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***In vitro* and *in vivo* activity of new strains of *Bacillus subtilis* against ESBL-producing *Escherichia coli*: an experimental study.**

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Running title: *B. subtilis* strains against ESBL-*E. coli*

ABSTRACT

Aims: The gastro-intestinal tract is a major reservoir of extended-spectrum beta-lactamase (ESBL) producing *E. coli*. *Bacillus* spores may be used as probiotics to decrease digestive colonization by ESBL-*E. coli*. Our aim was to assess the *in vitro* and *in vivo* activity of new *Bacillus* strains against ESBL-*E. coli*.

Methods and Results: We screened the *in vitro* activity of 50 *Bacillus* strains against clinical isolates of ESBL-*E. coli* and selected *B. subtilis* strains CH311 and S3B. Both strains decreased ESBL-*E. coli* titers by 4 log₁₀ CFU L⁻¹ in an *in vitro* model of gut content, whereas the *B. subtilis* CU1 strain did not. In a murine model of intestinal colonization by ESBL-*E. coli*, CH311 and S3B did not decrease fecal titers of ESBL-*E. coli*. Ten sequences of putative antimicrobial peptides were identified in the genomes of CH311 and S3B, but not in CU1.

Conclusions: Two new *B. subtilis* strains showed strong *in vitro* activity against ESBL-*E. coli*.

Significance and Impact of Study: Despite strong *in vitro* activities of new *B. subtilis* strains against ESBL-*E. coli*, intestinal colonisation was not altered by curative *Bacillus* treatment even if their spores proved to germinate in the gut. Thus this work underlines the importance of *in vivo* experiments to identify efficient probiotics. The use of potential antimicrobial compounds identified by genome sequencing remains an attractive alternative to explore.

Introduction

Third-generation cephalosporins and carbapenem-resistant Enterobacteriaceae are critical pathogens according to the World Health Organisation priority list of antibiotic resistant bacteria. The intestinal microbiota is a major reservoir of multidrug-resistant

Enterobacteriaceae (MDR-EB), and intestinal colonization by MDR-EB is promoted by intestinal dysbiosis (Sorbara *et al.* 2019). Intestinal colonization by MDR-EB may evolve from an asymptomatic carriage to various infections including urinary, gastrointestinal and bloodstream infections. Furthermore, the intestinal carriage of MDR-EB can lead to environmental contamination and transmission to healthy or diseased subjects. Hence, treatments to decrease the intestinal carriage of MDR-EB are crucial to limit the global spread of antimicrobial resistance. Various strategies have been assessed but none has proved to be effective so far (Tacconelli & Pezzani, 2019).

Probiotics may be considered to restore dysbiotic intestinal microbiota, to treat symptomatic intestinal infections and/or to decrease asymptomatic intestinal colonization by MDR-EB. Probiotic preparations, usually consisting of *Bifidobacteria*, *Lactobacilli*, *Enterococci* and/or *Streptococci* alter intestinal microbiota (Chae *et al.*, 2016) and showed variable efficacy in preventing experimental intestinal infections due to *E. coli* in animals (Fukuda *et al.* 2011; Guerra-Ordaz *et al.* 2014). Besides, their efficacy in humans on improving microbiome reconstitution after an antibacterial treatment has not been demonstrated, and a commercial probiotic preparation even delayed and decreased post-antibiotic restoration of the gut microbiota and prevented post-antibiotics reduction of antimicrobial resistance genes (Suez *et al.*, 2018; Montassier *et al.*, 2021). Furthermore, few randomized controlled trials have been conducted to assess if probiotics may decrease intestinal colonization by MDR-EB in humans (Newman & Arshad, 2020). Hua *et al.* found no effect of a commercial probiotic on rectal colonization by ESBL-producing bacteria in preterm newborns, although subgroup analysis suggested an effect in non breastfed patients (Hua, Tang & Mu, 2014). *Saccharomyces boulardii* and other commercial probiotic preparations consisting of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* (Vivomixx®) or *S. boulardii*, *L. acidophilus*, *B. animalis subsp. lactis* and *Lacticaseibacillus paracasei* (Bactiol Duo®) failed to significantly reduce intestinal colonization by Extended Spectrum Beta-Lactamase producing Enterobacteriaceae (ESBL-EB) in adult patients (Ljungquist *et al.*, 2020; Wieërs *et al.*, 2020). Hence, there is a need for probiotics that would reduce intestinal colonization by MDR-EB. *Bacillus* spp consists

of different species of spore-forming soil bacteria that are commonly ingested with vegetables, and reach a concentration of about 10^{4-5} colony-forming units (CFU) per gram of animal and human faeces (Elshaghabee *et al.* 2017). *Bacillus* spores are resistant to the acidity of gastric contents and are stable for long periods in various environments (Cutting 2011). After germination, vegetative cells of *Bacillus* produce antimicrobial compounds (AMC) that confer an antimicrobial activity against various pathogens (Caulier *et al.* 2019). Most of these compounds have bactericidal activity against gram positive bacteria but a few, such as Gramicidin S and Polymyxins, are active against gram negative bacteria (Mogi & Kita, 2009). Unfortunately, the latter antimicrobial peptides (AMPs) have toxicity and can induce colistin resistance in other enterobacteria (Halaby *et al.* 2013).

