Improving Phylogeny Reconstruction at the Strain Level Using Peptidome Datasets

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Abstract

Typical bacterial strain differentiation methods are often challenged by high genetic similarity between strains. To address this problem, we introduce a novel in silico peptide fingerprinting method based on conventional wet-lab protocols that enables the identification of potential strain-specific peptides. These can be further investigated using in vitro approaches, laying a foundation for the development of biomarker detection and application-specific methods. This novel method aims at reducing large amounts of comparative peptide data to binary matrices while maintaining a high phylogenetic resolution. The underlying case study concerns the Bacillus cereus group, namely the differentiation of Bacillus thuringiensis, Bacillus anthracis and Bacillus cereus strains. Results show that trees based on cytoplasmic and extracellular peptidomes are only marginally in conflict with those based on whole proteomes, as inferred by the established Genome-BLAST Distance Phylogeny (GBDP) method. Hence, these results indicate that the two approaches can most likely be used complementarily even in other organismal groups. The obtained results confirm previous reports about the misclassification of many strains within the B. cereus group. Moreover, our method was able to separate the B. anthracis strains with high resolution, similarly to the GBDP results as benchmarked via Bayesian inference and both Maximum Likelihood and Maximum Parsimony. In addition to the presented phylogenomic applications, whole-peptide fingerprinting might also become a valuable complementary technique to digital DNA-DNA hybridization, notably for bacterial classification at the species and subspecies level in the future.

Author Summary

Molecular based differentiation of bacterial species is important in phylogenetic studies, diagnostics and epidemiological surveillance, particularly where unusual phenotype makes the classical phenotypic identification of bacteria difficult. Typical bacterial
differentiation methods are often challenged by a high genetic similarity among strains. For decades, the technique of choice to classify and identify bacteria was DNA-DNA hybridization (DDH). The boosting of whole-genome sequencing technology facilitated the development of bioinformatics alternatives that could assist a much wider number of laboratories and are less biased to experimental errors. Currently, the Genome-to-Genome Distance Calculator web service, implementing the Genome-BLAST Distance Phylogeny (GBDP) method, provides the highest correlation to conventional DDH. Our methodology shows that whole peptide fingerprinting may complement the outputs of GBDP, i.e. experimental mass spectra may be used to cluster the bacteria, and more specifically it has been found useful for bacterial classification at the species and subspecies level. In addition, we present here how peptidome subsets obtained from in silico digestion of the peptidomes, is an efficient way to maintain the phylogenetic signal whilst reducing the total amount of data, making this methodology suitable for handling large data sets as in the case of epidemiologic studies.

This is a PLOS Computational Biology Methods paper.

Introduction

The most common techniques for bacterial classification and identification are conventional DNA:DNA hybridization (DDH) [1], comparison of 16S or 23S rRNA gene sequences or 16S–23S rRNA spacer regions [2], multi-locus sequence typing (MLST) [3] and rep-PCR fingerprinting [4], among others [5]. For decades, the technique of choice to identify and classify species has been DDH with a similarity value of 70% DDH as the species delimitation threshold [6]. In microbial taxonomy, DDH is mandatory whenever the 16S rRNA gene sequence similarity between two strains is above 97% for confirming that these do not belong to the same species. This threshold has recently been increased by proposing values of between 98.2 and 99.0%, depending on the phylum [7]. Conventional DDH has limitations, for instance, that it is only available in a few specialized molecular laboratories world-wide and it is particularly biased to experimental errors [8]. Due to this and because of the availability of whole-genome sequencing, this facilitated the development of bioinformatics alternatives to conventional DDH [9].

Here, the Genome-to-Genome Distance Calculator web service (GGDC; freely available at http://ggdc.dsmz.de/) currently provides the highest in silico correlation to conventional DDH–without sharing the aforementioned drawbacks—which is a crucial requirement for any such in silico method to maintain consistency in prokaryotic species delineation [10]. The GGDC server incorporates the latest version [10] of the Genome-BLAST Distance Phylogeny method (GBDP)—a highly optimized tool for the calculation of intergenomic distances—and estimates digital DNA-DNA hybridization values (dDDH values) from these distances under recommended settings [10]. Among other useful data, the dDDH values are reported along with confidence intervals, which are important for assessing the statistical uncertainty inherent to all model-based approaches [10]. In this way, GGDC can be reliably used for both species and subspecies delimitation [11].

The GBDP method incorporates several optimizations to avoid potentially biased results caused by elements such as paralogous genes or low-complexity regions. It is also robust against the use of incomplete genome sequences [10] and can be applied to both nucleotide
and amino acid data. Finally, it includes a pseudo-bootstrapping procedure [10] for the calculation of replicate intergenomic distances, which can be further used in phylogenetic applications to assess branch support values as shown earlier [11–13].

Matrix Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry (MALDI-TOF MS) has been applied as an alternative approach to identify and discriminate between species and strains [14–16]. This alternative is typically adopted when there is limited genetic variability within or across the species under study, and assumes the presence and detection of species/strain specific peptides through comparison of their mass-to-charge ratio. In this way this method supports species/strain differentiation. However, many of these differential peptides may not be detected due to their low abundance or other physicochemical properties, i.e., those methods are limited in such a way that it only explores a subset of the total peptidic variability.

To overcome this limitation, we have designed a novel in silico peptide fingerprinting methodology suitable for phylogeny inference. This methodology follows the same general principle of existing mass spectrometry approaches but it uses whole genome data and in silico protein digestion, i.e., it does not involve any conventional experimentation. Furthermore, the analysis stands on the shoulders of well-established software tools, namely PSortB [17], mzJava [18], SPECLUST [19] and MrBayes [20]. The aim is to be able to generate a valid and manageable list of peptides that are potentially specific to each strain. This list could then be further investigated using in vitro approaches, such as LC-MS/MS, towards the identification of biomarkers, strain specific peptides and the development of application-specific detection methods.

Our case study covers a subset of strains belonging to the Bacillus cereus group [21]. More precisely, the case study covers B. thuringiensis, B. anthracis and B. cereus (senso stricto) strains, which are known to share high genetic similarity [22]. Such strains are conventionally classified according to other features, such as their pathogenic potential or the presence of plasmids [23]. From a taxonomic point of view, separation of the three Bacillus species is still a subject of controversy among scientists. However, a recent large-scale whole-genome sequence-based study using GBDP elucidated the taxonomy within the B. cereus group and showed that B. thuringiensis, B. anthracis and B. cereus (senso stricto) species are indeed belonging to individual phylogenetic groups [12]. Other strains originally attributed to one of these three species, were either misclassified or belong to other novel species within the cluster. The results of the GBDP phylogenomic analysis serve as a good baseline, representative of what can currently be achieved with a state-of-the-art phylogenomic analysis as exemplified for the B. cereus group.

