Reference genome and comparative genome analysis for the WHO reference strain for *Mycobacterium bovis* BCG Danish, the present tuberculosis vaccine

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Abstract

**Background:** *Mycobacterium bovis* bacillus Calmette-Guérin (*M. bovis* BCG) is the only vaccine available against tuberculosis (TB). In an effort to standardize the vaccine production, three substrains, i.e. BCG Danish 1331, Tokyo 172–1 and Russia BCG-1 were established as the WHO reference strains. Both for BCG Tokyo 172–1 as Russia BCG-1, reference genomes exist, not for BCG Danish. In this study, we set out to determine the completely assembled genome sequence for BCG Danish and to establish a workflow for genome characterization of engineering-derived vaccine candidate strains.

**Results:** By combining second (Illumina) and third (PacBio) generation sequencing in an integrated genome analysis workflow for BCG, we could construct the completely assembled genome sequence of BCG Danish 1331 (07/270) (and an engineered derivative that is studied as an improved vaccine candidate, a SapM KO), including the resolution of the analytically challenging long duplication regions. We report the presence of a DU1-like duplication in BCG Danish 1331, while this tandem duplication was previously thought to be exclusively restricted to BCG Pasteur. Furthermore, comparative genome analyses of publicly available data for BCG substrains showed the absence of a DU1 in certain BCG Pasteur substrains and the presence of a DU1-like duplication in some BCG China substrains. By integrating publicly available data, we provide an update to the genome features of the commonly used BCG strains.

**Conclusions:** We demonstrate how this analysis workflow enables the resolution of genome duplications and of the genome of engineered derivatives of the BCG Danish vaccine strain. The BCG Danish WHO reference genome will serve as a reference for future engineered strains and the established workflow can be used to enhance BCG vaccine standardization.

**Keywords:** Tuberculosis, Live vaccines, BCG, Next-generation sequencing, Complete genomic sequence, Genetic differences, Tandem duplications

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Background
The BCG live attenuated TB vaccine is one of the oldest and most widely used vaccines in human medicine. Each year, BCG vaccines are administered to over 100 million newborns (i.e. 75% of all newborns on the planet). The original BCG strain was developed at the Pasteur Institute, through attenuation of the bovine TB pathogen M. bovis, by 231 serial passages on potato slices soaked in glycerol-ox bile over a time-span of 13 years [1]. After its release for use in 1921, this BCG Pasteur strain was distributed to laboratories around the world and different laboratories maintained their own daughter strains by passaging. Over the years, different substrains arose with different protective efficacy [2, 3]. The establishment of a frozen seed-lot system in 1956 and the WHO (World Health Organization) recommendation of 1966 that vaccines should not be prepared from cultures that had undergone > 12 passages starting from a defined freeze-dried seed lot, halted the accumulation of additional genetic changes [1]. In an effort to further standardize the vaccine production and to prevent severe adverse reactions related to BCG vaccination, three substrains, i.e. BCG Danish 1331, Tokyo 172–1 and Russia BCG-1 were established as the WHO reference strains in 2009 and 2010 [4]. Of these, the BCG Danish 1331 strain is the most frequently used one, and it also serves as a basis of most current ‘next-generation’ engineering efforts to improve the BCG vaccine or to use it as a ‘carrier’ for antigens of other pathogens [5, 6].

Complete genome elucidation of BCG strains is challenging by the occurrence of large genome segment duplications and a high GC content (65%). Therefore, no fully assembled reference genome is yet available for BCG Danish, only incomplete ones [7, 8], which hinders further standardization efforts. In this study, we set out to determine the completely assembled genome sequence for BCG Danish and meanwhile, to establish a current-generation sequencing-based workflow to analyze genomes of BCG Danish-derived engineered strains.

