SinR Controls Enterotoxin Expression in *Bacillus thuringiensis* Biofilms

Annette Fagerlund1, Thomas Dubois2,3, Ole-Andreas Økstad1, Emilie Verplaetse2,3, Nathalie Gilois2,3, Imène Bennaceur2,3, Stéphane Perchat2,3, Myriam Gominet4, Stéphane Aymerich2,3, Anne-Brit Kolstø1, Didier Lereclus2,3, Michel Gohar2,3

1 Laboratory for Microbial Dynamics (LaMDa) and Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, Oslo, Norway, 2Micalis, INRA (UMR1319), Domaine de Vilvert, Jouy-en-Josas, France, 3Micalis, AgroParisTech (UMR1319), Domaine de Vilvert, Jouy-en-Josas, France, 4Institut Pasteur, CNRS URA 2172, Unité de Biologie des Bactéries Pathogènes à Gram positif, Paris, France

**Abstract**

The entomopathogen *Bacillus thuringiensis* produces dense biofilms under various conditions. Here, we report that the transition phase regulators Spo0A, AbrB and SinR control biofilm formation and swimming motility in *B. thuringiensis*, just as they control biofilm formation and swimming motility in the closely related saprophyte species *B. subtilis*. However, microarray analysis indicated that in *B. thuringiensis*, in contrast to *B. subtilis*, SinR does not control an eps operon involved in exopolysaccharide production, but regulates genes involved in the biosynthesis of the lipopeptide kurstakin. This lipopeptide is required for biofilm formation and was previously shown to be important for survival in the host cadaver (necrotrophism). Microarray analysis also revealed that the SinR regulon contains genes coding for the Hbl enterotoxin. Transcriptional fusion assays, Western blots and hemolysis assays confirmed that SinR controls Hbl expression, together with PlcR, the main virulence regulator in *B. thuringiensis*. We show that Hbl is expressed in a sustained way in a small subpopulation of the biofilm, whereas almost all the planktonic population transiently expresses Hbl. The gene coding for SinI, an antagonist of SinR, is expressed in the same biofilm subpopulation as hbl, suggesting that hbl transcription heterogeneity is SinI-dependent. *B. thuringiensis* and *B. cereus* are enteric bacteria which possibly form biofilms lining the host intestinal epithelium. Toxins produced in biofilms could therefore be delivered directly to the target tissue.

Citation: Fagerlund A, Dubois T, Økstad O-A, Verplaetse E, Gilois N, et al. (2014) SinR Controls Enterotoxin Expression in *Bacillus thuringiensis* Biofilms. PLoS ONE 9(1): e87532. doi:10.1371/journal.pone.0087532

**Introduction**

*Bacillus subtilis* and pathogenic bacteria of the *Bacillus cereus* group (*B. cereus*, *B. thuringiensis* and *B. anthracis*) are all Gram-positive, flagellated, sporulating, and aerobic bacteria clustering closely in the phylogenetic tree of the *Bacillus* genus ([1]; http://www.patricbrc.org/portal/portal/patric/Phylogeny?cType = taxon&cId = 1396). They share a large number of transcriptional factors, including the sporulation regulator Spo0A, the stress response sigma factor σρ, and the phase-transition regulators SinI, SinR, CodY and AbrB [2]. However, there are also important differences in the regulatory pathways between *B. subtilis* and *B. cereus sensu lato*. For example, the stress regulator σρ is not activated in the same way in these species [3]: the two-component system DegU/DegS and the motility regulator SigD are absent from *B. cereus sensu lato* [2]; the virulence regulator PlcR, which promotes the transcription of numerous genes for extracellular enzymes and toxins and plays an important role in *B. cereus* and *B. thuringiensis* physiology [4–6], is absent from *B. subtilis*. These differences may well be the consequences of adaptation of these species to different ecosystems. *B. subtilis* is a saprophyte living on soil organic matter, whereas, *B. thuringiensis* is an entomopathogenic bacterium, genetically closely related to the human opportunistic pathogen *B. cereus* [7,8], and to the human pathogen *B. anthracis* [9].

Both *B. subtilis* and *B. thuringiensis*, or *B. cereus*, can form biofilms at air-liquid interfaces. Biofilms are widely found structures in which microorganisms are protected against various stresses, allowing them to persist in adverse environmental conditions. The regulatory pathways in *B. subtilis* leading either to biofilm formation or to sporulation share the same initial steps. The transcriptional regulator Spo0A controls entry into sporulation [10], and is required for biofilm formation [11]. Spo0A represses _abrB_ transcription [12] and promotes the transcription of _sinI_ [13], the product of which is the SinR antagonist SinI. Both AbrB and SinR repress the two polycistronic operons _tapA-sipW-tasA_ and _epsK-O_ [14,15]. The 15-gene _epsA-O_ operon is involved in the biosynthesis of the exopolysaccharide component of the biofilm matrix [16] and the three-gene _tapA-sipW-tasA_ operon is involved in the production of the protein component of the biofilm matrix [17,18]. An inhibitor of flagellar motility is encoded by the _epsE_ gene which is part of the _epsA-O_ operon [19]. Therefore, deletion of _sinR_ from *B. subtilis* results in an overproduction of biofilm and
in impaired motility, whereas deletion of sinI results in the reverse phenotype. A parologue of SinR, SlrR, is also involved in the control of biofilm formation and motility through its interaction with SinR [20,21].

How biofilm formation is regulated in B. thuringiensis or in B. cereus is still unknown. In B. anthracis, SinR strongly represses the sipW-tasA operon [22], but the effect of sinR deletion on biofilm formation has not been studied. The quorum sensing molecule AI-2 is produced by B. cereus and inhibits biofilm formation when added exogenously [23], and the transcriptional regulators PclR and CodY affect biofilm formation in the B. cereus reference strain ATCC 14579 [24–26]. PclR is the main virulence regulator in B. cereus [6] and CodY, which represses the biosynthesis of branched amino-acids, might also be involved in the pathogenicity of B. cereus [26–29]. These findings suggest a connection between biofilm formation and virulence in this species. Here we report an investigation of the roles of Spo0A, AbrB and SinI/SinR in biofilm formation in the B. thuringiensis strain 407, which produces dense pellicles at the air-liquid interface. We found that SinI/SinR had a large effect on biofilm formation. We therefore analyzed the B. thuringiensis sinR regulon, which was found to include the sipW-tasA operon, but surprisingly no eps operon. SinR was also found to control the transcription of genes required for the production of lipopeptides previously shown to be involved in the bacterial survival in the host [30], and the transcription of enterotoxin genes.