Currently, a method to infer bacterial taxonomy in silico through the use of peptidomes is missing. The development of such a method is appealing as it would complement GBDP analysis. Additionally, establishing the comparison and identification of unique peptides on an exemplary microbial data set would aid in the separation of closely related strains. Moreover, in silico peptidome fingerprinting is able to reduce whole proteome data into smaller binary matrices, which is of advantage when handling larger bacterial datasets. The amount of data may be decreased using different peptidome subsets without losing phylogenetic signal. Main results are discussed in this manuscript.

**Materials and Methods**

The following sections describe the methods and tools used in our peptidome-based strain-level genome comparison pipeline. These steps include the retrieval of proteins encoded in the comparison genomes, the prediction of the subcellular localization of the proteins, the digestion of proteins from different locations, the comparison of the peptides according to their mass and the subsequent computation of consensus peak sets. The software consisted of public, well-known tools and in-house customized scripts. Pipeline is depicted in Fig 1.
Genome and protein data retrieval

All the sequence data used in this study were retrieved from the BioProject collection of the National Center for Biotechnology Information (NCBI), using their public FTP site (ftp://ftp.ncbi.nih.gov/genomes/bacteria/) [24]. Our study focused on the complete genomes of *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis* whose BioProject accession numbers are listed in Table 1. Genetic data was obtained from ".fna" files, whereas proteomes for in silico digestion were obtained from ".faa" archives. *Bacillus subtilis* subsp. *natto* BEST195 was selected as an outgroup. For efficiency and to increase the flexibility in the analyses, protein data were stored in an in-house database.
Protein subcellular localization prediction

Subcellular localization defines the putative localization of the protein in the cell. This information is relevant because, for instance, extracellular proteins are used by the bacterium to communicate with its environment and thereby could help in bacterial differentiation. The subcellular localizations of the proteins were predicted using the standalone version of the PSortB v3.0 tool, following the developer guidelines [17]. The subsets corresponding to chromosomal proteins and plasmids were stored in the in-house database.

Peptidome generation

Bacterial proteomes were obtained for all the Bacillus strains used in this work. The open-source Java library mzJava from ExPASy (http://mzjava.expasy.org) supported protein digestion [18]. For the purposes of the present analysis, three proteases representing the major

Table 1. The Bacillus strains used in this study. Genome and protein data were retrieved from the Bio-Project collection of the NCBI in July, 2015.

| Bacillus strains                  | BioProject accession number |
|----------------------------------|----------------------------|
| B. anthracis str. A0248          | PRJNA59385                 |
| B. anthracis str. "Ames Antecesor" | PRJNA58083                |
| B. anthracis str. Ames           | PRJNA57909                 |
| B. anthracis str. CDC 684        | PRJNA59303                 |
| B. anthracis str. H901           | PRJNA162021                |
| B. anthracis str. Sterne         | PRJNA58091                 |
| B. cereus 03BB202                | PRJNA59299                 |
| B. cereus AH187                  | PRJNA58757                 |
| B. cereus AH820                   | PRJNA58751                |
| B. cereus str. ATCC 10987        | PRJNA57673                 |
| B. cereus str. ATCC 14579        | PRJNA57975                 |
| B. cereus B4264                   | PRJNA58757                 |
| B. cereus biovar anthracis str. CI 684 | PRJNA50615              |
| B. cereus str. E33L 10987        | PRJNA58103                 |
| B. cereus FB37/76 strain;F0837/76 | PRJNA83611                |
| B. cereus FRI-35                  | PRJNA173403                |
| B. cereus G9842                   | PRJNA58759                 |
| B. cereus NC7401                  | PRJNA82815                 |
| B. cereus str.Q1                   | PRJNA58529                 |
| B. subtilis subsp. natto BEST195 | PRJNA183001                |
| B. thuringiensis str. Al Hakam   | PRJNA58795                 |
| B. thuringiensis BMB171           | PRJNA49135                 |
| B. thuringiensis Bt407            | PRJNA177931                |
| B. thuringiensis HD 771           | PRJNA173374                |
| B. thuringiensis HD 789           | PRJNA173860                |
| B. thuringiensis MC28             | PRJNA176369                |
| B. thuringiensis serovar chinensis CT-43 | PRJNA158151              |
| B. thuringiensis serovar finitus YBT-020 | PRJNA158875              |
| B. thuringiensis serovar thungiensi str. IS5056 | PRJNA190186             |
| B. thuringiensis serovar konkuyan str. 97–27 | PRJNA58089             |
| B. thuringiensis serovar kurstaki str. HD73 | PRJNA189188              |
| B. thuringiensis YBT-1518         | PRJNA229419                |

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intestinal endoproteases were used: trypsin, chymotrypsin and pepsin (low specificity model, pH>2). Resulting peptides, denominated peptidomes, were also stored in the in-house database. Five different datasets were considered in our study: i) whole proteomes using GBDP for calculating intergenomic distances (GBDP), ii) peptides with a length > 28 amino acids obtained from cytoplasmic proteins (Cyto28-more), iii) peptides with a length comprised between 51 and 60 amino acids obtained from cytoplasmic proteins included in the pI range 4.5–5.5 (Cyto_PI_51–60), iv) peptides with a length higher than 60 amino acids obtained from cytoplasmic proteins included in the pI range 4.5–5.5 (Cyto_PI_60-more), and v) peptides obtained from extracellular proteins (Extracellular). For the four last subsets, three different methodologies were used to infer phylogenies, Bayesian (MB), Maximum Likelihood (ML) and Maximum Parsimony (MP).

Consensus peak set

The consensus peak set among all the strains was obtained in two steps. First, the list of the total peptides for each strain was subdivided based on peptide length for indexing purposes. Then, the molecular weight and isoelectric point of the selected peptides were calculated using an in-house customised tool adapted from the SIB Bioinformatics Resource Portal (http://web.expasy.org/compute_pi/). In the case of peptides obtained from extracellular proteomes, all peptides were kept for analysis.

SPECLUST, a public web-based tool, was used to identify representative and reproducible peak masses that are present in a collection of spectral profiles [18]. This tool calculates the mass difference between two peaks taken from different peak lists and determines whether or not the two peaks are identical, taking into account some measurement uncertainty (σ). In the present study, the measurement uncertainty was set empirically to 3.0 Da. In addition, the pairwise cut-off was set to 0.6, i.e., a peak was considered shared between two spectra if it was matched in the alignment of the spectra with a peak match score greater than 0.6 (corresponding to a 0.5 Da mass difference). The consensus spectra matrix was translated to a binary matrix (0s and 1s, representing absence or presence of a given peptide mass respectively) in NEXUS file format [25].

