IlsA, A Unique Surface Protein of *Bacillus cereus* Required for Iron Acquisition from Heme, Hemoglobin and Ferritin

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Abstract

The human opportunistic pathogen *Bacillus cereus* belongs to the *B. cereus* group that includes bacteria with a broad host spectrum. The ability of these bacteria to colonize diverse hosts is reliant on the presence of adaptation factors. Previously, an IVET strategy led to the identification of a novel *B. cereus* protein (IlsA, Iron-regulated leucine rich surface protein), which is specifically expressed in the insect host or under iron restrictive conditions in vitro. Here, we show that IlsA is localized on the surface of *B. cereus* and hence has the potential to interact with host proteins. We report that *B. cereus* uses hemoglobin, heme and ferritin, but not transferrin and lactoferrin. In addition, affinity tests revealed that IlsA interacts with both hemoglobin and ferritin. Furthermore, IlsA directly binds heme probably through the NEAT domain. Inactivation of ilsA drastically decreases the ability of *B. cereus* to grow in the presence of hemoglobin, heme and ferritin, indicating that IlsA is essential for iron acquisition from these iron sources. In addition, the ilsA mutant displays a reduction in growth and virulence in an insect model. Hence, our results indicate that IlsA is a key factor within a new iron acquisition system, playing an important role in the general virulence strategy adapted by *B. cereus* to colonize susceptible hosts.

Introduction

Iron is an essential element for most organisms, including bacteria, because it is involved in many cellular processes including aerobic respiration, amino acid and nucleotide biosynthesis [1,2]. Since free iron is highly toxic for the cells, its homeostasis is strictly regulated in living organisms. Protection against iron is achieved by iron sequestration in carrier proteins such as transferrin, lactoferrin, ferritin or as iron-binding to the heme in hemoproteins. Thus, the lack of free iron is an obstacle that bacteria must overcome, when invading a host. In order to scavenge iron from the host iron-binding proteins, bacteria have developed two principal high affinity iron-uptake systems, which are considered to be important virulence factors. One system is based on the secretion of siderophores that capture iron from iron-binding proteins by the virtue of a superior binding strength. The siderophores are then recognized by specific membrane anchored binding proteins and internalized into the cytosol [3,4]. The second system involves direct binding to host iron rich proteins via specific bacterial surface receptors which subsequently interact with membrane bound ABC transporters and permeases allowing iron transfer into the cytosol. These systems have been more studied in Gram-negative compared to Gram-positive bacteria [5–8]. The majority of these iron-uptake systems are under the control of the repressor Fur (Ferric uptake regulator) [3]. In Gram-positive bacteria, the best characterized system relying on bacterial surface proteins is the iron-regulated surface determinants (Isd) of *Staphylococcus aureus*. The Isd system implements cell wall proteins that act as hemoprotein receptors [9,10] because of the presence of several copies of the conserved NEAT domains (for NEAr iron Transport), which play a key role in heme and hemoproteins binding and transport [11,12]. An Isd system has been also studied in *Bacillus anthracis*. In addition to cell wall proteins, the *B. anthracis* Isd system uses secreted proteins that contain NEAT domains which act as hemophores, enabling heme acquisition from hemoglobin [13,14].

The Gram-positive, spore-forming and human opportunistic pathogen, *Bacillus cereus*, belongs to the *Bacillus cereus* group, which also includes the entomopathogen, *Bacillus thuringiensis*, and the etiological agent of anthrax in mammals, *B. anthracis*. These three closely related species share a large number of chromosomal determinants, whereas their host-specific toxins are carried on plasmids [15–17]. *B. cereus* is generally associated with human food poisoning, resulting from the diarrhea and the emetic toxins [18]. However, *B. cereus* can also cause serious infections such as endophthalmitis, pneumonia and meningitis [19–21]. In addition, a new *B. cereus* species was found to cause severe respiratory illness...
Iron is an essential compound for almost all living organisms, taking part in basic cellular homeostasis. Preventing access to iron sources for invading pathogens is one of the defense systems used by hosts to avoid pathogen colonization. To counteract this, pathogens have developed mechanisms to acquire nutrient iron during infection. Bacillus cereus is an opportunistic bacterium able to infect both insects and mammals; thus, it should have systems enabling iron uptake from these hosts. Here we describe, for the first time, a unique surface protein, called IlsA, which is essential for iron uptake from two very different iron binding molecules: ferritin and hemoglobin. IlsA is only produced in iron limited environments. We show that during insect infection, its expression is specific to insect hemocoel (blood), where ferritin is the major iron-binding molecule. Interestingly, the IlsA mutant has reduced survival in in vivo infection and in vitro when heme, hemoglobin and ferritin are the sole iron sources available. Thus, as IlsA is important for iron uptake from the major iron rich molecules in insects and mammals, we suggest that this new iron acquisition system may be a key factor that is evolutionarily adapted to infection of such diverse hosts.