Therefore, alternative *Bacillus* strains that could serve as a source for safer AMCs have been sought (Latorre *et al.* 2016; Guo *et al.* 2017; Caulier *et al.* 2019). Treatment with *Bacillus* spores has been shown to prevent various gastrointestinal infections, including infections due to *E. coli* in piglet, chicken, rabbit and mouse (La Ragione *et al.* 2001; La Ragione & Woodward, 2003; D'Arienzo *et al.* 2006; Tsukahara *et al.* 2013; Guo *et al.* 2017). *Bacillus* also abrogated asymptomatic intestinal colonization of methicillin resistant *S. aureus* in mice (Piewngam *et al.* 2018). However, whether *Bacillus* treatment decrease the intestinal colonization by MDREc remains unknown.

Consequently, we searched for new strains of *Bacillus* that would inhibit ESBL-producing *E. coli* (ESBL-*E. coli*) under in vitro conditions that mimic the gut environment. We also characterised their genome and tested their efficacy as a curative treatment of intestinal colonization by an ESBL-*E. coli* in a murine model.

Materials and Methods

Bacterial strains and media

Clinical isolates of ESBL-*E. coli* were collected at the University Hospital of Nantes. One of them was also resistant to carbapenems through an OXA-48 carbapenemase and was used

in the *in vitro* culture test and in the murine model of intestinal colonization. The reference strain for *Bacillus subtilis* was the commercially available CU1 strain (Lefevre *et al.* 2015). For bacterial numeration, we used CHROMID™ ESBL agar plates (Biomérieux) for ESBL-*E. coli*, and LB agar NaCl plates (75 g L⁻¹ NaCl) for *Bacillus* and *Staphylococcus* spp strains.

Isolation of *Bacillus* strains from the environment and identification

Samples were collected from different environmental sources such as soil, mice food, lake and river mud as well as faeces from a variety of organisms (human, mouse, dog and snail). Each sample was mixed with water. One ml of each mix was heated at 80°C for 20 min to kill vegetative cells, leaving *Bacillus* spores. Then they were spread on LB NaCl agar plates. For identification, 16S RNA genes were amplified with universal primers D88 (5'-AGAGTTTGATCCTGGCTCAG-3') and F17 (5'-CCGTCAATTCCTTTGAGTTT), and *gyrA* genes were amplified with primers *gyrA*-f (5'-CAGTCAGGAAATGCGTACGTCCTT) and *gyrA*-r (5'-CAAGGTAATGCTCCAGGCATTGCT). Sequences were blasted in NCBI.

Spores production

In order to produce large amounts of spores, an overnight culture of *Bacillus* in 10 ml LB medium was inoculated into a flask containing 400 ml of Difco Sporulation Medium (Monteiro *et al.* 2005). After 3 days of shaking at 37°C, most of the *Bacillus* bacteria were in a sporulated form. The culture was then centrifuged, washed and resuspended in 3 ml of sterile water. Titration of spores was carried out after heating this suspension for 20 min at 80°C and then stored (about 10¹⁰-10¹¹ spores ml⁻¹) at -80° C.

Colony diffusion assay

The *in vitro* inhibitory activity of *Bacillus* against ESBL-*E. coli* was screened as follows. Two µL of fresh *Bacillus* suspension (10⁶ CFU ml⁻¹) were inoculated on the surface of a dried LB or minimal medium with 0,2% inulin agar plates where an ESBL-*E. coli* isolate had been spread. Fifteen clinical isolates of ESBL-*E. coli* were used. After incubation for 24 hr at 37°C, the areas of inhibition around *Bacillus* colonies were determined. The *in vitro* activity of strains was

classified as strong, medium and weak for inhibition diameter higher than 20mm, between 10 and 20 mm, and lower than 10 mm, respectively.

Co-culture of *Bacillus* and ESBL-*E. coli* isolates in murine faeces

In order to mimic the culture conditions in colonic content (including low oxygen pressure), 50 mg of faeces from an antibiotic naive mouse were suspended in 1 ml of sterile water and shaken at 20 Hz (Mixer Mill MM 400, RETSCH's) for 5 min to make a homogenous suspension. Then, 100 µl of this suspension was mixed with suspensions of vegetative cells of *Bacillus* and ESBL-*E. coli* strains (in a 1:1 ratio) to obtain 10⁶ CFU ml⁻¹ of each bacterium (final volume, 1 mL), supplemented or not with 0.2% inulin (Bulk powders). Inulin was tested since it is a prebiotic which could be used by *Bacillus* as a carbon source. The 2 ml tubes (containing a 1ml suspension) were shaken horizontally for 24 hr at 37°C (orbital shaker-incubator ES-20, Grant bio). Titers of *E. coli* and *Bacillus* were assessed after incubation at 37°C for 24 hr and 48 hr, respectively. All experiments were done in triplicate.