Materials and Methods

Strains

Strains used in this study are listed in table S1. The acrystalliferous B. thuringiensis strain 407 Cry- (genome sequence at NCBI: NZ_CM000747) is genetically closely related to the B. cereus reference strain ATCC 14579 [31]; however, strain 407 forms thick biofilms, while ATCC 14579 is a poor biofilm producer. Locus tags listed below follow the annotations of the sequenced ATCC 14579 strain genome (NC_004622), and the corresponding locus tags in the sequenced 407 strain genome are given table S2.

Strain construction

The sinI-sinR locus in strain 407 was disrupted by insertion of a tetracycline resistance (TetR) cassette. A 937 bp HindIII–EcoRI fragment and a 758 bp tetracycline resistance (TetR) cassette. A 937 bp HindIII–EcoRI fragment was purified from pH1047S3 [32] as a 1.5 kb HindIII–EcoRI fragment, corresponding to the ring adhering at the air-liquid-solid interface. In the glass staining procedure and the biofilm mass determined corresponds to the ring adhering at the air-liquid-solid interface. In the glass tube assay, the entire biofilm is recovered. Biofilms were formed in the microtiter plate and glass tube assays measure different parts of the pellicle, thoroughly vortexed in 1 ml PBS, was measured. The DNA sequence containing the sinI-SOE-Fwd/psinI-Rev BamHI and phbl-sinI-SOE-Rev/hbl'-yfp, resulting in pHT304-18Z [35], the promoter and the hbl promoter region and lacZ. The DNA sequence containing the hbl promoter was amplified using primers Hbl_pHT304_FW and Hbl_pHT304_RV (table 1) and inserted into pHT304-18Z [35], to give pHT304-18OPw'-lacZ. The same DNA sequence was inserted into pHT304-18ZmCherry, resulting in pHT304-18ZmCherry. The DNA sequence containing the hbl promoter was amplified from pDG783 [34] with primers Apha3_pHT304_FW and Apha3_pHT304_RV (table 1) and inserted into pHT304-18ZmCherry, resulting in pHT304-18ZmCherry. The plasmid pHT304-18ZmCherry was constructed by digestion of between the sites EcoRI and KpnI of pHT304-18 [36], of the yfp gene amplified from pKL183 [37] using the primer pair Yfp-F and Yfp-R (table 1). The plasmid pHT304-18OPw'-yfp-Psad-yfp into monocytone simultaneously, in the same cell, hbl and sinI expressions, was constructed as follows. DNA fragments containing the promoter of sinI and hbl were amplified by PCR using the primers phbl-sinI-SOE-Fwd/psinI-Rev BamHI and phbl-sinI-SOE-Rev/Hbl_pHT304_RV, respectively (table 1). These fragments were annealed to each other through complementary overlapping sequences introduced in primers phbl-sinI-SOE-Fwd and phbl-sinI-SOE-Rev. A single DNA fragment containing the promoter elements of sinI and hbl in opposite directions was then generated by PCR amplification with the primers psinI-Rev BamHI and Hbl_pHT304_RV. The resulting 1225 bp fragment was digested with BamHI and cloned in the promoter free pHT304-18Z-yfp-mCherry digested with the same enzyme.

Electroporation was used to transfer pHT304-18OPw'-lacZ, pHT304-18OPw'-yfp, pHT304-18OPw'-yfp-yfp into 407 wild type or into 407 sinI strains. Beta-galactosidase specific activities were measured as described previously, and are expressed in units of beta-galactosidase per milligram of protein [38]. Beta-galactosidase was extracted from cells in biofilm obtained in glass tube assays (see below), and from planktonic cultures grown in LB medium at 30°C and 175 rpm. Three replicates were performed for each assay.

Biofilm and Enterotoxin in B. thuringiensis

Expression of the hbl operon in the 407 wild type and mutant strains was monitored using a transcriptional fusion between the hbl promoter region and lacZ. The DNA sequence containing the hbl promoter was amplified using primers Hbl_pHT304_FW and Hbl_pHT304_RV (table 1) and inserted into pHT304-18Z [35], to give pHT304-18OPw'-lacZ. The same DNA sequence was inserted into pHT304-18ZmCherry, resulting in pHT304-18ZmCherry. The DNA sequence containing the hbl promoter was amplified from pDG783 [34] with primers Apha3_pHT304_FW and Apha3_pHT304_RV (table 1) and inserted into pHT304-18ZmCherry, resulting in pHT304-18ZmCherry. The plasmid pHT304-18OPw'-yfp-Psad-yfp into monocytone simultaneously, in the same cell, hbl and sinI expressions, was constructed as follows. DNA fragments containing the promoter of sinI and hbl were amplified by PCR using the primers phbl-sinI-SOE-Fwd/psinI-Rev BamHI and phbl-sinI-SOE-Rev/Hbl_pHT304_RV, respectively (table 1). These fragments were annealed to each other through complementary overlapping sequences introduced in primers phbl-sinI-SOE-Fwd and phbl-sinI-SOE-Rev. A single DNA fragment containing the promoter elements of sinI and hbl in opposite directions was then generated by PCR amplification with the primers psinI-Rev BamHI and Hbl_pHT304_RV. The resulting 1225 bp fragment was digested with BamHI and cloned in the promoter free pHT304-18OPw'-yfp-mCherry digested with the same enzyme.

Electroporation was used to transfer pHT304-18OPw'-lacZ, pHT304-18OPw'-yfp, pHT304-18OPw'-yfp-yfp into 407 wild type or into 407 sinI strains. Beta-galactosidase specific activities were measured as described previously, and are expressed in units of beta-galactosidase per milligram of protein [38]. Beta-galactosidase was extracted from cells in biofilm obtained in glass tube assays (see below), and from planktonic cultures grown in LB medium at 30°C and 175 rpm. Three replicates were performed for each assay.