Author Summary

Iron is an essential compound for almost all living organisms, taking part in basic cellular homeostasis. Preventing access to iron sources for invading pathogens is one of the defense systems used by hosts to avoid pathogen colonization. To counteract this, pathogens have developed mechanisms to acquire nutrient iron during infection. Bacillus cereus is an opportunistic bacterium able to infect both insects and mammals; thus, it should have systems enabling iron uptake from these hosts. Here we describe, for the first time, a unique surface protein, called IlsA, which is essential for iron uptake from two very different iron binding molecules: ferritin and hemoglobin. IlsA is only produced in iron limited environments. We show that during insect infection, its expression is specific to insect hemocoel (blood), where ferritin is the major iron-binding molecule. Interestingly, the IlsA mutant has reduced survival in in vivo infection and in vitro when heme, hemoglobin and ferritin are the sole iron sources available. Thus, as IlsA is important for iron uptake from the major iron rich molecules in insects and mammals, we suggest that this new iron acquisition system may be a key factor that is evolutionarily adapted to infection of such diverse hosts.

Materials and Methods

Bacterial stains and growth conditions

Bacillus cereus strain ATCC 14579 (laboratory stock) was used throughout this study.

The mutant B. cereus ATCC 14579 ΔilsA was previously constructed by allelic exchange through a double cross-over event by introducing a tetracycline resistant cassette [32]. E. coli K12 strain TG1 was used as a host for cloning experiments. Dam−, Dcm− E. coli strains ET 12567 (laboratory stock) were used to generate unmutylated DNA for electro-transformation in B. cereus. For electro-transformation, B. cereus was grown in BHI (Brain Heart Infusion, Difco) broth. E. coli and B. cereus strains were transformed by electroporation as previously described [33,34]. E. coli BL-21 [F−, ompT, hsdR (ρB +, mB−)], gal strain was used for expression and purification of the Glutathione S-transferase (GST) fusion protein (GST-IlSAs). E. coli and B. cereus were cultured in LB (Luria–Bertani) broth, with vigorous shaking (175 rpm) at 37°C. Antibiotics for bacterial selection were used at the following concentrations: ampicillin (100 μg ml−1) for E. coli, erythromycin (10 μg ml−1) for B. cereus and tetracycline (10 μg ml−1) for B. cereus.

The iron chelator, 2,2’-dipyridyl and the iron sources, hemoglobin, heme, ferritin, transferrin and lactoferrin, were obtained from Sigma-Aldrich, St. Quentin Fallavier, France. The iron sources were used at a concentration (see below) that permits the growth of B. cereus. The ferric chloride (FeCl3) was used at a final concentration of 0.2 mM. All iron solutions were sterilized by passage through 0.22 μm pore size filter.

DNA manipulations

Chromosomal DNA was extracted from B. cereus cells with the Puregene DNA Purification Kit (Genent, Minneapolis, USA). Plasmid DNA was extracted from E. coli and B. cereus using QIAprep spin columns (Qiagen, France). For B. cereus, 5 mg ml−1 of lysozyme was added and cells were incubated at 37°C for 1 h. Oligonucleotide primers were synthesized by Prolog (Paris, France). PCRs were performed in a thermocycler PTG-100™ (MJ-Research, Inc., USA). Amplified fragments were purified using the QIAquick PCR purification Kit (Qiagen). Restriction enzymes (New England Biolabs, USA) and T4 DNA ligase (Invitrogen, USA) were used as recommended by the manufacturer. Digested DNA fragments were separated by electrophoresis on 1% agarose gels and extracted from gels using the QIAquick gel extraction kit (Qiagen).

Complementation of the ilsA mutant strain

The genetic complementation of the strain B. cereus ΔilsA was carried out as follows. A DNA fragment corresponding to the gene ilsA was amplified by PCR using the B. cereus ATCC 14579
genomic DNA as a template and the primers ilsA-forward (5'-AACTGCAGGCGCTTTTTATTTTGTACC-3') and ilsA-reverse (5'-CGGAATTCGTGAGGGCTACTAATCAGTTG-3'). The PCR product was digested with EcoRI and Pst1 restriction enzymes and inserted into the plasmid pHT304 by ligation using the T4 DNA ligase [35]. The resulting plasmid (pHT304VilsA) was amplified in E. coli and then introduced into the mutant strain B. cereus DilsA by electroporation.