Murine model of intestinal colonization by ESBL-*E. coli*

All experiments were approved by the Animal Experiment Committee of Pays de la Loire (France, authorization number APAFIS#18120) and ARRIVE Guidelines were followed. Six weeks old male Swiss mice (Janvier Labs, Saint-Berthevin, France) were kept in individual cages with free access to food and water. First, intestinal dysbiosis was induced with amoxicillin (0.5 g L⁻¹) in drinking water for 3 days. Pantoprazole (0.1 g L⁻¹) and inulin 0.2% (Bulk powders) were added to drinking water of all mice, from the experiment 1st day and the day of amoxicillin discontinuation, respectively. Pantoprazole was added because it is known to suppress the gastric acid production and to promote intestinal colonization by certain bacteria (Stiefel *et al.* 2006). ESBL-*E. coli* (10⁶ CFU) was instilled intragastrically the day of amoxicillin discontinuation. At one day post *E. coli* inoculation (dpi), 10⁹ spores of *Bacillus* CH311 were daily administered by intragastric instillation, for 4 days. The experimental protocol was slightly modified to assess activity of *Bacillus* S3B: amoxicillin was administered for 5 days, and ESBL-*E. coli* was inoculated 2 days after amoxicillin was stopped. Faecal samples were collected

daily. Faecal titers of *Bacillus*, *Staphylococci* and ESBL-*E. coli* were assessed by plating serially diluted faeces. There were 4-7 mice per group. No mouse was excluded from analysis. Researchers were aware of the group allocation during the experiment. To enumerate the total number of *Bacillus* (spores and vegetative cells) per gram of faeces, faeces were homogenized in sterile water before plating serial dilutions in LB NaCl agar and incubating at 37°C for 24 h. To enumerate the total number of spores, suspensions were heated for 20 min at 80°C and then spread on the same medium. The difference in the number of CFU between the non heat-treated and heat-treated suspensions was considered as the number of spores that germinated. The spores of S3B and CH311 in frozen stocks were used as control to check the efficiency of this procedure.

Genome sequencing and assembly

Whole-genome sequencing was performed using the Illumina MiSeq platform, with a paired-end library (2 x 150 bp paired-end read mode). Illumina reads were trimmed using Trimmomatic (Bolger, Lohse & Usadel, 2014), quality filtered using the Fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and then assembled using the SPAdes software (Bankevich *et al.* 2012; Nurk *et al.* 2013). SIS and GapFiller version 1.10 (Boetzer & Pirovano, 2012; Nadalin, Vezzi & Policriti, 2012) were used to improve the initial set of contigs, and the closest complete genome was used as the reference to order and orient the contigs. The draft genome sequences of CH311 and S3B were deposited in NCBI under accession numbers JAAUXF000000000 and JAAUXE000000000 respectively.

Genome annotation, comparative genomics and phylogeny

The nucleotide sequences of CH311 and S3B strains were also submitted to ResFinder 3.2 servers (<https://cge.cbs.dtu.dk>) for identification of acquired resistance genes. All annotated proteins with unknown function were submitted to Deep-AmPEP30 (Yan *et al.* 2020) to predict peptides with antimicrobial activities. The genomes of 423 *B. subtilis* strains were downloaded from Genbank (<http://www.ncbi.nlm.nih.gov/genome>), redundant sequenced strains and very poor genome quality was removed resulting in a total number of 405 genomes. The 405 *B.*

subtilis genomes were submitted to CSIphylogeny (<https://cge.cbs.dtu.dk/services/CSIPhylogeny/>). The core genome polymorphic sites were retrieved and phylogenetic tree constructed using Fasttree (Price, Dehal & Arkin, 2010). To normalize genes predictions, all genomes were annotated using Prokka (Seemann, 2014). The pangenomic analysis was performed using Roary software (Page *et al.* 2015). The gene homologies between the strains were assessed with >98% nucleotide identity and >80% sequence coverage cutoffs.

Statistical analyses

Means were reported with standard deviations. Difference in mean titers were tested with t test or ANOVA according of the number of compared groups. The efficacy of treatments was assessed using linear mixed effect models, where faecal titer was the dependent variable, treatment and time being the fixed independent variable, and mouse the random effect. Statistical analyses were performed with R 3.6.3, R Foundation for Statistical Computing, Vienna, Austria.