Biofilm assays

The ability of 407 wild type and mutant strains to form biofilms in PVC (polyvinylchloride) microtiter plates was measured as described previously [23]. The method used to obtain biofilm in glass tubes was similar to that used in microtiter plates, with the following differences. Sterilized 6 ml glass tubes were inoculated with 2 ml of the cultures diluted to an OD_{600} of 0.01, and incubated for 48 h. The 2 ml culture medium was then removed using a Pasteur pipette and the OD_{600} of the ring and of the pellet, thoroughly vortexed in 1 ml PBS, was measured. The microtiter plate and glass tube assays measure different parts of the biofilm: in the microtiter plate assay, the pellet is lost during the staining procedure and the biofilm mass determined corresponds to the ring adhering at the air-liquid-solid interface. In the glass tube assay, the entire biofilm is recovered. Biofilms were formed in glass tubes for binocular microscopy observation, for beta-galactosidase assays, for fluorescence microscopy observation or for flow cytometry experiments.

Swimming assays

The swimming ability of the 407 mutant strains was determined on LB 0.3% agar plates. Strains were grown in LB medium at
### Table 1. Primers used in this study.

| Primer            | sequence*                                      | restriction site |
|-------------------|------------------------------------------------|------------------|
| Sin1              | CCGAAGCTTTTACGAAACTGTTAACC                     | HindIII          |
| Sin2              | CCGAATTCGCAAGTCTAGTAAAGTGA                   | EcoRI            |
| Sin3              | GGCCTGATGCTGTGTTGTC                        | XbaI             |
| Sin4              | CCGGATCTGTATACGAAACTACATTAGC                   | BamHI            |
| Sin11             | GGCCTGATGCTGTGTTGTC                        | XbaI             |
| Sin12             | CCGAATTCGCAAGTCTAGTAAAGTGA                   | EcoRI            |
| Sin14             | CCGAATTCGCAAGTCTAGTAAAGTGA                   | EcoRI            |
| Sin17             | GGTCTGATGCTGTGTTGTC                        | XbaI             |
| Sin18             | CCGGATCTGTATACGAAACTACATTAGC                   | BamHI            |
| AbrB1FW           | CCAAGCTTTGGGCTGCTTAAATCTCTCTACTCCGGCG        | HindIII          |
| AbrB1RV           | GCCTAGAGACACATATGGATATTGGGATATGATATGATATGATGA | XbaI             |
| AbrB2FW           | AAAGTGCAGCACTATAGGACACTATGATATTGGGATATGATATGC | PstI             |
| AbrB2RV           | CCGGATCTGTATACGAAACTACATTAGC                   | BamHI            |
| Hbl_pHT304_FW     | CCAAGCTTTGGGCTGCTTAAATCTCTCTACTCCGGCG        | HindIII          |
| Hbl_pHT304_RV     | CCGGATCTGTATACGAAACTACATTAGC                   | BamHI            |
| Yfp_F             | GGGGATCCACATAGGCGAGAACTATATGATATTGGGATATGATATG | NsiI             |
| Yfp_R             | CCGGATCTGTATACGAAACTACATTAGC                   | EcoRI            |
| Apha3_pHT304_FW   | CCAAGCTTTGGGCTGCTTAAATCTCTCTACTCCGGCG        | HindIII          |
| Apha3_pHT304_RV   | CCGGATCTGTATACGAAACTACATTAGC                   | BamHI            |
| psiI-Rev BamHI    | CCGGATCTGTATACGAAACTACATTAGC                   | BamHI            |
| phbl-sinI-SOE-Fwd | aacatctataatTCTAGAAataattgctatcatctg         | none             |
| phbl-sinI-SOE-Rev | aacatctataatTCTAGAAataattgctatcatctg         | none             |
| Hbl_pHT304_RV     | CCGGATCTGTATACGAAACTACATTAGC                   | BamHI            |

The primers were used to delete the sinI, sinR, sinI-sinR and abrB genes from the strain 407, or to create transcriptional fusions between the promoters of hbl or of sinI and lasZ, yfp or mcherry on the phHT304-18 plasmid.

* underlined sequences indicate the location of restriction sites, and lower case letters indicate overlapping sequences complementary to Phbl (not underlined) or to PsiI (underlined).

The microarrays used contain 70-mer oligonucleotide probes designed to detect open reading frames (ORFs) in *B. anthracis* strain Ames, *B. anthracis* strain A2012, *B. cereus* strain ATCC 14579, and *B. cereus* strain ATCC 10987 [39]. Only probes with 93% identity or greater to a transcript/feature sequence of 407 were included in the analysis. Of the predicted genes of the 407 genome, 1719 did not have corresponding probes on the array. However, among these genes, 1165 were annotated as hypotheticals (68%), and 761 were on contigs 00213 and 00060, which have later been shown to be plasmid-borne [40]. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-1806.

### Hemolysis assays

The various strains were grown in LB medium at 37°C until they reached an OD₆₀₀ of 1. A 5 μl drop of each culture was spotted on an agar plate and incubated at 30°C for 12 hours. Each experiment was repeated four times.

### Microarray analysis

Cells used for microarray analysis were grown in bactopeptone medium at 30°C and 250 rpm. Samples were collected 2 hours after entry into stationary phase (t2). Entry into stationary phase (t0) was determined as the breakpoint of the growth curve, i.e. the time point when the slope of the growth curve starts to decrease, which usually occurs in the 407 strain around OD 2.5 in these culture conditions. RNA isolation and cDNA synthesis, labeling and purification were performed as described previously [6]. Microarray slides were printed at the microarray core facility of the Norwegian University of Science and Technology (NTNU). Design, printing, prehybridization, hybridization and scanning of the slides and analysis of the data was as previously described [6]. The microarray experiments were based on four slides, all being true biological replicates. Genes with false discovery rate corrected p-values < 0.05, and for which differential expression between the sinR-negative and sinR-positive strains was at least two-fold, were considered to be repressed (fold change FC > 2.0) or induced (FC < 0.5) by the deleted gene.