IlsA purification and antibody production

Glutathione S-transferase (GST) and GST-IlsA were purified as recombinant proteins from E. coli. The expression plasmid pGST-ilxA was constructed by PCR amplification of the ilxA sequence from the B. cereus genome using the primer pair ilxA-forward-2 (5'-CGGAATTCGTGAGGGCTACTAATCAGTTG-3') and ilxA-reverse-2 (5'-CCCCTGGAGTTATTTCTTTATGCAATTAGC-3'). The DNA fragment was digested with EcoRI/XhoI, cloned into pGEX6P1 (Amersham Biosciences) digested with the same restriction enzymes and transferred into E. coli Bl-21 by electroporation. E. coli Bl-21 strain harboring pGEX6P1-ilxA was grown to log phase in LB medium with ampicillin at 37°C. The expression of the fusion protein GST-IlsA was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM and the cultures were incubated for 3 hours at 37°C. The bacteria were collected following centrifugation at 7700 rpm for 15 min in 50 ml tubes and suspended in 1X PBS containing 1% Triton X-100. Bacteria were lysed on ice by sonication using a Branson sonicator 250. Bacterial lysate was centrifuged at 7600 rpm for 15 min at 4°C, and the pellet containing the fusion protein IlsA-GST was solubilized in 50 mM Tris (pH 8.0) containing 1 M urea. After solubilization in 8 M urea overnight at 4°C, the insoluble pellet was removed by centrifugation at 13000 rpm for 20 min at 4°C. The supernatant
containing the solubilized protein was dialyzed against buffer containing Tris 50 mM (pH 9.0) and 1 M urea, for about 5 hours with stirring at 4°C to remove the residual detergent. The solubilized and dialyzed fusion protein was loaded onto a glutathione Sepharose 4B column equilibrated with 1X PBS. After binding the fusion protein, the column was washed with PBS 1X and the target protein GST-IlsA was eluted with 10 mM reduced glutathione.

Purified GST-IlsA was then dialyzed against the PreScission protease buffer (50 mM Tris HCl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) at 4°C overnight. The GST was then cleaved using the PreScission protease at a concentration of 2 Units μl⁻¹ for 4 hours at 5°C. After digestion, the mix was separated on a 10% gel SDS PAGE and the IIsA protein band was cut from the gel and used to raise anti-IlsA antibodies (Proteogenix, Oberhausbergen, France). Purification of IIsA was monitored by SDS PAGE electrophoresis (Figure S1). The GST was prepared using the strain E. coli B212 harbouring the plasmid pGEX6P1. After induction using IPTG, the bacteria were lysed by sonication and GST present in the cytosol of the bacteria was purified using the glutathione Sepharose 4B column as described above (Figure S1).

Immunofluorescence analysis
For the localization of IIsA at the bacterial surface, overnight cultures of B. cereus wild-type, B. cereus ΔilsA and the complemented strain B. cereus ΔilsA pHT304ΔilsA were grown in LB medium or in LB medium treated with 150 μM 2,2'-dipyridyl at 37°C and used immediately. Bacteria from stationary phase culture (10⁸) concentrated in 50 μl, were washed twice in phosphate buffered saline (PBS 1X) and fixed with 4% paraformaldehyde dissolved in PBS 1X on a cover slip. After fixation, bacteria were washed in PBS and then labelled with an anti-IlsA polyclonal antibody diluted at 1:500 in 1% BSA. The labelled bacteria were then washed two times with PBS and incubated with an anti-rabbit secondary antibody Alexa 488-conjugated at a dilution of 1:500 in 1% BSA. Bacterial DNA was also labelled with DAPI at a dilution of 1:300 in 1% BSA. Labelled bacteria were then stuck with Mowiol to a glass slide and dried at 37°C for 30 min. Preparations were examined under a Zeiss Axiosvert 135 microscope. Image acquisition from the Zeiss microscope was carried out with a cooled charge-coupled device camera (Princeton), and the images were processed with Metamorph software (Universal Imaging Corporation).

Growth assay
B. cereus cultures were grown overnight under low iron conditions by inoculating strains in LB medium supplemented with 200 μM 2,2'-dipyridyl. Overnight cultures were centrifuged and washed twice in LB medium supplemented with 500 μM 2,2'-dipyridyl. Washed bacteria were then inoculated to a final optical density (OD) of about 0.01 into LB medium containing 2,2'-dipyridyl (450 μM) only or supplemented separately with hemoglobin (2 μM), hemin (16.5 μM), ferritin (0.3 μM), transferrin (1.5 μM) or lactoferrin (1.5 μM). Knowing that both the content of iron per molecule and the biochemical structure largely vary among these sources; the concentration of these iron sources varied among these sources; the concentration of these iron sources largely

Heme detection by chemiluminescence
Hemin was dissolved immediately before use in a minimal volume of 0.1 M NaOH and diluted with phosphate buffer (Na₂HPO₄ 0.1 M, pH 7.4) to a concentration of 10⁻⁴ M. Twenty
microliters of purified GST-IlsA (2.5 x 10^{-7} M) were incubated with heme or with the buffer at room temperature for 30 min. Purified GST (2.5 x 10^{-7} M) was also used as negative control. Mixtures were separated by non-denaturing 8% PAGE in the absence of SDS at 4 °C and the proteins were electrotransferred to a nitrocellulose membrane. The presence of heme complexed to IlsA was detected by chemiluminescence due to the heme peroxidase activity [36], using the Pierce ECL Super signal system.