Results

Identification of Bacillus strains from the environment and screening for inhibitory activity against ESBL-E. coli

Fifty different *Bacillus* strains were isolated from environmental samples. Based on their 16S RNA and *gyrA* genes sequences, they were identified as *B. subtilis*, *B. licheniformis* or *B. pumilus*. We screened their inhibitory activity against 15 clinical isolates of ESBL-*E. coli* isolates using the colony diffusion assay. Nine *Bacillus* isolates inhibited growth of ESBL-*E. coli* isolates (Table 1). Among these, two *Bacillus subtilis* strains CH311 and S3B, isolated from dog and snail faeces respectively, presented the highest inhibition against 12 or 13 different strains of clinical isolates of ESBL-*E. coli*. For several strains, inhibition diameters were higher in minimal medium with inulin than in LB (data not shown).

In vitro activity against ESBL-E. coli

In order to assess the activity of these strains in conditions mimicking the gut environment, co-cultures of an ESBL-*E. coli* and *Bacillus* strains were performed in faeces suspension with or without inulin, and ESBL-*E. coli* titers were determined after 24 hr. *Bacillus* titers increased after 24-h culture in the medium without inulin and remained steady in the medium with inulin (Fig. 1A). However, after a 24-h culture, titers of *Bacillus* isolates in the medium without inulin showed a high inter-isolate variability, whereas they did not when cultivated with inulin.

After a 24-h culture without *Bacillus*, mean ESBL-*E. coli* titers increased in the medium with or without inulin. ESBL-*E. coli* titers after a 24-h co-culture with *Bacillus* without inulin were significantly different from the control for one *Bacillus* isolate (isolate Bac2, 7.4 ± 0.3 vs $8.1 \pm 0.3 \log_{10}$ CFU ml⁻¹, t test P-value, 0.04, Fig.1B). The combination of *Bacillus* and inulin was more effective, as 5 among 11 *Bacillus* strains significantly decreased ESBL-*E. coli* titers, the latter ranging from 3.7 ± 0.5 to $4.8 \pm 1.0 \log_{10}$ CFU ml⁻¹. Regardless of the culture conditions, the commercial *B. subtilis* strain (CU1) did not show inhibition (Fig 1AB). For subsequent *in vivo* experiments, we selected the 2 most active strains on ESBL-*E. coli* (*B. subtilis* CH311 and S3B). Of note, these strains also had strong *in vitro* activity against *S. aureus* (data not shown).

In vivo* activity in a murine model of intestinal colonization by ESBL-*E. coli

Then, we assessed the activity of curative treatment with *Bacillus* spores on ESBL-*E. coli* fecal titers. First, we tested the *B. subtilis* S3B strain. Between the 2nd and the 5th day after starting treatment with *Bacillus* S3B (i.e. between 3 and 6 days post *E. coli* inoculation), fecal titers of *Bacillus* in treated mice exceeded those of control mice by $3.0 \pm 0.2 \log_{10}$ CFU g⁻¹ (P-value < 0.00001, Fig. 2A). Although *Bacillus* titers decreased when *Bacillus* administration was discontinued, they were still higher than controls at 6 dpi ($4.5 \pm 0.2 \log_{10}$ CFU g⁻¹). Germination rates of a *Bacillus* spores in faeces are reported in Table 2. During the same period, neither ESBL-*E. coli* or commensal *Staphylococcus* titers were significantly altered (differences with control, $-0.2 \pm 0.7 \log_{10}$ CFU g⁻¹, P-value, 0.82; and $-0.3 \pm 0.7 \log_{10}$ CFU g⁻¹, P-value 0.64, respectively, Fig. 2B and 2C).

Second, we tested the activity of *B. subtilis* CH311. Fecal titers obtained during and after treatment with *Bacillus* CH311 were slightly higher than those with *Bacillus* S3B. Indeed, between the 1st and the 5th day after starting treatment with *Bacillus* CH311, *Bacillus* fecal titers were higher by $3.7 \pm 0.4 \log_{10} \text{ CFU g}^{-1}$ in comparison with control mice (P-value < 0.00001, Fig. 2D). Meanwhile, *Staphylococcus* faecal titers were lower by $1.3 \pm 0.3 \log_{10} \text{ CFU g}^{-1}$ (P-value < 0.00001; Fig. 2E). However, ESBL-*E. coli* titers were not significantly altered by *Bacillus* treatment (difference with control, $-0.5 \pm 0.6 \log_{10} \text{ CFU g}^{-1}$, P-value, 0.42, Fig. 2F).