The microarray data were analyzed using GeneSpring GX 7.3 software (Agilent Technologies, Inc., Santa Clara, CA). The data were imported into GeneSpring GX 7.3 and normalized using the Median-Median method. A two-tailed Student’s t-test was applied to determine the significance of the gene expression changes. The fold change (FC) for a given gene was calculated as the ratio of the median expression level of the strain to that of the control. A gene was considered to be differentially expressed if the FC was greater than 2.0.

The authors used microarrays to explore the transcriptional responses of the *B. thuringiensis* 407 strain to various challenging conditions. The data were analyzed using GeneSpring GX 7.3 software (Agilent Technologies, Inc., Santa Clara, CA). The data were imported into GeneSpring GX 7.3 and normalized using the Median-Median method. A two-tailed Student’s t-test was applied to determine the significance of the gene expression changes. The fold change (FC) for a given gene was calculated as the ratio of the median expression level of the strain to that of the control. A gene was considered to be differentially expressed if the FC was greater than 2.0.

The authors used microarrays to explore the transcriptional responses of the *B. thuringiensis* 407 strain to various challenging conditions. The data were analyzed using GeneSpring GX 7.3 software (Agilent Technologies, Inc., Santa Clara, CA). The data were imported into GeneSpring GX 7.3 and normalized using the Median-Median method. A two-tailed Student’s t-test was applied to determine the significance of the gene expression changes. The fold change (FC) for a given gene was calculated as the ratio of the median expression level of the strain to that of the control. A gene was considered to be differentially expressed if the FC was greater than 2.0.

The authors used microarrays to explore the transcriptional responses of the *B. thuringiensis* 407 strain to various challenging conditions. The data were analyzed using GeneSpring GX 7.3 software (Agilent Technologies, Inc., Santa Clara, CA). The data were imported into GeneSpring GX 7.3 and normalized using the Median-Median method. A two-tailed Student’s t-test was applied to determine the significance of the gene expression changes. The fold change (FC) for a given gene was calculated as the ratio of the median expression level of the strain to that of the control. A gene was considered to be differentially expressed if the FC was greater than 2.0.

37°C with shaking at 175 rpm until the culture reached an OD₆₀₀ of 1. A 5 μl drop of each culture was spotted on an agar plate and incubated at 30°C for 12 hours. Each experiment was repeated four times.
Hi = \frac{Ha - Cs}{Cs}

were Hi is the hemolytic index, Ha the hemolysis area and Cs the colony size at the end of the incubation time. Results are presented as means of four independent experiments.

Western blot analysis

 Cultures were grown in bactopeptone medium at 30°C and 250 rpm, and culture supernatants were collected by centrifugation at t2. SDS-PAGE was carried out using 12% acrylamide gels, and silver stained according to Blum et al. [42]. Proteins were blotted onto Immun-Blot PVDF membranes (Bio-Rad), and nonspecific binding was blocked with 5% nonfat milk. The HblB (binding) component was detected using monoclonal antibody 2A3 diluted 1:15 [43], kindly provided by Dr Erwin Martlbauer (Ludwig-Maximilians-Universität, Munich, Germany). Peroxidase-conjugated AffiniPure Goat-anti-mouse IgG (Jackson Immuno Research Laboratories Inc) were used at 0.8 μg/ml as secondary antibody, and bands were detected using the SuperSignal West Femto Substrate (Pierce) and quantified using ImageJ [41]. After subtraction of the background, the mean gray value for each band was normalized to the intensity of the band in the 407 wild type sample, arbitrarily defined as 1.

Flow cytometry experiments

 Biofilms recovered from glass tubes assays were homogenized by aspirating/pushing ten times through a 26-gauge needle. Planktonic cultures, or homogenized biofilms, were mixed with an equal volume of ice-cold, 0.2 μm-filtered PBS containing 8% formaldehyde, washed with ice-cold PBS and resuspended in TEG (Tris...
20 mM EDTA 10 mM glucose 0.5M pH 7.2). Fluorescence was recorded on a CyFlow SL flow cytometer (Partec GmbH, Münster, Germany). YFP fluorescence was measured by using a solid blue-laser emitting at 488 nm, a 620-nm Long Pass Dichroic Mirror and a 590-nm band pass filter (565–615). mCherry fluorescence was measured with a solid yellow-laser emitting at 561 nm combined to a 585-nm band pass filter. Gating on FSC/SSC was used to discriminate bacteria from the background. For each sample, at least 40,000 gated events were measured. Data were collected with the FlowMax software (Partec GmbH, Münster, Germany) and analysed with the Wecasel 2.0 software (WEHI, USA).

Results

Spo0A, AbrB and SinI/SinR control biofilm formation and swimming motility

Deletion of abrB, sinI or sinI-sinR did not result in significant changes in growth (figure S1a and S1b). In contrast, the spo0A mutant strain grew more slowly (figure S1a), and the sinR mutant strain grew poorly (figure S1b). This is true in B. anthracis strain Sterne, where it has previously been described that deletion of sinR do not impair bacterial growth [22].

The effect of spo0A, abrB, sinI and sinR on biofilm formation and on motility was similar to B. subtilis as previously reported. In microtiter plates and in glass tubes, Spo0A promoted biofilm formation while AbrB repressed this phenotype (figure 1A). Both mutants had no effect on swimming motility on 0.3% LB agar plates (figure 2). The sinI mutant was highly motile but unable to form biofilms, the sinR mutant was non-motile and overproduced biofilms and the sinI-sinR mutant was highly motile and overproduced biofilms. The architecture of the wild type strain biofilm, viewed from above in glass tubes, appeared as a thick ring sticking to the tube wall, surrounding a floating pellicle on which protrusions could be seen (figure 1B). This architecture was similar in the abrB and the sinI-sinR mutants, whereas the sinR mutant produced a thick ring and a flat pellicle (figure 1B). In contrast, the spo0A mutant produced no ring and no pellicle, and the sinI mutant displayed a small ring but no pellicle.