In vivo expression using the gfp reporter gene

In order to follow the expression of ilsA in vivo, we constructed a plasmid carrying the pilsA-gfp transcriptional fusion. The plasmid pH7T315-gfp was obtained by cloning the gene gfp-mut1' into the plasmid pH7T315 [35] between XhoI and HindIII restriction sites. The gfp-mut1' was amplified from the template plasmid pN8 of Listeria monocytogenes [37] by using the primers F-gfp (5'-GCTCTAGAGAAAGGAGGTATTAAATGAGTAAAGGAGAAGAACT-T-3') and R-gfp (5'-CCCAAGCTTTTATTTTGATATCATTCCATGCCA-3'). The ilsA promoter region (pilsA) was generated by PCR with oligonucleotide pairs Fw-pilsA (5'-CCGGAATTCCGCCTTTTATTATTTTGATATCATTCCATGCCA-3') and Rv-I1 (5'-CCGCTGT-ACAGTGTTCCTGGT) using the pH7T304-pStu2'-1 from the IVET screen [32] as a template. The PCR fragments were digested by EcoRI and XhoI and were inserted between the EcoRI and XhoI restriction sites of pH7T315-gfp plasmid to give the plasmid pH7T315-pilsA-gfp. The resulting plasmid pH7T315-pilsA-gfp was cloned in E. coli and was then used to transform B. cereus. The strain harboring the plasmid pH7T315-pilsA-gfp was used to infect the lepidopteran larvae of G. mellonella orally in order to follow the kinetics of ilsA expression in vivo. The insect larvae were injected with 5 x 10^9 mid-log phase bacteria (OD_{600} = 1) in association with 3 μg of Cry1C toxin and incubated at 37°C, according to the protocol previously described [38]. Larvae were dissected and bacteria were recovered from the insect gut at 9 hours after infection and from the hemocoel at 24 hours after infection. The bacteria were then subjected to bright field (DIC) and epifluorescence microscope observation (NIKON Eclipse E600 equipped with UV mercury disposal and observed with an FTC filter). For a positive control we used the B. cereus strain harbouring the plasmid pH7T315-pgstA-gfp which contains a transcriptional fusion between the constitutive promoter pgstA obtained by PCR amplified from the pGDG783 plasmid [39] and the gfp gene (C. Nielsen-LeRoux, unpublished data).

Growth kinetics of B. cereus in the hemocoel of G. mellonella

The growth (number of bacteria) was assessed at 2, 6 and 24 hours after injecting doses of 500 mid-log phase bacteria of the wild type or the ilsA mutant strains into the hemocoel of the larvae G. mellonella. At each time-point, three alive larvae were recovered from the insect hemocoel and their bacterial count was determined by plating serial dilutions on LB medium (standard conditions) (Figure 1B i-vi) or the mutant strain B. cereus ilsA grown in iron depleted medium (Figure 1B vii-xi). Transformation of the B. cereus ilsA mutant with the plasmid pH7T304-ilsA restored the production of IlsA at the surface of the bacteria grown in iron-depleted medium (Figure 1B x-xii). In addition, we observed an accumulation of IlsA on the division site of the bacteria. These results indicate that IlsA is present on the surface of the bacteria and only in iron depleted conditions, which is in agreement with the iron regulated expression of ilsA [32].

B. cereus uses hemoglobin, hemin and ferritin as iron sources

The ability of B. cereus ATCC 14579 to utilize host iron sources has not been previously investigated. To determine which iron sources B. cereus is able to use, we measured its ability to grow on hemoglobin, hemin, ferritin, transferrin and lactoferrin, in iron depleted LB medium (Figure 2). B. cereus growth was very weak in iron depleted condition, but the addition of either hemoglobin, or hemin, or ferritin to the iron depleted medium increased significantly the growth kinetics of B. cereus. In contrast, transferrin and lactoferrin addition to iron depleted medium yielded only growth of B. cereus similar to that in iron depleted medium.

This result indicates that B. cereus is able to use hemoglobin, hemin and ferritin as iron sources for growth, whereas transferrin and lactoferrin cannot be used. IlsA is required for efficient iron acquisition from hemoglobin, hemin and ferritin

To test the hypothesis that IlsA is important for iron scavenging from the host iron sources used by B. cereus, the growth rates of the wild-type and ilsA mutant strains were compared after inoculation in iron depleted medium where either hemoglobin, hemin or ferritin were provided as the sole iron source. Bacterial growth was measured by spectrophotometric analysis of culture samples at an optical density of 600 nm (OD_{600}) (Figure 3). Iron depleted LB did
not support the growth of the wild-type and the ΔilsA mutant strains, confirming that iron is an essential nutrient for *B. cereus* growth. In contrast, the wild-type strain grew well in iron depleted LB that had been supplemented with hemoglobin, hemin or ferritin, while *B. cereus* lacking *ilsA* showed significant growth defects in these media. However, the mutant strain grew as the wild-type when iron was provided in its inorganic form (FeCl₃). Therefore, IlsA is not required for the uptake of inorganic iron. To verify that this phenotype was specifically due to *ilsA*, the mutant strain was complemented with a plasmid harboring *ilsA*. The growth of the bacteria was restored to the wild-type level when hemoglobin, hemin or ferritin was added to iron depleted LB. These data indicate that IlsA is very important for efficient growth of *B. cereus* under iron-restricted conditions; they suggest that IlsA is involved in iron acquisition from the host during infection.