Genome sequencing of S3B and CH311 strains

Genomes of S3B and CH311 strains were sequenced in order to (i) determine whether they are novel strains and (ii) try to identify the genes responsible for the strong *in vitro* inhibitory activity against *E. coli*. Comparative genomics statistics are summarized in table 3. To specify the relationship between the isolates, we performed a pangenomic analysis of the 405 *B. subtilis* genomes. We predicted a total of 894 orthologous genes representing the core genome (genes shared by all strains) and 46,942 orthologous genes and singletons representing the accessory genome (genes encoded in one or more isolates, but not in all). Strain CH311 shared 86% of gene content with strain GXA-28 and 85% with strain H19. The strain S3B shared 97% of gene content with strains MSP1 and MSP5. The strains CH311 and S3B shared 83% of their gene content. We further analysed SNPs in the core genome and detected 38,016 polymorphic sites between all *B. subtilis* genomes. For strain CH311 we detected 1,521 SNPs with the closest strain H19. For strain S3B we detected 140 SNPs with the closest strains MSP1 and MSP5. We also detected 2,198 SNPs between strains CH311 and S3B. Altogether, phylogenetic and comparative genomic analyses demonstrated a polyclonal population, suggesting that CH311 and S3B are phylogenetically distinct from other strains (Fig. S1 available as supplementary data).

We then searched 50 antimicrobial compounds (AMCs) encoding genes (Sumi *et al.*, 2015; Caulier *et al.*, 2019) within the annotated genomes of the two newly identified CH311 and S3B *B. subtilis* strains. Nine different antimicrobial compounds were detected in both strains,

including 4 genes reported to have a mild inhibitory activity against Gram-negative bacteria (Table 4). Of note, these 4 genes have also been reported in CU1 genome. Then, we pursued this analysis and all peptides with unknown function were submitted to the software Deep-AmPEP30 (Yan *et al.*, 2020) to predict potential new peptides with anti-microbial activities. As presented by the table 3, we found respectively 5 and 9 peptides in the genomes of CH311 and S3B with a probability above 0.7. All of them are different but 4 predicted peptides from CH311 genome are also present in the CU1 genome. Thus sequences of putative antimicrobial peptides only found in CH311 and S3B are presented in the figure S2 (available as supplementary data).

Discussion

From our environmental sample screening, we isolated new *Bacillus* strains. About 10% of them displayed a strong *in vitro* activity against ESBL-*E. coli* not only in the colony diffusion assays but also in conditions that mimic faecal microbiota. Thus most of the collected strains of *Bacillus* did not show such bactericidal activity, as did the reference CU1 *Bacillus* strain and in agreement with previous reports (Caulier *et al.*, 2019).

The *in vitro* activities of the newly isolated *Bacillus* in gut mimicking conditions were strongly related to the presence of inulin, albeit *Bacillus* grew well in faeces without this oligosaccharide. However, without addition of these *Bacillus*, inulin 0.2% alone was not able to decrease ESBL-*E. coli* titers in a suspension of antibiotic naïve mouse faeces. The *in vitro* efficacy of *Bacillus* with inulin on ESBL-*E. coli* may be mediated by the fermentation of inulin by *Bacillus*, production of short chain fatty acids (SCFAs) and colonic acidification. Indeed, *Bacillus* produces SCFAs, mainly acetate, and the combination of pH under 5,75 and SCFAs proved to be deleterious for *E. coli* survival (Nakano *et al.* 1997; Sorbara *et al.* 2019). However, this mode of action was not predominant in our experiments, as pH after a 24-h co-culture ranged between 6 and 7 (data not shown). Alternatively, inulin may induce the synthesis of antimicrobial compounds by *Bacillus* as it was shown for the antifungal compound Bacillomycin

(Qian *et al.* 2015). It is thus likely that the *in vitro* bactericidal activities of *Bacillus* were due to the production of AMCs.

Despite using inulin and the strains S3B and CH311 that exhibited the most potent *in vitro* activity, we could not decrease ESBL-*E. coli* faecal titers in a mouse model of intestinal colonization. Several causes could explain this result. First, inulin concentration in the gut was not assessed and we cannot affirm that it was high enough to induce the activity of *Bacillus*. Second, the intestinal transit time of the mouse is as short as a few hours, which quickly eliminates this genus of the intestinal flora, as shown by the rapid decrease of *Bacillus* titers in our mice faeces. Third, although the *Bacillus* strains proved to germinate at quite high level, they are subjected to harsh environmental conditions including low oxygen tension and high faeces concentration. Inhibitors may reduce growth and/or gene expression of *B. subtilis* in comparison to what we observed in our *in vitro* experiments. As the bactericidal activity of *Bacillus* on *Staphylococci* was previously described in mice gut (Piewngam *et al.*, 2018), we tested it as a control of the metabolic activity of our strains in these conditions. Activity against endogenous *Staphylococci* was found for the CH311 strain, but not for S3B, suggesting that AMCs are differentially expressed in the gut. This result suggests that the lack of activity of *Bacillus* in our murine model is likely to be caused by insufficient expression/activity of the AMCs.