Tree reconstruction based on consensus peak set data

MrBayes, the model-based phylogenetic inference tool using Bayesian statistics, was utilised to generate a consensus tree [20]. The consensus binary file obtained from the previously generated SPECLUST consensus file was used as input. The phylogeny was inferred through the restriction data type implemented in MrBayes (with state 0 or 1 representing the absence or presence of a consensus peptide throughout the strain peptidomes). For the purpose of our study, we assumed that the frequencies of these two possible states had a Dirichlet (1.00, 1.00) prior parameter. Bayesian analysis was performed in two independent runs using four Markov chains and 1,000,000 generations. When necessary, the number of generations was incremented for chain convergence diagnosis. The potential scale-reduction factor, printed at the end of the analysis, was used as convergence diagnosis. A majority-rule consensus tree (50%) was obtained after discarding the initial 25% of the trees (burnin = 250), where the log-likelihood values of the analysis (log probability of the data given the parameter values) are frequently not yet stabilized. Using this command, MrBayes plots the number of generations (each corresponding to a phylogenetic tree) versus its log probability. Usually, the first sampled trees show trends towards increasing or decreasing log-likelihood values, which results in inadequate sampling from the posterior probability distribution. Maximum likelihood (ML) and maximum parsimony (MP) phylogenies were inferred using the DSMZ phylogenomics pipeline [11]. A multiple sequence alignment was created
with MUSCLE [26], and ML and MP trees were inferred from it with RAxML [27] and TNT [28], respectively. For ML, rapid bootstrapping in conjunction with the autoMRE bootstop-ping criterion [29] and subsequent search for the best tree was used; for MP, 1000 bootstrapping replicates were used in conjunction with tree-bisection-and-reconnection branch swapping and ten random sequence addition replicates.

GBDP-based phylogenomic analysis including (sub-)species clustering

A whole-genome phylogeny (based on the proteome data) was inferred using the latest version of the Genome-BLAST Distance Phylogeny (GBDP) method [11,30]. Here, pairwise proteome comparisons (including pseudo-bootstrap replicates) were done under the greedy-with-trimming algorithm and further recommended settings [13]. The tree was inferred using FastME v2.07 with TBR post-processing [31]. The species and subspecies clustering was conducted on the nucleotide data (i) with the help of the Genome-to-Genome Distance Calculator (GGDC), (ii) established (sub-)species distance cut-offs [11,12], and (iii) the OPTSIL clustering tool [32], in analogy to a recent study [12].

Tree visualization and annotation

The Interactive Tree Of Life (iTOL) web-based tool was utilised to visualize the phylogenetic trees [33]. Using the tree files generated previously, the annotation was performed, highlighting the BCG (Bacillus Cereus Group) notation as reported before by Li et al. [12]. Posterior probabilities or branch support values were included when equal or above 60%.

Inferred trees comparison

The inferred trees were compared amongst themselves and with the pseudo-bootstrapped whole-proteome GBDP phylogeny [13]. The topological comparison was based on pairwise weighted Robinson-Foulds distances, which were calculated using the RaxML tool [27,34]. Visualisation was supported by the packages ggplot [35] and ggdendro [36] for the statistical language R [37].

Results and Discussion

The results obtained with our in silico peptidome-based strain comparison pipeline are presented in Fig 2. These results reflect the analysis of the complete genomes of B. anthracis, B. cereus and B. thuringiensis, using B. subtilis subsp. natto BEST195 as outgroup (Table 1). We adopted the nomenclature proposed by Liu et al. regarding the taxonomy of Bacillus cereus [12]. Briefly, these authors used a GBDP analysis to separate 224 Bacillus cereus strains into 30 clusters. Eleven of these clusters represented already described species, whereas 19 clusters supported the proposal of putative novel species. These clusters were annotated as Bacillus cereus groups (BCG), and we have used such annotation in the present discussion.

Our analysis of the results is focused on intra-cluster strain homogeneity and the unexpected or incongruent grouping of certain strains. The rationale behind inter-cluster strain allocation agreement between the different approaches is that strain specialisation or evolution is likely to affect the peptidome composition of the subcellular locations differently.

GBDP phylogenomic analysis

As illustrated in Fig 3, the GBDP proteome tree recovered all species with high support and showed insignificant subspecies conflicts. Most notably, this tree has an average branch support of 84.7% (Table 2) and confirms previous results of a nucleotide-based GBDP analysis [12].
Moreover, the OPTSIL clustering method [32] yielded eight species clusters as well as ten subspecies clusters (excluding the outgroup of *B. subtilis*). For instance, the cluster BCG01 contained some "*B. cereus*" and "*B. thuringiensis*" strains, which in fact belong to *B. anthracis* based on the dDDH estimates (see Supplementary S3 File). In turn, cluster BCG03 (*B. cereus*) included two "*B. thuringiensis*" strains: "*B. thuringiensis* BMB171" and "*B. thuringiensis* serovar kurstaki HD73". This is in accordance with a recent study on the taxonomic situation of the *B. cereus* group [12].

In summary, three major groups were identified: (i) BCG01 containing traditional and anomalously assigned strains of *B. anthracis*, (ii) a group encompassing the three related BCG03 (*B. cereus*), BCG04 (*B. thuringiensis*) and BCG17 and, (iii) a group formed by BCG10, BGC12 and BCG20 comprising three potential novel species [12]. Finally, "*B. thuringiensis* MC28" was classified into BCG09, which has been proposed as a novel species [12].

**Assessment of peptidome datasets**

The phylogenies of the peptidome datasets resulting from all possible combinations of the three human proteases were evaluated based on MB, ML and MP criteria (see Supplementary S1 File). We also investigated proteins with different subcellular location as a possible way of reducing the amount of proteomic data input. In the case of extracellular proteins, all the
resulting peptides were used in the analysis, but in the case of cytoplasmic peptidomes, the high number of peptides was further reduced by means of amino acid length and pI value filtering. Specifically, we considered three length bins, i.e. 28-more, 51–60 and 60-more amino acids, and those proteins with a pI between 4.5 and 5.5, which corresponds to the pI exhibited by most of the housekeeping and metabolic enzymes, as deduced from 2 dimensional electrophoresis experiments [38]. In addition, genes coding for many of these proteins, such the β-subunit of RNA polymerase (rpoB), the β-subunit of ATP synthase F₀F₁ (atpD), or the chaperonin GroEL (groEL) are frequently used in multilocus sequence typing approaches [39]. Interestingly, this pI range do not correspond with the normal cytoplasmatic pH in mesophilic organisms such as Escherichia coli or Bacillus subtilis, which is slightly alkaline (7.0–7.8) over an external pH ranges of 5.0–9.0 [40–44] was determined by means of a flow cytometry with the fluorescent probe 5(and 6-)carboxyfluoresce in ester. As an example, we can say that the dataset including peptides with more than 60 amino acids comprised approximately 1,000 peptides per strain (Suppl. S2 File), which contrasts with the 320,000–411,000 peptides obtained after proteome digestion for the different strains concerned in this study, and results in an obvious reduction of data input.