Microarray analysis of the sinI, sinR and sinI-sinR mutants reveals the SinR regulon

We used microarray analysis of strain 407 to identify genes mediating the effects of sinI and sinR on biofilm formation. A comparative microarray analysis of the sinI mutant and of the wild type strain 407, in the early stationary growth phase, revealed 421 repressed genes in the mutant, many of which are associated with the translation machinery (data not shown). The large decrease in the growth rate of strain 407 upon deletion of sinR is consistent with this result. To overcome this problem, the transcriptome in the presence and absence of sinR was analyzed in a sinI-deletion background. Indeed, both sinI and sinI-sinR mutant strains grew similarly to the wild type strain (figure S1b).

From this analysis, 32 genes appeared to be repressed by SinR two hours after the onset of stationary phase (42) (Table 2). The B. cereus homologue of B. subtilis sipW and two tase homologues (BC1278, BC1279 and BC1281), as well as the Hbl enterotoxin genes BC3102 (hblA encoding the binding component HblB) and BC3104 (hblC encoding the lytic component HblL2) were highly repressed by SinR (Table 2). The kstEABC operon (BC2450–BC2453), recently shown to be involved in the biosynthesis and export of a non-ribosomal lipopeptide [30], was also found to be SinR-regulated. Deletion of the kst locus abolished the capability to form biofilms either in microtiter plates or in glass tubes (figure 1). The SinR regulon also included as many as nine genes coding for proteins of unknown functions, some of them being strongly repressed by the sinR deletion. In addition, two motility genes, encoding the chemotaxis protein CheA (BC1628) and the flagellar basal body rod protein FlgC (BC1642), were slightly repressed by SinR (signal ratio 2.5 and 2, respectively), but this moderate repression probably does not explain the large opposite effect of SinR on motility. Genes possibly involved in detoxification processes (BC2230, BC3076, BC3078, BC4272), in sugar metabolism (BC2950, BC2854, BC3759), in DNA recombination (BC2556) or degradation (BC1072), in peptidoglycan turnover (BC5254), and in energy production (BC3142) were also identified as being regulated by SinR in strain 407 at t2 (table 2). Finally, SinR repressed a gene (BC2410) encoding a PlcR-controlled transcriptional regulator [6].

hbl expression is controlled by SinR

Microarray analysis suggested that expression of the Hbl enterotoxin may be controlled by SinR. The Hbl enterotoxin is already known to be controlled by the virulence transcriptional regulator PlcR [44,45]. To confirm that the hbl genes were also under SinR regulation, we used a transcriptional fusion between the hblC (BC3104; the first gene of the hblCDA operon) promoter region and the lacZ gene. As already described [46], expression of hblC in the wild type strain increased sharply after t0 and reached a plateau at t2 (figure 3A). Deletion of sinI did not abolish hblC expression, but reduced it greatly, resulting in a ratio of expression of 2.3 at t4 (figure 3A). The effect of SinR on extracellular HblB (encoded by hblA; the third gene of the operon) was then assessed by Western immunoblotting (figure 3C). The amount of extracellular HblB component produced by the sinR mutant strain was higher than that of the wild type, whereas no band could be detected in the sinI mutant. Hence, SinR repressed HblB production while SinI had the reverse effect. Hbl enterotoxins have hemolytic activity, and these toxins are the major hemolysins acting on sheep blood [47]. Therefore, we studied sinI, sinR and sinI-sinR deletion mutants by hemolysis assays on sheep blood agar plates (figure 3D). The hemolytic activity of the sinI mutant strain was much lower than that of the wild type strain. In contrast,
deletion of sinR had no effect on hemolytic activity, and deletion of both sinI and sinR resulted in higher hemolytic activity.

**hbl** is expressed in the biofilm produced by strain 407

The co-regulation of biofilm formation and hbl expression by SinR suggests that hbl could be expressed by strain 407 in biofilm. To test this, we produced biofilms in glass tubes with the wild type strain 407 transformed with pHT304-18VP\(hbl'^{-}\)-lacZ, the plasmid carrying the P\(hbl'^{-}\)-lacZ transcriptional fusion, and we followed hbl expression in the floating pellicle and the planktonic bacterial population right underneath (figure 3B): hbl was expressed in the biofilm, peaking after 48 to 72 hours of culture, when the biofilm has reached its maximal development in glass tubes. This expression, despite decreasing from the peak at 48 h, was sustained and lasted for more than 120 hours, whereas hbl expression in planktonic cultures was shut down after t8 (data not shown). Therefore, *B. cereus* biofilms are persistent structures in which the bacteria produce significant amounts of hbl over long periods of time.

**hbl** is expressed by a small subpopulation of biofilm cells

To determine the proportion of *B. cereus* cells expressing hbl enterotoxin genes, we used a transcriptional fusion between the promoter region of hbl and the yellow fluorescence protein gene yfp. Strain 407 expressing yfp under the control of the hbl promoter was grown in planktonic cultures and in biofilms, and samples were harvested when hbl expression reached a plateau or was maximal as determined with lac\(\beta\)-fusions (figure 3A and 3B: t2 for planktonic cultures and 48 h for biofilms). As shown by flow cytometry, the hbl promoter was active in 90% of bacteria in