Genome sequencing of CH311 and S3B showed that these strains potentially expressed at least nine antimicrobial compounds. Four of these AMCs are reported to have inhibitory activity against Gram-negative bacteria, but these effects are often described as mild and all of them are also potentially produced by the CU1 strain that showed no *in vitro* activity against *E. coli*. Only two compounds, Subtilin and Kanosamine, were found in CH311 and/or S3B and not in the CU1 strain but they have no activity against Gram-negative bacteria (Sumi *et al.* 2015; Caulier *et al.* 2019). Therefore, it was not possible to attribute the inhibitory activity against ESBL-*E. coli* to any of these compounds, although it cannot be excluded that it could be due to different levels of gene expression of the gram-negative-active AMCs. In addition, some *Bacillus* AMCs likely remain unidentified and those that have been detected using Deep-

AmPEP30 must be investigated. Thus, further biochemical analyses are required to identify the origin of the bactericidal activity of these new strains of *Bacillus* against *E. coli*.

In conclusion, we described two new strains of *Bacillus* exhibiting an *in vitro* activity against ESBL-*E. coli*. Further studies are needed to elucidate their mechanisms of action and to assess if they could be used to treat infections or decrease colonization due to MDR *E. coli*.

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Conflict of interest

No conflict of interest declared.

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- 1) Substantial contribution to conception and design or the acquisition and analysis of data, Murad Ishnaiwer, Yihienew Bezabih, François Javaudin, Mohamed Sassi, Eric Batard and Michel Dion
- 2) Drafting or critically revising the manuscript: Murad Ishnaiwer, Yihienew Bezabih, Eric Batard, Pascale Bemer, François Javaudin, Mohamed Sassi, and Michel Dion.

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Figure 2: Effect of treatment with *Bacillus* on fecal titers of *Bacillus*, commensal *Staphylococci* and ESBL-*E. coli*. Fecal titers of *Bacillus* (A and D), *Staphylococcus* (B and E) and ESBL-*E. coli* (C and F). Mice were treated with spores of *B. subtilis* S3B (left) or *B. subtilis* CH311 (right). The experimental design is shown at the top of the figure: mice were treated with amoxicillin before being inoculated with ESBL-*E. coli*. Spores of *B. subtilis* S3B and CH311 were inoculated from 1 to 3 dpi, and from 1 to 4 dpi, respectively. Grey lines and circles, control; black lines and circles, *Bacillus* treatment.

Table 1 *Bacillus* strains: identification and screening for activity against ESBL-*E. coli*

| Strain | Origin | Identification method | Identification | Level of activity against ESBL <i>E. coli</i> (number of <i>E. coli</i> strains)* | | | | |
|--------------|--------------|-----------------------|-------------------------|--|--------|------|--------------|-------|
| | | | | strong | medium | weak | any activity | total |
| CU1 | commercial | - | <i>B. subtilis</i> | 0 | 0 | 0 | 0 | 15 |
| A532 | Bogoria Lake | 16S RNA | <i>B. licheniformis</i> | 0 | 0 | 0 | 0 | 15 |
| S2 | Snail faeces | gyrA | <i>B. subtilis</i> | 2 | 4 | 6 | 12 | 15 |
| S3B | Snail faeces | gyrA | <i>B. subtilis</i> | 6 | 3 | 3 | 12 | 15 |
| S28 | Snail faeces | 16S RNA | <i>B. pumilus</i> | 4 | 4 | 3 | 11 | 15 |
| CH311 | Dog faeces | gyrA | <i>B. subtilis</i> | 10 | 1 | 2 | 13 | 15 |
| Bac2 | Mice Aliment | gyrA | <i>B. subtilis</i> | 4 | 0 | 5 | 9 | 15 |
| Bac4 | Mice Aliment | gyrA | <i>B. subtilis</i> | 2 | 2 | 6 | 10 | 14 |
| MD1 | Human faeces | gyrA | <i>B. subtilis</i> | 2 | 0 | 4 | 6 | 15 |
| Mi1 | Mice faeces | gyrA | <i>B. subtilis</i> | 3 | 0 | 5 | 8 | 15 |
| Mi2 | Mice faeces | gyrA | <i>B. subtilis</i> | 2 | 5 | 4 | 11 | 15 |

*Note. Activity was assessed using a colony diffusion assay on LB agar against 15 clinical isolates of ESBL-*E. coli*. A532 strain was kept to provide a negative control.

