the y-axis. Each assay was repeated at least twice independently and a representative graph is shown for each experiment. (TIF)

Figure S4 Expression of the P_{*nprA*}-*lacZ* chromosomal fusions into the wt (circles) and the *lappB* strains (squares). Time on the x-axis is given relative to the transition to stationary phase (t₀). β -Galactosidase activity in Miller units (MU) is plotted on the y-axis. Assays were repeated at least three times independently and a representative graph is shown. (TIF)

Table S1 NprR is not involved in the pathogenicity of *Bt*. (DOC)

Table S2 The Δ RX strain is unable to sporulate in the insect host. These experiments were done at 30°C. The percentages were calculated as 100 \times the ratio between heat-resistant spores ml⁻¹ and viable cells ml⁻¹. For both strains, the OD₆₀₀ at t₀ in HCT medium was 2.2 \pm 0.1 and that in LB was 2.6 \pm 0.1. n is the number of independent sporulation efficiency measurements. Results are given as mean \pm SEM. (DOC)

Table S3 NprR is a pleiotropic regulator. The NprR-regulated genes can be classified into four functional groups encoding: stress resistance proteins (in purple); an oligopeptide permease (in green); a NRPS system (in orange); and food supply proteins (in blue). This table includes genes more than four times differentially regulated in Δ RX strain relative to the wt strain at t₃ in LB medium, as confirmed by qRT-PCR (expression ratios). Locus tags for the *Bc* ATCC 14579 microarray probes are listed. (DOC)

Table S4 Primers used for construction of recombinant strains and transcriptional fusions. Restriction sites are underlined. (DOC)

Table S5 Plasmids used in this study. (DOC)

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Author Contributions

Conceived and designed the experiments: TD KF ABK DL. Performed the experiments: TD KF SP CL CB NR PJ. Analyzed the data: TD KF CNL MG PJ NR ABK DL. Contributed reagents/materials/analysis tools: TD KF. Wrote the paper: TD KF ABK DL.

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