IlsA binds hemoglobin and ferritin

To address the question of whether IlsA can directly interact with hemoglobin and ferritin, we performed binding studies using the purified recombinant protein GST-IlsA. In these assays, increasing amounts of hemoglobin and ferritin were immobilized on ELISA plates (hemin cannot bind to these plates) and IlsA was added. The amount of bound IlsA was detected using anti-IlsA polyclonal antibody. By comparing the signal intensities generated by the binding of IlsA to the different proteins, we detected a slightly stronger binding of IlsA to hemoglobin ($K_{d} = 3.6 \pm 1$ nM) relative to ferritin ($K_{d} = 10 \pm 3$ nM) (Figure 4A). However, the Student T-test showed no significant difference ($p < 0.05$) for IlsA to bind hemoglobin and ferritin.

To investigate whether binding also occurs in a real time interaction, we used the Surface Plasmon Resonance (SPR) system. The interaction between chip immobilized GST-IlsA and different host iron-rich molecules (hemin, hemoglobin, ferritin and transferrin) under flow conditions shows typical SPR curves (Figure 4B). These SPR curves are from one experiment, but the assays were run 5 times on 3 different chips for which the curves...
shape were similar to the ones shown. Computational analysis showed that our data don’t fit into a typical association-dissociation model with a 1:1 molar ratio. This is probably resulting from protein multivalencies and potential steric hindrance of the various sizes of the ligands (ferritin 440 KDa, hemoglobin 68 KDa and heme 651 Da). Then, quantitative information about the relative stability of the complex, was deduced from the dissociation phase only, independent from the analyte concentration. Then, assuming a simplified kinetic scheme, the indicative kinetic dissociation constants \(k_{off}\) (expressed in \(s^{-1}\)) were calculated: \(1.71 \pm 0.83 \times 10^{-3}\) for ferritin, \(1.48 \pm 0.98 \times 10^{-3}\) for hemoglobin and \(1.34 \pm 0.44 \times 10^{-3}\) for heme. Thus, the results showed similar \(k_{off}\) values for the three iron sources. These findings are in accordance with the ELISA experiments showing that soluble ferritin and hemoglobin bind to immobilized IlsA, but transferrin did not (result not shown). The lack of direct interaction between IlsA and transferrin could be expected, because the growth experiment (Figure 2) indicated that this protein was not used by \(B.\) cereus as an iron source.

IlsA binds heme

To determine whether IlsA in solution binds to heme or another component of hemoglobin, we incubated purified GST-IlsA and GST (negative control) with hemin (\(10^{-4}\) M) and analyzed the binding reaction on a non-denaturing polyacrylamide gel system (Figure 5A). This allows the separation of free heme, purified proteins and heme-loaded proteins without dissociating heme from...
the proteins. Proteins were transferred to a nitrocellulose filter, and heme peroxidase activity was detected by chemiluminescence. This method is the most sensitive test available for detecting heme bound to proteins [36]. Our results showed that GST-IlsA could bind heme-iron, whereas GST alone cannot. This is in agreement with the result of the multiple sequence alignment of the NEAT domain of IlsA with those found in Isd proteins of *S. aureus* (Figure 5B). Indeed, this comparison indicates that the three conserved residues (Ser82, Tyr166 and Tyr170) present in IsdA, which are essential for heme interaction [11,12], are also present in IlsA. Furthermore, the residues Val157, Ile159 and Val161 that form the hydrophobic heme-pocket in IsdA are either conserved in IlsA or replaced with similar hydrophobic amino acids. The conservation in IlsA NEAT domain of amino acid residues known to interact with heme, suggests that IlsA binds heme through the NEAT domain.

**ilsA** is strongly expressed in the hemocoel of infected larvae

The IVET screen showed that *ilsA* was strongly expressed during infection of the insect larvae [32]. However, it was not determined at which stage of the infection and in which tissue *ilsA* expression occurred. To determine where and when *ilsA* is expressed in vivo, larvae were infected with *B. cereus* harbouring a plasmid that contained a transcriptional fusion of the promoter p*ilsA* and the gfp reporter gene. Bacteria recovered from the larvae at various times post-infection were examined under brightfield (Figure 6i–ii) and epifluorescence microscopy (Figure 6iii–vi). No fluorescence was observed in bacteria isolated from the intestine of living larvae after nine hours of infection (FIGURE 6iii). In sharp contrast, *B. cereus* bacteria isolated from the hemocoel of dying larvae after 24 hours of infection, showed strong fluorescence (FIGURE 6iv), thus indicating that *ilsA* is highly expressed in the hemocoel. Indeed, iron is not accessible to bacteria in the hemocoel due to the presence of proteins that bind iron such as transferrin and ferritin [43,44]. *B. cereus* containing the plasmid (pHT315-paphA3'-gfp) with the constitutively expressed gfp gene, showed a strong expression in both the intestine and hemocoel of the infected larvae (Figure 6v–vi).