Table 2 Percentage of spores and vegetative cells of *Bacillus* in faeces

| isolate | CH311 | S3B |
|------------------------------------|--|---|
| total cells, CFU/g | $2.4 \cdot 10^6 \pm 1.8 \cdot 10^6$ | $1.2 \cdot 10^5 \pm 8.2 \cdot 10^4$ |
| Spores, CFU/g (%) | $1.4 \cdot 10^6 \pm 1.4 \cdot 10^6$ (51% \pm 12%) | $5.6 \cdot 10^3 \pm 3 \cdot 10^3$ (5% \pm 2%) |
| Vegetative cells, CFU/g (%) | $1 \cdot 10^6 \pm 5 \cdot 10^5$ (49% \pm 12%) | $1.2 \cdot 10^5 \pm 7.9 \cdot 10^4$ (95% \pm 2%) |

*Note. Faeces were sampled in mice treated with *B. subtilis* S3B and CH311 3 and 2 days after inoculation of ESBL-*E. coli*. *Bacillus* cells were enumerated in faeces before (total cells) and after 20 min heating at 80°C (spores). Vegetative cells were estimated as the difference between total cells and spores.

Table 3 Genomic and comparative genomic statistics

| | CH311 | S3B |
|--|---|---|
| Genome properties | | |
| Genome size (bp) | 4,190,428 | 4,038,907 |
| Number of contigs | 5 | 6 |
| Total genes | 4,377 | 4,138 |
| Protein-coding genes | 4,209 | 3,995 |
| Comparative genomics | | |
| Number of unique genes | 48 | 8 |
| Closest genomes (Number of SNPs ; % of shared orthologous genes) | H19 strain (1,521 SNPs ; 85.8%) | MSP1 and MSP5 strains (140 SNPs ; 97.4%) |
| Genome annotation | | |
| Number of genes with unknown function | 712 | 552 |
| Number of predicted antimicrobial peptides with Deep-AmPEP30 , product probability > 0.7 | 5 | 9 |
| Predicted antibiotic resistance genes (Phenotype) | <i>mph</i> (K) gene (spiramycin, telithromycin) | <i>mph</i> (K) gene (spiramycin, telithromycin) |
| | <i>aadK</i> gene (streptomycin) | <i>aadK</i> gene (streptomycin) |
| | <i>tet</i> (L) gene (doxycycline, tetracycline) | |

Table 4 Genes associated with antimicrobial compounds in 3 strains of *Bacillus subtilis*

| antimicrobial compound | inhibition spectrum | | | gene detected in strain | | |
|------------------------|------------------------|------------------------|-------|-------------------------|-------|-----|
| | Gram positive bacteria | Gram-negative bacteria | Fungi | CU1 | CH311 | S3B |
| Subtilin | + | | + | | | + |
| Subtilosin-A | + | + | | + | + | + |
| Surfactin | + | | + | + | + | + |
| Bacilysin | + | + | + | + | + | + |
| Bacillaene | + | + | | + | + | + |
| Plipastatin | | | + | + | + | + |
| Bacillibactin | | | + | + | + | + |
| Kanosamine | | | + | | + | + |
| Bacilysocin | | + | + | + | + | + |

*Note. Ribosomal peptide is indicated in bold character. +, target of the AMC or gene coding for peptide or AMC synthesising enzyme.

Supporting information

Figure S1: (A) Phylogenetic tree of 405 strains of *B. subtilis*. The 405 *B. subtilis* genomes were submitted to CSIphylogeny (<https://cge.cbs.dtu.dk/services/CSIPhylogeny/>). The core genome polymorphic sites were retrieved and phylogenetic tree constructed using Fasttree. (B) Phylogenetic tree of 10 closest *B. subtilis* genomes to S3B and CH311. According to core genome SNPs analysis, 10 closest genomes were selected for phylogenetic analysis. Maximum likelihood (ML) phylogeny was constructed using iqtree2 with 100 non-parametric bootstrap iterations (Nguyen *et al.* 2015).