**Table 2. Microarray results for the 407 sinRsinI mutant compared to the 407 sinI mutant.**

| Locus tag   | Gene Producta | Ba b | SR c |
|------------|---------------|------|------|
| BC1278     | Signal peptidase I, SipW | GBA1287 | 36.8 |
| BC1279     | TasA homologue | GBA1288 | 26.0 |
| BC1281     | Calyxin CalY | GBA1290 | 88.6 |
| BC1072     | endonuclease/exonuclease/phosphatase family protein | GBA1075 | 3.6 |
| BC2556     | DNA integration/recombination/inversion protein | NH | 2.4 |
| BC3102     | Enterotoxin binding component precursor Hbl | NH | 8.9 |
| BC3104     | Enterotoxin lytic component HblL2 | NH | 3.9 |
| BC0418     | hypothetical protein | NH | 9.8 |
| BC1280     | hypothetical protein | NH | 5.4 |
| BC2409     | hypothetical protein | NH | 3.9 |
| BC2875     | hypothetical protein | NH | 4.2 |
| BC3283     | hypothetical protein | NH | 2.7 |
| BC3290     | hypothetical protein | NH | 2.2 |
| BC3697     | hypothetical protein | NH | 3.2 |
| BC4216     | hypothetical protein | NH | 8.1 |
| BC4259     | hypothetical protein | NH | 3.1 |
| BC1628     | chemotaxis protein CheA | NH | 2.5 |
| BC1642     | flagellar basal body rod protein FlgC | NH | 2.1 |
| BC2230     | macrolide-efflux protein MFS-1 family | NH | 2.4 |
| BC3076     | acetyltransferase | NH | 4.4 |
| BC3078     | aminoglycoside 3'-phosphotransferase | NH | 5.4 |
| BC4272     | superoxide dismutase | NH | 2.7 |
| BC2854     | aldo-keto-oxidoreductase | NH | 6.2 |
| BC2960     | Glycosyl transferase | NH | 2.9 |
| BC3759     | 6-phospho-beta-glucosidase | NH | 3.0 |
| BC2410     | TetR family transcriptional regulator | NH | 2.2 |
| BC3142     | NADPH-dependent oxidoreductase | NH | 2.2 |
| BC5234     | N-acetylmuramoyl-L-alanine amidase | NH | 2.2 |
| BC2450     | macrolide-efflux protein MFS-1 family | NH | 4.1 |
| BC2451     | peptide synthetase | NH | 1.3 |
| BC2452     | peptide synthetase | NH | 1.1 |
| BC2453     | peptide synthetase | NH | 2.2 |

Genes are grouped into functional families.
a and b: locus tag and gene product, respectively, according to the annotation of the ATCC14579 genome.
c: homologues in the SinR regulon of *B. anthracis* (Pflughoeft et al., 2011); NH: no homologues.
d: SR, microarray signal ratio, computed as the signal for the sinkinR mutant divided by the signal for the sinI mutant.
doi:10.1371/journal.pone.0087532.t002

expression in planktonic cultures was shut down after t8 (data not shown). Therefore, *B. cereus* biofilms are persistent structures in which the bacteria produce significant amounts of hbl over long periods of time.
planktonic cultures in early stationary phase and in 33% of bacteria in homogenized 48 h-old biofilms (figure 4A). In contrast, the apha3 constitutive promoter was active in 88% of bacteria in 48 h-old biofilms (figure 4B), showing that the heterogeneity of hbl expression in biofilms was not consecutive to non-viable bacterial cells or to plasmid loss in this culture condition. In addition, colonies recovered from 48 h-old biofilms formed with the 407 strain carrying the pHT304-18V
\text{P}_{\text{hbl}'}-\text{yfp}, and transferred to LB- and erythromycin-LB plates, were 100% resistant to erythromycin, the pHT304 resistance marker (3 independent experiments). Flow cytometry also revealed that hbl was on average transcribed at a lower level in the biofilm than in planktonic cultures (figure 4A). These results are supported by epifluorescence microscopy, which showed that almost all bacteria in planktonic cultures expressed hbl whereas only a few expressed it in biofilms (figure 4C). By using a plasmid carrying both the P_{sal}’-yfp and the P_{sal}’-mcherry transcriprional fusions, we have monitored the expression of hbl and sin in the same cells in 48 h-old biofilms. We found that 16% of the bacteria expressed hbl (figure 5A), which is in the same range as our previous results. Furthermore, flow cytometry and microscopy observation revealed that almost all bacteria expressing hbl also expressed sinI (figure 5A and 5B). In addition, 12% of the bacteria expressed sinI but not hbl.

**Discussion**

We deleted from *B. thuringiensis* the genes encoding Spo0A, AbrB and SinR, which are regulators of the transition phase of growth. These regulators were previously shown to control biofilm formation and swarming motility in *B. subtilis*. We report here that, in *B. thuringiensis*, SinR represses biofilm formation and is required for swimming motility, whereas SinI has the reverse effect. Consequently, the SinI/SinR antirepressor/repressor pair is likely to act as a switch between biofilm formation and swimming motility, as it does in *B. subtilis* between biofilm formation and swarming motility [48]. In addition, Spo0A is required in *B. thuringiensis* for biofilm formation and AbrB represses this phenotype, and neither of these regulators affects motility. These findings suggest that the regulation of biofilm formation and of motility by Spo0A, AbrB, and SinI/SinR show similarities in *B. cereus* and in *B. subtilis*. Similarities between the two species for control of biofilm formation is supported by the presence of the sipW-tacA operon in their respective SinR regulons. The sipW-tacA operon was shown to be required for the production of the proteic...
component of the biofilm matrix in *B. subtilis*, and to be directly controlled by SinR in *B. anthracis* [22].

However, within the 32 genes included in the *B. thuringiensis* SinR regulon, only *sipW* and *tasA* are shared with the *B. subtilis* SinR regulon reported previously [15]. *B. thuringiensis* and *B. cereus* display a chromosomal conserved locus (genes BC5267 to BC5278 in *B. cereus* strain ATCC14579) similar to the epsAO locus which in *B. subtilis* is involved in the biosynthesis of the exopolysaccharide component of the biofilm matrix. A 120 bp antitermination RNA element named EAR is found, in *B. subtilis*, exclusively only in the epsAO locus [49], and a corresponding element is predicted to be present in the BC5267–BC5278 locus, consistent with these loci being homologous. But while the *B. subtilis* epsAO genes are repressed by SinR, the *B. thuringiensis* BC5267–BC5278 orthologs are not.

Conversely, the *B. thuringiensis* - but not the *B. subtilis* - SinR regulon includes genes required for the production of a lipopeptide. This lipopeptide, kurstakin, is required for biofilm formation. In *B. subtilis*, production of the lipopeptide surfactin, also important for biofilm formation [16], is controlled by the two-component system ComA-ComP [50]. This two-component system is not present in bacteria of the *B. cereus* group, but it was recently shown that kurstakin production is activated by the NprR cell-cell communication system during stationary phase [30]. In addition to its role in biofilm formation, kurstakin was shown to be required for bacterial survival in the host cadaver [50].