Fig 3. Whole proteome-based phylogenomic tree, including species and subspecies cluster information. This analysis was based on the GBDP algorithm and rooted with Bacillus subtilis. Numbers above branches are greedy-with-trimming pseudo-bootstrap support values from 100 replicates [13] and only support above 60% is shown. The leaf labels refer to the current NCBI nomenclature, whereas the BCG groups represent the recently revised names [12].

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Table 2. Summary data on the different phylogenetic trees. Specifically, the average support obtained by the different methods and the size of the character matrices used.

| Dataset       | MrBayes | RaxML | TNT  | GBDP | # characters |
|---------------|---------|-------|------|------|--------------|
| Cyto.PI.51–60 | 67.03   | 50.03 | 61.59| ~    | 229          |
| Extracellular | 80.03   | 65.93 | 72.93| ~    | 530          |
| Cyto.PI.60-more| 84.13   | 76.07 | 79.48| ~    | 1166         |
| Cyto28-more   | 93.33   | 83.79 | 84.52| ~    | 1696         |
| GBDP          | ~       | ~     | ~    | ~    | 84.7         |

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So, the hereafter presented results relate to the extracellular peptide dataset (Fig 4), the cytoplasmic dataset containing peptides with 28 or more amino acids (Fig 5), and the cytoplasmic datasets containing peptides with 51–60 amino acids or more than 60 amino acids and pI values within the range 4.5–5.5 (Figs 6 and 7, respectively). Interestingly, other filtering criteria, doi:10.1371/journal.pcbi.1005271.g004

Fig 4. Bayesian tree based on the [Extracellular] dataset. Peptides were obtained from extracellular proteins. Bayesian analysis was performed in two independent runs using four Markov chains and 1,000,000 generations [58]. A majority-rule consensus tree (50%) was obtained after discarding the initial 25% of the trees and only support above 60% is shown. The leaf labels refer to the current NCBI nomenclature, whereas the BCG groups represent the recently revised names [12].

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Fig 5. Bayesian tree based on [Cyto28-more] dataset. It contains peptides with a length higher or equal than 28 amino acids obtained from cytoplasmic. Bayesian analysis was performed in two independent runs using four Markov chains and 1,000,000 generations [58]. A majority-rule consensus tree (50%) was obtained after discarding the initial 25% of the trees and only support above 60% is shown. The leaf labels refer to the current NCBI nomenclature, whereas the BCG groups represent the recently revised names [12].

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such as charge to mass amino acid ratio, may be implemented as mean as reducing the proteomic input.

Phylogenomic inference based on peptidome datasets

The four peptide subsets were loaded in MrBayes and used to infer phylogenies. At the end of the Bayesian analysis, the average standard deviation of split frequencies after 1e06 generations suggested a good convergence of the analyses, as in all cases it was lower than 0.01 (Cyto28-more: 0.004; Cyto_PI_51–60: 0.007; Cyto_PI_60-more: 0.004; Extracellular: 0.006). Convergence of the analyses was confirmed by calculating the potential scale reduction factor (PSRF) of the total tree length (TL) and the stationary phase frequencies (pi) of the two possible states of our binary model (0 or 1). In all cases the PSRF values converged to 1.000–1.001 at the end of the analysis, indicating a good phylogenetic tree sampling from the posterior distribution.

A summary of the results of the phylogenetic inference is found in Table 2. The ML analyses yielded and subsequently used “Uncorrected+GAMMA” as best model during the inference. Since the ML, MP and MB trees were very similar within each peptidome dataset in terms of weighted topological distance (see below), only the MB-based trees are shown while discussing the different datasets. The remaining ML and MP trees are shown in Suppl. S1 File.

Assessing conflicts among the inferred phylogenies

Pairwise weighted Robinson-Foulds distances supported the assessment of topological differences among the five trees at the light of the four methods of analysis (Fig 2). More specifically, the differences observed between the trees inferred from whole proteomes (GBDP analysis) and Cyto28-more, cyto_PI_60-more cyto_PI_51–60 and Extracellular subsets (applying the
MB, ML and MP criteria). Significance of conflict between two trees was assumed when a bipartition implied by one tree was found incompatible with a bipartition implied by the other tree, with both receiving \( \geq 95\% \) support. Similarly, disagreement with the monophyly of a species or subspecies was only considered if the conflicting branches had \( \geq 95\% \) support.

As an initial observation we can say that the MB [Cyto\_PI\_60-more] and [Cyto28-more] trees showed no significant conflict with the GBDP tree. However, there are some interesting discrepancies between several trees. For example, in contrast to ML and MP trees, the MB [Cyto28-more] tree (Fig 5) showed significant conflict in terms of subspecies assignments within BCG01 (\emph{B. anthracis}) cluster. Another example is the conflict between the MB [Extracellular] tree and some of the MB cytoplasmic trees regarding the placement of "\emph{B. thuringiensis} serovar *kurstaki* HD73". Specifically, in the [Extracellular] tree (Fig 4) the "\emph{B. thuringiensis} serovar *kurstaki* HD73" is placed next to the BCG04 (\emph{B. thuringiensis}) cluster with high support while in the MB [Cyto\_PI\_60-more] (Fig 6) and [Cyto28-more] (Fig 5) trees it is part of the BCG03 (\emph{B. cereus}) group. Likewise, the MB [Cyto\_PI\_51–60] tree (Fig 6) significantly deviated from the GBDP proteome tree by placing \emph{B. anthracis} H9401 as sister group of all other highly virulent \emph{B. anthracis} strains instead of as sister group of \emph{B. anthracis} CDC 684; and, by
forming a well-supported group (96%) comprising “B. thuringiensis MC28”, the cluster BCG04 (B. thuringiensis) and the cluster BCG17 (B. thuringiensis). Noteworthy, these arrangements received no support in the ML and MP analyses of the [Cyto_PI_51–60] dataset. See Supplementary S1 File for details.

Possible biological reasons behind conflicting phylogenetic grouping

The comparison of the peptidome-based phylogenetic trees allowed us to gain a better understanding about the information provided by the different sets of peptides. The four peptide subsets produced similar results regarding the identification of quite unrelated strains (e.g., B. subtilis subsp. natto BEST195), and established a species grouping as close as the one suggested by Liu et al. using 224 genomes of strains belonging to the B. cereus group [12]. Classically, B. thuringiensis strains have been considered an insect pathogen, affecting mainly members of the orders Lepidoptera, Diptera and Coleoptera [23]. Spores from these strains include large crystal protein inclusions, which are cleaved by the insect mid-gut proteases producing the active toxin forms. The action of this toxin leads to the complete destruction of the intestinal epithelium.