IlsA is important for bacterial growth and adaptation of *B. cereus* in the hemocoel

Growth of the wild-type and Δ*ilsA* mutant strains was assessed by homogenization of whole larvae at several time points after
Figure 6. Analysis of ilsA expression in vivo. Microscopic observations by Bright-field DIC (panels iii–vi) and epifluorescence (panels iii–vi) were performed on B. cereus carrying pH7315-pilsA’gfp isolated at 9 hours after infection from the intestine of alive larvae (panels iii–vi) and at 24 hours after infection from the hemocoel of dead larvae (panels ii, iv). Green bacteria indicate expression of gfp due to the activation of the ilsA promoter. B. cereus carrying pH7315-ppha3’gfp was used as a control for its constitutive expression of GFP (panels v, vi).

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Discussion

The ability of living organisms to sequester free iron is not only necessary for protection against iron toxicity but it is also an innate resistance mechanism of hosts (mammals or insects) to fight infections [45,46]. Iron incorporated into heme is the most abundant iron source in the mammalian host, being mainly associated with the oxygen-carrying molecule, hemoglobin, sequestered within erythrocytes. Ferritin, the major iron storage protein is a large molecule, composed of several subunits, containing about 4500 ferric ions per molecule, is present not only in mammals but also in almost all living organisms including insects, plants (phytoferritin) and bacteria (bacterioferritin) [47]. In insects, notably in G. mellonella, ferritin is present in high amounts in hemolymph and in hemocytes and may play a role in iron transport in addition to iron storage [44,48]. Thus, free iron is not available and bacteria are iron restricted whatever their hosts. Therefore, they must be able to chelate and actively take up any available iron in order to grow and successfully infect their hosts. It has been shown that expression of the majority of genes involved in iron acquisition and metabolism, such as those coding for surface receptors, transport proteins, some hemolysins and cytotoxins, are controlled by the repressor Fur [1,3]. In this study, we show that B. cereus can use hemoglobin, hemin and ferritin as iron sources through the surface receptor IlsA that also belongs to the Fur regulon. We show that B. cereus strain ATCC 14579 is not able to use lactoferrin or transferrin for its growth, which is in accordance with Sato et al. 1999 [30,31] and in disagreement with Park et al. 2005 [29]. This result suggests that there might be strain variations, related to iron acquisition, but also that this B. cereus strain might be unable to cope with the antibacterial activity of lactoferrin [49], since higher concentrations of lactoferrin affected its growth. Hemoglobin and ferritin are intracellular iron rich proteins and must be released from cells in order to be used by B. cereus for its growth. It has been shown that B. cereus produces a large variety of cytotoxic proteins (Hbl, Nhe, CytK, Clq and HlyII) able to lyse various eukaryotic cells including erythrocytes [50–52]. Expression of these cytotoxic proteins with the exception of hemolysin II (HlyII), is regulated by the pleiotropic regulator PlcR [24,53]. Then, all these factors may contribute to release hemoglobin, heme and ferritin from cells following cell lysis. These iron-binding molecules can then be used for B. cereus growth after direct binding to the surface receptor, IlsA. Our results show that purified recombinant IlsA can bind hemoglobin and ferritin in vitro. In addition, IlsA also binds heme alone, suggesting that the interaction between IlsA and hemoglobin is due to its interaction with heme. How IlsA binds heme is currently unknown; however, studies from other NEAT proteins have identified conserved tyrosine residues and hydrophobic residues that form a heme pocket, which mediates heme binding [11,12]. These residues are also present in the IlsA NEAT domain, suggesting that IlsA mediates heme binding through this NEAT. The interaction with ferritin is much less evident, although it has previously been reported that the NEAT protein IsdA in S. aureus binds to several non-heme host proteins, including the ferric iron carrier transferrin [54], and several matrix and plasma proteins [55]. IlsA is a more complex protein than IsdA because of the LRRs domains. These domains are found in a large range of proteins with different functions and are known to bind structurally unrelated protein ligands [56,57]. In Gram positive bacteria, only few LRR-containing proteins have been characterized to date,

injection into the hemocoel of G. mellonella (Figure 7A). A dose of ~500 mid-log phase bacteria (estimated as colonies forming units (cfu)) was injected and used as the start point. After 2 hours of injection, the number of the ΔilsA bacteria in the insect larvae decreased drastically (75.5 cfu), while the number of the wild-type bacteria remains stable (530 cfu). After 6 hours, B. cereus ΔilsA had a growth of about 10-fold less (39.4×10^3 cfu) than the B. cereus wild-type strain (50.3×10^4 cfu). After 24 hours, the growth of the mutant strain (18.5×10^3 cfu) was about 2.5-fold less than the B. cereus wild-type strain (50.7×10^3 cfu). The growth of B. cereus ΔilsA was significantly different (p<0.05) from the B. cereus wild-type strain at 2 hours and 24 hours after injection.