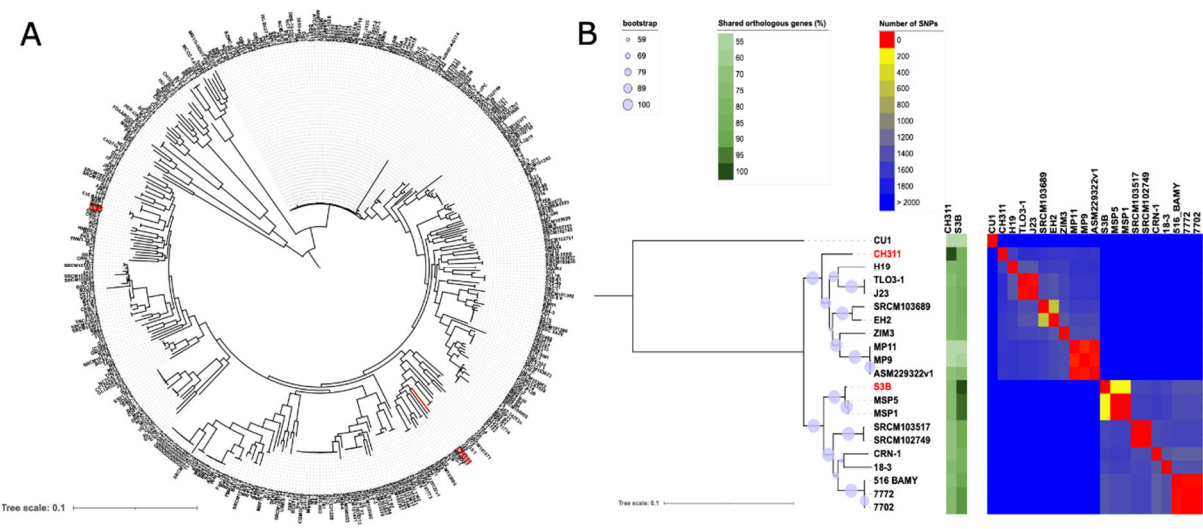


Figure S2: Sequences of putative antimicrobial peptides of strains CH311 and S3B that were not present in the strain CU1, as determined by the software Deep-AmPEP30

CH311

MTIYEQIKDALKNKINELVSPQEVKKTLEKEYGTNPDSIILSDYCYNRYNKGISFNKHLFEYMN
RSSYKYLGENSLYTGLIFRKSKEGDEKIVIVGEWVNGVKSLEASVTNNQINDQAEIISKEQLV
NLYNEYNQILR

S3B

1. MIQYASESINLPGEIAFKDVREIFFYQIAKISCFYFLLFCAIFAAVNFINGWPRIVYGSDA
LNLFMMSLIIVMSVLFLLLLLLLLLYVKFSRAYKKNERMKSRTYTLNQEGIRICSKKYDLIFNW
NEITAVFEYKNIFRINTSSSQYIAIPKHFFHSEVEMNRFKEIILKNTETKKLKFKKDQH
2. MKILGVTGVILICLLAISVLMQGFSLTKAVYNNMSSFKMTTFAEWVILFFVLVLV
REMYVIYKSKKKNP
3. MTEKKQQNKPENPEHNDLTDPIPNELKENMNDEKHKRQQRDNSQSERDYDTK
4. MFRIFKMSFAVIIIILALIAFNTEHTSVIQSVMLVFLGAVMFMQGLEERKKENDGSGA
FNIYTAVFVWSVSLIGFTLHII
5. MVREAAMLHIKEGLEQEFEDAFRQAAPISGMKGYITHSLSKCMEETHKYLLLVEWE
TLEDHTEGFRGSSEYQEWKALLHRFYTPFPTVEHFQDV
6. MLQTPIGRLRTMGFIEGMSLLILLFIAMPLKYWAGLPLAVTIVGSVHGGLFILYLLVLAY
ATFSVKWPLKWSAAGFVAAPVFPFNFLYDRGLRKYK
7. MNYKGITLLCVMMLLFSAIASFPVSAQAKDQDAGILIIYSTLDGKESSQVKMLDLAG
HFTSHVTVKKDLDEASDFKGDHVIYYGQTKRKLKSLISGVKNPVVAIGYNAGQINQF
SGLSLARKENVFQVHSRSEKADVLESGLNVLSISGLKGTALYTFKADEGTTHSFIWTKKKG
NVYIGLTNLLNDNLIVAKQLREAFGEKAGTTLLYLRLEDISPMSDEKLLLQAGTYLHKRHPFIL
AVIPVYLNPELGDKVYLSDKPKMVKVLKQLQSMGGSIIHGYTHAYRYSETGEGFEFWDK
ADQPITSGNAEDPPSILEKEQDFPNEQAYHSYLEPFREKEETYTKQLTRAIEDLTSSGLYPL
AFEAPHYTMSEYGYQIASQYFTSIFGQVQLSSTTWKTSGAPPFVTAPSMHGMTLYPETIGF
VDTSKQNPLGEMEERISQMIDFEGGVAGGFYHPYLGMYLPELVDQMERIPDSEWLDLKKKT
KQTVKTDKVEIHTSGDGTIQVKNGVSPIDEFFDHRQTPEKALWILSAVVLLFVIMFVSYTFY
LRATLKKRIFKERRSLG
8. MLSFLVSLVVAIVIGLIGSAIVGNRLPGGIFGSMIAGLIGAWIGHGLLGTWGPSLAGFAI
FPAIIGAAIFVLLGLIFRGLRKEAMPWSMKDYPASLKNLEKPVRKKAIDIANAMIDEGYEEGR
APIATSKAKEWAENASTDEIDDFLTHDDETERDADPSSGSGPELMNKAEHVIKHKKGWAVK
AEGAKRVSEIKDTKKEAIERAKEIAAHKGIEVIVHLADGSVQRKIKTGS