In turn, the BCG03 cluster corresponds to B. cereus, which is an opportunistic human pathogen and food-borne bacterium that causes two forms of poisoning, one characterised by diarrhea and abdominal pain, and the other involving nausea and vomiting [45,46]. Some “B. thuringiensis” strains also clustered in BCG03, because they share certain genetic similarity with B. cereus ATCC 14579T, namely genetic regions such as a putative polysaccharide capsule cluster [47].

B. anthracis (BCG01 cluster) is the etiological agent of anthrax, a fatal disease for herbivores and mammals that is best known for its use as biological weapon [48]. Strains from this species can be classified according to different phenotypical tests. For instance, these strains are non-motile, penicillin-sensitive, and produce an extracellular capsule of poly-γ-D-glutamic acid [49]. Toxins responsible for anthrax symptoms and other virulence factors necessary for complete virulence are codified into two large plasmids, denominated pXO1 and pXO2 [50]. Two strains of “B. thuringiensis” also clustered within the BCG01: “B. thuringiensis Al Hakam”, and “B. thuringiensis serovar konkurian”. Indeed both strains have been shown to be more related to the B. anthracis cluster. The genome of these strains contain no homologues of the known B. thuringiensis insecticidal genes cry, cyt, or vip and, even if these ever existed, the plasmid(s) encoding for these genes may have been lost during in vitro culture [50,51]. Therefore, classification of these two strains as B. thuringiensis strains may not be correct, as previously reported in [12].

Other cluster identified in our analysis was BCG17, a putative novel species. This contained “B. cereus G9842” together with other two “B. thuringiensis” strains. The G9842 strain was isolated from stool samples of an emetic outbreak that involved three individuals in Nebraska (1996) and the genome was sequenced by the J. Craig Venter Institute (http://www.ncbi.nlm.nih.gov/bioproject/17733). The isolate was characterised by MLST typing using the MLSTDB scheme as sequence type 56 (http://pubmlst.org/bcereus/). Interestingly, the sequence type 56 was quite unrelated to the major clade of pathogenic B. cereus isolates and was suggested as representative for a novel pathogenicity group within the B. cereus group [52]. Peptidome fingerprinting confirms the new affiliation to B. thuringiensis. The peptidome of strain G9842, shared a high homology with the other “B. thuringiensis strains”, so it is plausible that these two isolates lost the plasmids containing the insecticide genes and acquired certain virulence factors, which allow them to act as pathogens in the human host.

Finally, phylogenetic techniques consistently grouped “B. thuringiensis serovar finitimus” individually, and it has been proposed as representative for the novel species BCG20 [12]. This strain contains several cry genes encoding for crystal proteins and located in two plasmids [53]. The chromosome of this strain has been shown to be closer phylogenetically to B.
anthracis Ames than to B. cereus ATCC 14579T [12,54]. Given the close distance of “B. thurini-
giensis serovar finitus” to the other BCG groups containing “B. cereus” strains, such as
BCG10 and BCG12, we speculate that this strain may be a B. cereus strain that acquired the
plasmids from a B. thuringiensis donor.

Peptidome analysis benchmarking

Another important aspect of the evaluation of our peptidome similarity method is the com-
putational complexity induced by each processing step and the resulting processing time eventu-
ally, although available computational power will be of course decisive. The running time of
future in silico experiments can thus be extrapolated, especially that of significantly larger
datasets. We computed the processing time of each of the main steps for the whole Bacillus
dataset (i.e. 32 genomes) and for four subsets, representing a large dataset (i.e. 24 genomes), a
medium-large dataset (i.e. 16 genomes), a medium-small dataset (i.e. 8 genomes) and a small
dataset (i.e. 4 genomes, which is the smallest possible dataset that one can use for phylogenetic
inference). In particular, we randomly sampled without replacement four sets of 24, 16, 8 and
4 genomes, and calculated the average running time. Here, we present the average times, but
details on the different runs can be found in S4 File.

Table 3 summarises the running times taken by the steps of protein localization, which is
performed by PsortB, and protein digestion, which is performed by ExPASy MzJava. Protein
localization is the most time consuming task and, in particular, the processing of larger data-
sets may take several days. Although this may be considered somewhat time consuming, this
step enables further filtering of the peptide dataset that, in turn, may reduce considerably the
data matrices to be computed and speed up the subsequent steps of analysis.

The running times of steps leading to the generation of the NEXUS files are negligible com-
pared to those of previous steps (Table 4). For most of the sample sets both steps took less than
15 minutes to execute. A large running time (> 2 hours) was observed for the SPECLUST run
over the whole dataset of cytoplasmic peptides with 28 or more amino acids, which comprises
a total of 121,632 peptides.

Concluding remarks

One of the potential applications of our pipeline is to accept, as input, experimental peptide
mass profiles. If traced back, our application allows detection of differential peptide profiles,
providing a robust tool to discriminate not only strain-specific peptides, but true intraspecies
differences among a set of biological replicates or even microorganism-phenotype variations
such as those occurring between biofilm and planktonic populations. In this regard, the nega-
tive effect of certain peptide families on bacteria through different mechanisms is well known
[55,56]. In this regard, our pipeline will just provide a candidate peptide list, but experimental

| Dataset  | PSortB average running time | MZJava average running time | TOTAL average running time |
|----------|-----------------------------|----------------------------|----------------------------|
| All genomes* | 56:42:07                  | 0:49:45                    | 57:31:52                   |
| 24 genomes  | 42:37:48 +/- 0:35:24      | 0:37:26 +/- 0:00:49        | 43:15:14 +/- 0:35:19      |
| 16 genomes  | 28:19:39 +/- 0:15:17      | 0:24:37 +/- 0:00:57        | 28:44:16 +/- 0:15:57      |
| 8 genomes   | 13:51:25 +/- 0:24:28      | 0:12:42 +/- 0:01:10        | 14:04:07 +/- 0:25:12      |
| 4 genomes   | 6:53:02 +/- 0:09:10       | 0:06:16 +/- 0:00:35        | 7:03:08 +/- 0:13:43       |

*No replicates were performed.

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approaches such as MS/MS experiments will never detect peptides that are inhibiting own bacterial growth. Rather, such experimental approaches will validate the presence of those certain strain-specific peptides, either free or most probably encoded in a “carrier protein”.