Our transcriptional analysis also revealed that SinR repressed the hbl enterotoxin genes. The transcription of *hbl* genes is promoted by the virulence regulator PlcR [44,45]. The products of these genes are active against the rabbit intestine [51] and may be responsible for the diarrheal symptoms associated with *B. cereus*-dependent gastroenteritis [7]. Transcriptional fusion experiments confirmed that SinI/SinR controls hbl transcription, and Western blotting and hemolysis assays also showed that SinI/SinR regulates the production of Hbl enterotoxin components. The SinR regulon includes another gene controlled by PlcR: BC2410. The product of BC2410 is a transcriptional regulator, the targets of which are still unknown, but which might be involved in bacterial pathogenesis [6]. Therefore, SinR in *B. cereus* controls virulence factors that are part of the PlcR regulon, in addition to biofilm formation and motility. These findings suggest that

**Figure 4. Heterogeneity of hbl expression in planktonic cultures and in biofilms.** A: Expression from the hbl promoter was monitored in planktonic cultures and in biofilms by epifluorescence microscopy through a transcriptional fusion to yfp. Cell limits are shown by the membrane stain FM4-64 (red). B: Flow cytometry analysis of bacteria expressing Pphbl-yfp in planktonic cultures or in biofilms, shown as histogram plot. The blue-filled curve shows biofilm data, the yellow-filled curve shows planktonic cultures data and the unfilled dashed curve shows data from bacteria lacking yfp. C: Flow cytometry analysis of bacteria expressing Papha3’-yfp in biofilms (blue-filled curve) compared to bacteria lacking yfp (unfilled dashed curve), shown as histogram plot.
doi:10.1371/journal.pone.0087532.g004
inactivation of SinR by SinI both serve to trigger biofilm formation and to enhance enterotoxin production.

We assessed the expression of hbl in biofilms using lacZ fusions. We found that this expression was sustained and lasted for more than 48 h but was moderate as compared to the strong expression of hbl in planktonic cultures. We determined hbl expression at the cell level using yfp fusions; most of the bacteria in planktonic cultures expressed hbl, while only a small subpopulation of cells expressed it in biofilms. This heterogeneity in the expression of hbl in biofilms is likely to be a consequence of the heterogeneity in the expression of sinI, since we found that bacteria expressing hbl also expressed sinI. SinI-dependent heterogeneity of genes expression in biofilms has been described in B. subtilis, where the sipW-tasA operon is expressed in the same subpopulation as sinI, whereas sinR is expressed in the whole biofilm bacterial population [32].

The B. thuringiensis SinR regulon shares only 4 genes with the B. anthracis SinR regulon as determined previously [22]; the homologue of B. subtilis sipW and two homologues of tasA, and a gene encoding an endonuclease. The 28 other genes of the B. thuringiensis SinR regulon have not been identified as components of the B. anthracis SinR regulon, indicating possible differences between these two species for the role of SinR. More specifically, the inhAI gene, encoding a metalloprotease, has previously been reported to be SinR-dependent in B. anthracis [22]. This gene is also present in B. thuringiensis and B. cereus, where it is likely to play an important role in the bacterial pathogenesis [53,54]. Although inhAI was previously reported to be controlled by Spo0A, AbrB and SinR in the 407 strain [55], we do not confirm, by microarray analysis, the role of SinR in the regulation of inhAI transcription. However, the previous study used an overexpression of sinI or of sinR on high copy plasmids to investigate the role of SinR in the control of inhAI, which might have introduced bias in that study.

The sinI-sinR mutant produced more biofilm and was more hemolytic than the sinR mutant, which was unexpected. If the function of SinI is only antagonizes SinR, then sinI-sinR and sinR mutants should have similar phenotypes, and give similar results. The observed difference could suggest that Sin also acts on another regulator, different from SinR. In B. subtilis, biofilm formation is stimulated by SirR, a paralogue of SinR [21,56], and SirR interacts with SlrA, a paralogue of SinI [57]. Possibly, SinI in B. thuringiensis inactivates both SinR and a putative paralogue of SinR.

In the present work, we have shown that B. thuringiensis and B. subtilis have similarities and differences in the control of biofilm formation. The SinR regulon of the two species have only two genes in common under the conditions studied. In B. thuringiensis, the SinR regulon includes the hbl gene and the krsEABC locus, which are involved in the interaction of the bacterium with its host. The Hbl toxin complex is cytotoxic and causes damage to the intestinal tract [7], and kurstakin is required for biofilm formation and for bacterial survival in the host cadaver. Therefore, SinR co-regulates both biofilm formation and part of the infectious process in B. thuringiensis, which makes sense if we consider that this pathogen can settle in heterologous biofilms [58], and therefore potentially integrate into the biofilm microbiota lining the host intestinal epithelium. In this biofilm, toxins could be delivered directly to their target tissue.

Supporting Information

Figure S1 Growth curves. The various strains were grown in LB medium at 37°C and 175 rpm. The OD was measured at 600 nm and plotted against time. a: wild-type strain (black circle), spo0A mutant (white circle), abrB mutant (white triangle). b: wild-type strain (black circle), sinR mutant (white circle), sinI mutant (white triangle), sinI—sinR mutant (white inverted triangle). (DOC)
Table S1 | Strains used in this study.

Table S2 | Correspondence between the locus tags in the ATCC14579 and in the 407 strains. No ortholog annotated in B. thuringiensis 407 and no DNA with similarity to the indicated B. cereus ATCC 14579 ORF present in the B. thuringiensis 407 genome. Could potentially be a result of missing data in the draft B. thuringiensis 407 sequence.

Acknowledgments

The flow cytometer was acquired with the financial support of the DIM Astrea program, and bioinformatics analyses were performed using computing services available from the Norwegian EMBlnet node.

Author Contributions

Conceived and designed the experiments: AF OAO TD M. Gohar EV. Performed the experiments: AF TD NG IB SP M. Gominet M. Gohar EV. Analyzed the data: AF OAO M. Gohar. Wrote the paper: AF OAO SA AK DL M. Gohar.