To test if the growth defect of the mutant strain affects the virulence of B. cereus in the hemocoel, we analyzed virulence tests where bacteria were directly injected into the hemocoel of G. mellonella. Where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella, where bacteria were directly injected into the hemocoel of G. mellonella.

results indicate that IlsA is important for the growth and the survival of B. cereus in the hemocoel following injection or natural injury.
especially in *Listeria monocytogenes*, *Streptococcus pyogenes* and *Enterococcus faecalis*, where they are involved in the interaction with the host [57]. Thus, it is tempting to speculate that the LRR domains of IlsA are involved in the binding to ferritin. To our knowledge, IlsA is the first LRRs protein that has a function in iron uptake. A recent study revealed that a surface protein of *Candida albicans*, Als3, is essential for iron acquisition from ferritin by binding to the fungal hypha [58]. However, there is no obvious structural homology, between Als3 (family of agglutinin like adhesion molecules) and IlsA. Moreover, the authors did not report any direct *in vitro* binding between ferritin and Als3. Thus, in depth structural studies related to the interaction between IlsA and ferritin are needed to elucidate this aspect.

Our results suggest that IlsA is a key protein of a new iron/heme acquisition system in *B. cereus*. To achieve the transport of ferric iron from ferritin and heme across the cell wall and the cytoplasmic membrane into the cytoplasm, IlsA has to interact with other bacterial partners. The iron-regulated surface determinants (Isd) involved in heme acquisition and transport has been studied in several Gram-positive bacteria especially in *S. aureus* and *B. anthracis*. In *S. aureus*, the Isd system is composed from cell wall anchored proteins acting as hemoprotein receptors that are in vicinity of an iron transporter ABC systems [9,10]. Unlike *S. aureus*, the heme uptake system Isd in *B. anthracis* depends on secreted NEAT domains proteins in the extracellular environment and also on those located in the bacterial envelope which enable heme transfer to an ABC transporter system and then into the cytoplasm [13,14,13].

The heme scavenging strategy evolved by *B. cereus* seems to be different from those described in *S. aureus* and *B. anthracis*, since it involves the surface protein IlsA, which contains SLH domains that enable its binding to peptidoglycan [59]. Homologs of IlsA are found in *B. anthracis* (belonging to Bsl surface proteins), but none of them possess the three conserved domains (NEAT, LRR, SLH) at the same time like IlsA. In addition to three SLH domains, BslK presents a NEAT domain, while BslL contains LLR domains [60]. Then, since *B. anthracis* can use heme as an iron source via the Isd uptake system [61], it might suggest that IlsA homologs in *B. anthracis* do not function in the same way as in *B. cereus*. Thus, on the basis of our studies and those from other Gram-positive bacteria, we propose a model (Figure 8) where IlsA interacts with NEAT proteins belonging to the Isd system to ensure heme transport through the bacterial envelope into the cytoplasm.

In *s silico* analysis of the *B. cereus* ATCC 14579 genome reveal, in addition to IlsA, several genes (Bc4547, Bc4548, Bc4549) encoding NEAT domain proteins. The protein encoded by

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**Figure 7.** IlsA is required for *B. cereus* infection in the model *G. mellonella*. (A) Effect of ilsA mutation on the growth of *B. cereus* after intrahemocoelic injection in *G. mellonella*. Wild-type and the mutant ΔilsA strains were injected separately into the hemocoel of last-instar larvae at a dose of 5 x 10^9 mid-log phase bacteria. Bacteria were counted at various times after injection (2, 6 or 24 h) from living larvae. Time 0 h is the real dose of each strain injected in the hemocoel. Results are the mean of bacterial counts from nine different larvae and error bars indicate standard errors of the means. (B) Effect of ilsA mutation on the virulence after intrahemocoelic injection in *G. mellonella*. Last-instar larvae were injected with various doses (10^2 to 10^5) of mid-log phase bacteria. Mortality was evaluated after 24 h of injection of *B. cereus* wild-type (bold histogram) and *B. cereus* ΔilsA (grey histogram). Results are mean values of four independent experiments and error bars indicate the standard errors of the means.