Generation of a potential strain-specific peptide list together with its experimental identification, may facilitate development of different approaches focused on the identification of given strain, such as a dairy starter or a probiotic that has to be traced through the human gut during clinical intervention studies. This can be accomplished, for instance, with the use of high-resolution mass spectrometers or antibody-based protocols targeting these specific peptides.

Table 4. Summary data on the NEXUS generation process for Bacillus cereus benchmarking. Running time is displayed using hours:minutes:seconds format.

| Dataset          | Subcellular location | Isoelectric Point | Peptide Size | Specclus average running time | MrBayes average running time | TOTAL average running time |
|------------------|----------------------|-------------------|--------------|-------------------------------|----------------------------|--------------------------|
| All genomes      | Extracellular        | All               | All          | 0:22:11                       | 0:13:51                    | 0:36:02                  |
|                  | Cytoplasmatic        | 4.5–5.5           | 50–60        | 0:05:47                       | 0:05:42                    | 0:11:29                  |
|                  |                      | 50–60             |              | 0:18:24                       | 0:04:27                    | 0:22:51                  |
|                  |                      | 4.5–5.5           | 60-more      | 0:05:13                       | 0:17:41                    | 0:22:54                  |
|                  |                      | 60-more           |              | 0:16:38                       | 0:23:33                    | 0:40:11                  |
|                  |                      | 4.5–5.5           | 28-more      | 0:20:41                       | 0:16:33                    | 0:37:14                  |
|                  |                      |                   |              | 2:08:16                       | 0:22:08                    | 2:30:24                  |
| 24 genomes       | Extracellular        | All               | All          | 0:10:01                       | 0:09:50                    | 0:19:51                  |
|                  | Cytoplasmatic        | 4.5–5.5           | 50–60        | 0:03:10                       | 0:04:29                    | 0:07:40                  |
|                  |                      | 50–60             |              | 0:11:13                       | 0:03:36                    | 0:14:49                  |
|                  |                      | 4.5–5.5           | 60-more      | 0:04:07                       | 0:13:14                    | 0:17:21                  |
|                  |                      | 60-more           |              | 0:13:20                       | 0:17:32                    | 0:30:52                  |
|                  |                      | 4.5–5.5           | 28-more      | 0:11:13                       | 0:12:45                    | 0:23:58                  |
|                  |                      |                   |              | 1:13:45                       | 0:16:32                    | 1:30:17                  |
| 16 genomes       | Extracellular        | All               | All          | 0:05:05                       | 0:06:23                    | 0:11:28                  |
|                  | Cytoplasmatic        | 4.5–5.5           | 50–60        | 0:02:23                       | 0:03:48                    | 0:06:11                  |
|                  |                      | 50–60             |              | 0:07:55                       | 0:02:02                    | 0:09:57                  |
|                  |                      | 4.5–5.5           | 60-more      | 0:02:09                       | 0:08:29                    | 0:10:38                  |
|                  |                      | 60-more           |              | 0:05:13                       | 0:10:56                    | 0:16:09                  |
|                  |                      | 4.5–5.5           | 28-more      | 0:04:38                       | 0:07:57                    | 0:12:35                  |
|                  |                      | 28-more           |              | 0:31:54                       | 0:11:13                    | 0:43:07                  |
| 8 genomes        | Extracellular        | All               | All          | 0:01:22                       | 0:01:57                    | 0:03:19                  |
|                  | Cytoplasmatic        | 4.5–5.5           | 50–60        | 0:00:54                       | 0:01:35                    | 0:02:29                  |
|                  |                      | 50–60             |              | 0:03:17                       | 0:01:21                    | 0:04:38                  |
|                  |                      | 4.5–5.5           | 60-more      | 0:01:15                       | 0:02:17                    | 0:03:32                  |
|                  |                      | 60-more           |              | 0:04:04                       | 0:02:26                    | 0:06:31                  |
|                  |                      | 4.5–5.5           | 28-more      | 0:01:47                       | 0:01:52                    | 0:03:39                  |
|                  |                      | 28-more           |              | 0:12:05                       | 0:02:04                    | 0:14:09                  |
| 4 genomes        | Extracellular        | All               | All          | 0:00:39                       | 0:00:47                    | 0:01:25                  |
|                  | Cytoplasmatic        | 4.5–5.5           | 50–60        | 0:00:59                       | 0:00:37                    | 0:01:36                  |
|                  |                      | 50–60             |              | 0:01:33                       | 0:00:37                    | 0:02:10                  |
|                  |                      | 4.5–5.5           | 60-more      | 0:00:27                       | 0:00:35                    | 0:01:01                  |
|                  |                      | 60-more           |              | 0:01:26                       | 0:00:42                    | 0:02:08                  |
|                  |                      | 4.5–5.5           | 28-more      | 0:00:35                       | 0:00:49                    | 0:01:24                  |
|                  |                      | 28-more           |              | 0:02:46                       | 0:01:01                    | 0:03:47                  |

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Whereas our bioinformatic approach will reliably produce the same results, conventional methods might yield different results even if applied on the same organisms, due for instance to phenotype-variations or the use of transient input data. In addition, the big advantage of the in silico method is accuracy, reproducibility and speed, whereas the disadvantage is that it might not get the experimental peptidome as we simply consider all proteins encoded in a genome and not only those that are actively produced by the organism while being measured.

Overall, results show that our phylogenetic method based on peptidome similarity, as opposed to genome-sequence homology, is complementary to the proteome-based GBDP analysis. Most notably, our peptidome-based phylogeny analysis supported already reported taxonomic discrepancies within the \textit{B. cereus} group. Our peptidome-based method has the advantage of reducing larger amounts of proteomic data to small matrices (by a factor of 320) without losing too much phylogenetic signal. Our pipeline can be also applied to other peptide datasets originated from viruses, eukaryotic species or even metaproteomes with the inclusion of few modifications regarding the prediction of the protein subcellular location. This could be of interest for developing more efficient applications aimed at managing very large bacterial datasets, such as those generated in epidemiologic studies.

**Supporting Information**

**S1 File.** Set of additional phylogenetic trees (ML and MP) based on the digestion of one or two of the endoproteases considered in this study.
(PDF)

**S2 File.** Peptide distribution by subcellular localization and length. Ranges with few instances (e.g., loose ranges of extracellular peptides) and with high abundance (e.g., cytoplasmic proteins consisting of 11 to 20 amino acids) were not helpful for comparison. The analysis focused on extracellular peptides and cytoplasmic peptides consisting of 51 to 60 amino acids and above.
(PDF)

**S3 File.** All pairwise digital DDH values regarding the 32 genomes dataset as calculated under the recommended settings of the GGDC 2.1.
(ODS)

**S4 File.** Peptidome analysis benchmarking.
(XLSX)

**Author Contributions**

**Conceived and designed the experiments:** BS AL FFR.

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**Analyzed the data:** BS JPMK AL MG.