References

1. Priest FG (1993) Systematics and Ecology of Bacillus. In: Sonnenfeld AL, Hoch JA, Losick R, editors. Bacillus subtilis and Other Gram-Positive Bacteria. Washington: American Society for Microbiology. pp. 3–16.
2. Ivanova N, Sorek A, Anderson J, Gallner N, Candelon B, et al. (2003) Genome sequence of Bacillus anthracis and comparative analysis with Bacillus anthracis. Nature 423: 87–91.
3. Van Schaik W, Abee T (2005) The role of sigmaH in the stress response of Gram-positive bacteria – targets for food preservation and safety. Curr Opin Biotechnol 16: 218–224.
4. Lerechus D, Agaisse H, Gominet M, Salamitou S, Sanchis V, et al. (2006) Two-dimensional electrophoresis analysis of the extracellular protocine of Bacillus cereus reveals the importance of the PlcR regulon. Proteomics 2: 784–791.
5. Leontaritis M, Okstad OA, Gohar M, Fosset A, et al. (2000) Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis - One species on the basis of genetic evidence. Appl Environ Microbiol 66: 2627–2630.
6. Paget PJ, Hiltbert DW (2004) Sporulation of Bacillus subtilis. Curr Opin Biotechnol 7: 379–390.
7. Hamon MA, Lazazzera BA (2001) The sporulation transcription factor Spo0A is required for biofilm development in Bacillus subtilis. Mol Microbiol 42: 1199–1210.
8. Strauch M, Webb V, Spiegelman G, Hoch JA (1998) The Spo0A protein of Bacillus subtilis is a repressor of the absB gene. Proc Natl Acad Sci USA 87: 1801–1803.
9. Shahfikari SH, Manfie-Mulec I, Strauch MA, Smith I, Leighton T (2002) Postexponential regulation of sin operon expression in Bacillus subtilis. J Bacteriol 184: 564–571.
10. Hamon MA, Stanley NR, Britton RA, Grossman AD, Lazazzera BA (2004) Identification of AbsR-regulated genes involved in biofilm formation by Bacillus subtilis. Mol Microbiol 52: 647–660.
11. Hamon MA, Stanley NR, Britton RA, Grossman AD, Lazazzera BA (2004) Identification of AbsR-regulated genes involved in biofilm formation by Bacillus subtilis. Mol Microbiol 52: 647–660.
12. Branda SS, Gonzalez-Pastor JE, Derven E, Ehrlich SD, Losick R, et al. (2004) Genes involved in formation of structured multicellular communities by Bacillus subtilis. J Bacteriol 186: 3976–3979.
13. Brandt SS, Chu F, Kearns DB, Losick R, Kolter R (2006) A major protein component of the Bacillus subtilis biofilm matrix. Mol Microbiol 59: 1229–1238.
14. Romero D, Vlamakis H, Losick R, Kolter R (2011) An accessory protein required for anchoring and assembly of amyloid fibres in B. subtilis biofilms. Mol Microbiol 80: 1155–1160.
15. Blair KM, Turner L, Winkelmann JT, Berg HC, Kearns DB (2008) A molecular clutch disperses flagella in the Bacillus subtilis biofilm. Science 320: 1636–1638.
16. Koya Y, Koyama K (2000) Shk/ShkA controls the initiation of biofilm formation in Bacillus subtilis. Mol Microbiol 36: 1409–1410.
17. Chu F, Kearns DB, Mcloon A, Chai Y, Kolter R, et al. (2008) A novel regulatory protein governing biofilm formation in Bacillus subtilis. Mol Microbiol 68: 1117–1127.
18. Bhogesh KJ, Sumby P, Koscher TM (2011) Bacillus anthracis sin locus and regulation of secreted proteases. J Bacteriol 193: 651–639.
19. Auger S, Kim E, Aymerich S, Gohar M (2006) Autoinducer 2 affects biofilm formation by Bacillus subtilis. Appl Environ Microbiol 72: 937–941.
20. Hsueh YH, Somers EB, Lerechus D, Wong AC (2006) Biofilm Formation by Bacillus subtilis is Influenced by PlcR, a Pleiotropic Regulator. Appl Environ Microbiol 72: 5089–5092.
21. Hsueh YH, Somers EB, Wong AC (2008) Characterization of the codF gene and its influence on biofilm formation in Bacillus cereus. Arch Microbiol 189: 557–565.
49. Irnov I, Winkler WC (2010) A regulatory RNA required for antitermination of biofilm and capsular polysaccharide operons in Bacillales. Mol Microbiol 76: 559–575.
50. Msadek T (1999) When the going gets tough: survival strategies and environmental signaling networks in Bacillus subtilis. Trends Microbiol 7: 201–207.
51. Beecher DJ, Schoeni JL, Wong AC (1995) Enterotoxic activity of hemolysin BL from Bacillus cereus. Infect Immun 63: 4423–4428.
52. Chai Y, Chu F, Kohler R, Losick R (2008) Bistability and biofilm formation in Bacillus subtilis. Mol Microbiol 67: 254–263.
53. Ramarao N, Lereclus D (2005) The InhA1 metalloprotease allows spores of the B. cereus group to escape macrophages. Cell Microbiol 7: 1357–1364.
54. Dalhammar G, Steiner H (1984) Characterization of inhibitor A, a protease from Bacillus thuringiensis which degrades attacins and cecropins, two classes of antibacterial proteins in insects. Eur J Biochem 139: 247–252.
55. Grandvalet C, Gominet M, Lereclus D (2001) Identification of genes involved in the activation of the Bacillus thuringiensis inhA metalloprotease gene at the onset of sporulation. Microbiology 147: 1805–1813.
56. Kobayashi K (2007) Bacillus subtilis pellicle formation proceeds through genotypically defined morphological changes. J Bacteriol 189: 4920–4931.
57. Chai Y, Kohler R, Losick R (2009) Paralogous antirepressors acting on the master regulator for biofilm formation in Bacillus subtilis. Mol Microbiol 74: 876–887.
58. Houry A, Gohar M, Deschamps J, Tischenko E, Aymerich S, et al. (2012) Bacterial swimmers that infiltrate and take over the biofilm matrix. Proc Natl Acad Sci USA 109: 13088–13093.