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Bc4548 contains a signal peptide, but lacks obvious anchoring motif. This protein, which has 98% identity with IsdX1 of *B. anthracis* [13,14], may function as a secreted hemophore that captures heme from hemoglobin. The proteins encoded by Bc4547 and Bc4549 with the anchoring motifs NSKTA and NPKTG, respectively, may be the substrates of the putative sortase B (Bc4543) and be located in variable positions throughout the peptidoglycan. These proteins have 87% identity with IsdX2 [14] and 98% identity with IsdC of *B. anthracis* [61] respectively. Similar to *S. aureus* IsdC [62,63] and *B. anthracis* IsdC [61], we propose that Bc4549 occupies a critical position enabling the transfer of heme to the iron permease system (Bc4544, Bc4545, Bc4546), followed by transport across the bacterial membrane. Finally, a putative intracellular monooxygenase, encoded by Bc4542 degrades heme to liberate iron to be used by *B. cereus*. The presence of IlsA is crucial for this iron-uptake system, since its disruption abolishes the ability of *B. cereus* to grow despite the presence of hemoglobin, hemin and ferritin. The mechanism by which IlsA permits the uptake of iron from ferritin is certainly not the same as from heme. We speculate that IlsA might be able to destabilize the ferritin structure, via a possible interaction with the IlsA LRR domains.

The structural modification may permit other factors, such as reductases [64] or proteases [65] to liberate iron (Fe^{3+}) that can then be captured by siderophores, such as petrobactin or bacillibactin [26] and transferred by an iron uptake-system into the cytosol. Studies are ongoing to investigate this hypothesis.

To understand the contribution of IlsA in the pathogenesis of *B. cereus*, we analyzed its expression during infection using *gfp* transcriptional fusion. The results showed that *ilsA* is highly expressed in the hemocoel where free iron is not accessible to bacteria, being bound to proteins such as ferritin [44]. IlsA is also expressed in collected hemocoel and when bacteria are directly injected into the hemocoel (results not shown), indicating that the expression of *ilsA* is not depending on a dying insect. Then, expression of *ilsA* could be initiated in response to iron depleted conditions and/or due to bacterial sensing of ferritin, thus enabling capture of iron from this source and subsequent use by *B. cereus* for growth and survival within *G. mellonella*. Moreover, disruption of *ilsA* decreases the growth and the virulence of *B. cereus* after injection into the hemocoel. However, the effect of IlsA on the virulence of *B. cereus* was larger following infection by oral route [32], compared to what we found here, following injection...
into the hemocoel. This can be explained by a combination of several factors among which one might rely on the activation of the ferritin production. Assuming when the bacteria are entering the hemocoel from the intestinal lumen, the innate sensing (production of antimicrobial peptides, ferritin etc.) has been turned on for a while [66] and thus less free iron is available. In this situation, the dcl mutant cannot grow as well as the wild-type. A second point is reliant to the dose dependent in vivo growth capacity of the dcl mutant, reported here (Figure 7B). Indeed, unpublished histopathological studies (Nielsen-LeRoux et al.), have shown that bacteria, from the intestinal lumen, enter the hemocoel by small numbers which can be considered equivalent to a low dose of hemocoel injected bacteria.

Together, these observations indicate that IlsA is an important factor required for adaptation within an insect host especially during the early stages of infection, as soon as the bacteria enter the hemocoel and before iron ions are released from degraded tissues. Thus, IlsA is the first host iron receptor in B. cereus shown to contribute to in vivo growth by using hemoglobin, heme and ferritin as an iron source, as well as contributing to virulence in vivo. To our knowledge, it is also the first time that an extracellular pathogen has been shown to use ferritin for its growth in physiological conditions that are correlated with host infection. The intracellular bacteria Neisseria meningitidis has been reported to degrade ferritin by manipulating the cellular machinery and the lysosomal activity [67] and L. monocytogenes was reported to use ferritin in vitro via a reductase activity [64].

In this study, we provide evidence that IlsA is necessary and sufficient to mediate interaction and iron acquisition from several iron-binding proteins. The exceptional structure of IlsA (NEAT and LRRs) probably explains its ability to bind to these different ligands. Moreover, since IlsA-like proteins are restricted to B. cereus group bacteria, it may be a concrete example of how bacteria create molecules adapted to their particular surface structure. Indeed, the SLH domain of IlsA permits binding to the peptidoglycan via a mechanism often found in this bacterial group [68]. Further investigations are needed for a complete understanding of the mechanism of iron-uptake through IlsA.

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Supporting Information

Figure S1 Purification of IlsA. Coomassie-stained 10% SDS-PAGE analysis of the purified GST-IlsA and GST proteins from recombinant E. coli. Lane M, molecular weight markers in KDa. Purified GST-IlsA and GST with apparent molecular weights shown on the gel, was loaded in lane 1 and 2 respectively.

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Author Contributions

Conceived and designed the experiments: ND MG MK CNL. Performed the experiments: ND CB JV HB. Analyzed the data: ND DL CNL. Contributed reagents/materials/analysis tools: MG JV HB MK. Wrote the paper: ND CNL. Helpful discussions: MG. Co-directeur of PhD thesis for N. Daou: MK. Participated in discussion and writing, head of lab where experiments were performed: DL.