**Contributed reagents/materials/analysis tools:** FFR.

**Wrote the paper:** ABM BS AL JPMK.

**References**

1. Priest F. DNA homology in the genus Bacillus In: The Aerobic Endospore-forming Bacteria (Berkeley R. C.W. and Goodfellow M., Eds.). Acad Press London. 1981; 35–57.
2. Bavykin SG, Lysov YP, Zakhariev V, Kelly JJ, Jackman M, Stahl DA, et al. Use of 16S rRNA, 23S rRNA, and gyrB gene sequence analysis to determine phylogenetic relationships of Bacillus cereus group microorganisms. J Clin Microbiol. 2004; 42: 3711–3730. doi: 10.1128/JCM.42.8.3711-3730.2004 PMID: 15297521

3. Helgason E, Bourasse NJ, Meisal R, Cauwants DA, Kolste AB. Multilocus Sequence Typing Scheme for Bacteria of the Bacillus cereus Group. Appl Environ Microbiol. 2004; 70: 191–201. doi: 10.1128/AEM.70.1.191-201.2004 PMID: 14711642

4. Cherif A, Brusetti L, Borin S, Rizzi A, Boudabous A, Khayami-Horani H, et al. Genetic relationship in the “Bacillus cereus group” by rep-PCR fingerprinting and sequencing of a Bacillus anthracis-specific rep-PCR fragment. J Appl Microbiol. 2003; 94: 1108–1119. PMID: 12752821

5. Vandamme P, Pot B, Gillis M, de Vos P, Kersters K, Swings J. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev. 1996; 60: 407–38. PMID: 8801440

6. Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, et al. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. Int J Syst Bacteriol. 1987; 37: 463–464.

7. Meier-Kolthoff JP, Göker M, Spröer C, Klenk H-P. When should a DDH experiment be mandatory in microbial taxonomy? Arch Microbiol. 2013; 195: 413–418. doi: 10.1007/s00203-013-0888-4 PMID: 23591456

8. Auch AF, von Jan M, Klenk H-P, Göker M. Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. Stand Genomic Sci. 2010; 2: 117–134. doi: 10.4056/sigs.531120 PMID: 21304684

9. Stackebrandt E, Frederiksen W, Garrity GM, Grimont PAD, Kämpfer P, Maiden MCJ, et al. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int J Syst Evol Microbiol. 2002; 52: 1043–7. doi: 10.1099/00207713-52-3-1043 PMID: 12054223

10. Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics. 2013; 14: 60. doi: 10.1186/1471-2105-14-60 PMID: 23432962

11. Meier-Kolthoff JP, Hahnke RL, Petersen J, Scheuner C, Michael V, Fiebig A, et al. Complete genome sequence of DSM 30083T, the type strain (U5/41T) of Escherichia coli, and a proposal for delineating subspecies in microbial taxonomy. Stand Genomic Sci. 2014; 9: 2. doi: 10.1186/1944-3277-9-2 PMID: 25780495

12. Liu Y, Lai Q, Göker M, Meier-Kolthoff JP, Wang M, Sun Y, et al. Genomic insights into the taxonomic status of the Bacillus cereus group. Sci Rep. Nature Publishing Group; 2015; 5: 14082. doi: 10.1038/srep14082 PMID: 26373441

13. Meier-Kolthoff JP, Auch AF, Klenk HP, Göker M. Highly parallelized inference of large genome-based phylogenies. Concurrency Computation Practice and Experience. 2014. pp. 1715–1729.

14. Alves G, Wang G, Ogurtsov AY, Drake SK, Gucek M, Suffredini AF, et al. Identification of Microorganisms by High Resolution Tandem Mass Spectrometry with Accurate Statistical Significance. J Am Soc Mass Spectrom. 2016; 27: 194–210. doi: 10.1007/s13361-015-1271-2 PMID: 26510657

15. Singhal N, Kumar M, Kanaujia PK, Virdi JS. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front Microbiol. 2015; 6: 791. doi: 10.3389/fmicb.2015.00791 PMID: 26300860

16. Zhang L, Smart S, Sandrin TR. Biomarker- and similarity coefficient-based approaches to bacterial mixture characterization using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Sci Rep. 2015; 5: 15834. doi: 10.1038/srep15834 PMID: 26537565

17. Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R, et al. PSORTb 3.0: Improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics. 2010; 26: 1608–1615. doi: 10.1093/bioinformatics/btp249 PMID: 20472543

18. Horlacher O, Nikitin F, Allocco D, Mariethoz J, Mülter M, Lisacek F. MzJava: An open source library for mass spectrometry data processing. J Proteomics. 2015;

19. Johansson P, Alm R, Emanuelsesson C. SPECLUST: a web tool for clustering of mass spectra. J Proteome Res. 2006; 785–792. Available: http://home.theplu.se/~markus/publications/papers/lu_tps_07_14.pdf

20. Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics. 2001; 17: 754–755. PMID: 11524383

21. Soufiane B, Côtre J-C. Bacillus weihenstephanensis characteristics are present in Bacillus cereus and Bacillus mycoides strains. FEMS Microbiol Lett. 2013; 341: 127–137. doi: 10.1111/1574-6968.12106 PMID: 23413955
22. Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, et al. Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis—one species on the basis of genetic evidence. Appl Environ Microbiol. 2000; 66: 2627–2630. PMID: 10831447

23. Schnepf E, Crickmore N, Van Rie J, Lereclus D, Baum J, Feitelson J, et al. Bacillus thuringiensis and its pesticidal crystal proteins. Microbiol Mol Biol Rev. 1998; 62: 775–806. PMID: 9729609

24. Wheeler DL, Chappey C, Lash AE, Leipe DD, Madden TL, Schuler GD, et al. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 2004; 32: 1792–1797. doi: 10.1093/nar/gkh340 PMID: 15034147

25. Maddison DR, Swofford DL, Maddison WP. NEXUS: an extensible file format for systematic information. Syst Biol. 1997; 46: 590–621. PMID: 11975335

26. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32: 1792–1797. doi: 10.1093/nar/gkh340 PMID: 15034147

27. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014; 30: 1312–1313. doi: 10.1093/bioinformatics/btu033 PMID: 2451623

28. Goloboff PA, Farris JS, Nixon KC. TNT, a free program for phylogenetic analysis. Cladistics. 2008; 24: 774–786.

29. Patterson NL, Alipour M, Bininda-Emonds ORP, Moret BME, Stamatakis A. How Many Bootstrap Replicates Are Necessary? J Comput Biol. 2010; 17: 337–354. doi: 10.1089/cmb.2009.0179 PMID: 20377449

30. Henz SR, Huson DH, Auch AF, Nieselt-Struve K, Schuster SC. Whole-genome prokaryotic phylogeny. Bioinformatics. 2005; 21: 2329–35. doi: 10.1093/bioinformatics/bti324 PMID: 15166018

31. Desper R, Gascuel O. Fast and accurate phylogeny reconstruction algorithms based on the minimum-evolution principle. J Comput Biol. 2002; 106: 687–705.

32. Göker M, García-Blázquez G, Voglmayr H, Tellería MT, Martín MP. Molecular taxonomy of phytopathogenic fungi: A case study in Peronospora. PLoS One. 2009; 4.

33. Letunic I, Bork P. Interactive Tree Of Life (iTOl): an online tool for phylogenetic tree display and annotation. Bioinformatics. 2007; 23: 127–128. doi: 10.1093/bioinformatics/btl529 PMID: 17050570

34. Salichos L, Stamatakis A, Rokas A. Novel information theory-based measures for quantifying incongruence among phylogenetic trees. Mol Biol Evol. 2014; 31: 1261–71. doi: 10.1093/molbev/msu061 PMID: 24509691

35. Wickham H. Ggplot2: Elegant Graphics for Data Analysis. 2nd edition. New York: Springer; 2009.

36. de Vries A, Ripley B. RDevelopment Core Team: R: a language and environment for statistical computing. 2015.

37. The R Core Team. R: a language and environment for statistical computing. 2015.

38. Sánchez B, Champomier-Vergès M-C, Collado M del C, Anglade P, Baraige F, Sanz Y, et al. Low-pH adaptation and the acid tolerance response of Bifidobacterium longum biotype longum. Appl Environ Microbiol. 2007; 73: 6450–9. doi: 10.1128/AEM.00886-07 PMID: 17720838

39. Glaeser SP, Kempfer P. Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. Systematic and Applied Microbiology. 2015. pp. 237–245.

40. Slonczewski JL, Rosen BP, Alger JR, Macnab RM. pH homeostasis in Escherichia coli: measurement by 31P nuclear magnetic resonance of methylphosphonate and phosphate. Proc Natl Acad Sci. 1981; 78: 6271–6275. PMID: 7031646

41. Zilberstein D, Agnon V, Schuldiner S, Padan E. Escherichia coli intracellular pH, membrane potential, and cell growth. J Bacteriol. 1984; 158: 246–252. PMID: 632589

42. Wilks JC, Slonczewski JL. pH of the cytoplasm and periplasm of Escherichia coli: Rapid measurement by green fluorescent protein fluorimetry. J Bacteriol. 2007; 189: 5601–5607. doi: 10.1128/JB.00615-07 PMID: 17720838

43. Shioi JI, Matsura S, Imae Y. Quantitative measurements of proton motive force and motility in Bacillus subtilis. J Bacteriol. 1980; 144: 891–897. PMID: 6254950

44. Spilimbergo S, Bertucco A, Basso G, Bertolini G. Determination of extracellular and intracellular pH of Bacillus subtilis suspension under CO2 treatment. Biotechnol Bioeng. 2005; 92: 447–451. doi: 10.1002/bit.20606 PMID: 16025536

45. Drobniowski FA. Bacillus cereus and related species. Clinical Microbiology Reviews. 1993. pp. 324–338. PMID: 8269390

46. Kolste AB, Lereclus D, Mock M. Genome structure and evolution of the Bacillus cereus group. Curr Top Microbiol Immunol. 2002; 264: 95–108. PMID: 12012872

47. Rasko DA, Altherr MR, Han CS, Ravel J. Genomics of the Bacillus cereus group of organisms. FEMS Microbiol Rev. 2005; 29: 303–29. doi: 10.1016/j.femsre.2004.12.005 PMID: 15808746
48. Jernigan DB, Raghunathan PL, Bell BP, Brechner R, Bresnitz EA, Butler JC, et al. Investigation of bioterrorism-related anthrax, United States, 2001: Epidemiologic findings. Emerg Infect Dis. 2002; 8: 1019–1028. doi: 10.3201/eid0810.020353 PMID: 12396809

49. Hoffmaster AR, Ravel J, Rasko DA, Chapman GD, Chute MD, Marston CK, et al. Identification of anthrax toxin genes in a Bacillus cereus associated with an illness resembling inhalation anthrax. Proc Natl Acad Sci U S A. 2004; 101: 8449–8454. doi: 10.1073/pnas.0402414101 PMID: 15155910

50. Okinaka R, Cloud K, Hampton O, Hoffmaster A, Hill K, Keim P, et al. Sequence, assembly and analysis of pX01 and pX02. J Appl Microbiol. 1999; 87: 261–262. PMID: 10475962

51. Klee SR, Brzuskiewicz EB, Nattermann H, Brüggemann H, Dupke S, Wollherr A, et al. The genome of a Bacillus isolate causing anthrax in chimpanzees combines chromosomal properties of B. cereus with B. anthracis virulence plasmids. PLoS One. 2010; 5: e10986. doi: 10.1371/journal.pone.0010986 PMID: 20634886

52. Jolley KA, Maiden MCJ. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics. 2010; 11: 595. doi: 10.1186/1471-2105-11-595 PMID: 21143983

53. Zhu Y, Shang H, Zhu Q, Ji F, Wang P, Fu J, et al. Complete genome sequence of Bacillus thuringiensis serovar finitimus strain YBT-020. Journal of Bacteriology. 2011. pp. 2379–2380. doi: 10.1128/JB.00267-11 PMID: 21398543

54. Ivanova N, Sorokin A, Anderson I, Galleron N, Candelon B, Kapatral V, et al. Genome sequence of Bacillus cereus and comparative analysis with Bacillus anthracis. Nature. 2003; 423: 87–91. doi: 10.1038/nature01582 PMID: 12721630

55. Laughlin TF, Ahmad Z. Inhibition of Escherichia coli ATP synthase by amphibian antimicrobial peptides. Int J Biol Macromol. 2010; 46: 367–374. doi: 10.1016/j.ijbiomac.2010.01.015 PMID: 20100509

56. Azim S, McDowell D, Cartagena A, Rodriguez R, Laughlin TF, Ahmad Z. Venom peptides cathelicidin and lycotoxin cause strong inhibition of Escherichia coli ATP synthase. Int J Biol Macromol. 2016; 87: 246–251. doi: 10.1016/j.ijbiomac.2016.02.061 PMID: 26930579

57. Murtagh F, Legendre P. Ward’s Hierarchical Agglomerative Clustering Method: Which Algorithms Implement Ward’s Criterion? J Classif. 2014; 31: 274–295.

58. Huelsenbeck JP, Ronquist F. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics. 2001; 17: 754–755. PMID: 11524383