Results
General genomic features of the whole genome sequence for BCG Danish 1331 (07/270)
The BCG Danish 1331 (07/270) strain genome sequence was assembled by combining second (Illumina) and third (PacBio) generation sequencing technologies in an integrated bioinformatics workflow (Fig. 1, see Methods). Ambiguous regions were locally reassembled and/or experimentally verified (Additional file 1: Table S1). In all cases, the experimental validation confirmed the assembly, demonstrating that this integration of sequencing data types and bioinformatics workflow is adequate for high-GC mycobacterial genomes. The single circular chromosome is 4,411,814 bp in length and encodes 4084 genes, including 4004 genes encoding for proteins, 3 genes for rRNA (5S, 16S and 23S), 45 genes for tRNA, 1 tmRNA gene (ssrA), 1 ncRNA gene (rnpB) and 30 pseudogenes (Fig. 2a). Compared to the reference genome sequence of BCG Pasteur 1173P2, 42 SNPs were identified, including 24 non-synonymous SNPs, 9 synonymous SNPs and 9 SNPs in the intergenic region (Additional file 1: Table S2). For all the genes containing missense and/or nonsense SNPs, we attempted to validate the SNPs via PCR and Sanger sequencing (26 SNPs affecting 19 genes) (Additional file 1: Table S3). In all cases where the validation experiment yielded interpretable quality results (i.e. not hindered by highly repetitive and/or highly GC-rich regions), these mutations were all validated (15 SNPs affecting 15 genes), demonstrating that the generated genome has very high per-base accuracy. Genetic features determinative for the BCG Danish sub-strain, as described by Abdallah et al. [8], were identified, including the region of difference (RD) Denmark/Glaxo and the DU2 type III, that was completely resolved in the assembly (Fig. 2a-b). Additionally, a 1 bp deletion in Mb3865 and a 465 bp insertion in PE_PGRS54 compared to BCG Pasteur were found. The organization of 2 repeats (A and B) in PE_PGRS54 has been reported to differ between the BCG strains [9]. We report a A-A-B-B-B-B-B organization for BCG Danish in contrast to BCG Tokyo (A-A-B-B-B-B-B) and BCG Pasteur (A-B-B-B-B-B-B). Previously, two separate genetic populations for BCG Danish 1331 have been described, which differ in the SenX3-RegX3 region (having 2 or 3 repeats of 77 bp) [10]. For BCG Danish 1331 07/270, we documented only 3 repeats of 77 bp (Additional file 1: Figure S1). Two features described by Abdallah et al. [8] to be determinative for BCG Danish were not identified, namely the rearrangement of the fadD26-pssA gene region and a 894 bp deletion in Mb0096c-Mb0098c. In addition, a 399 bp instead of a 118 bp insertion was detected in leuA, giving 12 direct repeats of 57 bp, as in the Pasteur strain (previously denoted as S-RD13 [11]). These three regions were characterized by the presence of inherent repeat structures. Furthermore, these genome regions contained assembly gaps in the assembly for BCG Danish published with the study of Abdallah et al. [8, 12], so it is likely that our long-read based genome is more accurate in these challenging regions.

The DU1 in BCG strains
Two large tandem chromosomal duplications characterize the BCG strains; the DU2 and DU1. While four different forms of the DU2 exist, the DU1 is supposed to be exclusively present in BCG Pasteur [11, 13, 14]; it spans the chromosomal origin of replication or oriC (dnaA-dnaN region) and encodes key components of the replication initiation and cell division machinery. Surprisingly, we
detected a DU1-like duplication of 14,577 bp in BCG Danish (Fig. 2). This finding was validated by performing a copy-number analysis of genes in and surrounding the DU1-like duplication (Fig. 2d). To adapt an unambiguous terminology, we considered all duplications spanning the oriC as DU1, while specifying the strain in which the duplication was found. Investigation of other publicly available data for BCG Danish did not show presence of a DU1 (Figs. 2c and 3), indicating that only the Danish 1331 substrain deposited as the WHO reference at the National Institute for Biological Standards and Control (NIBSC) contains this duplication. Additional inconsistencies in DU1 presence/absence were detected by reanalyzing publicly available data [12, 15–20] (Figs. 2c and 3): in contrast to what is concluded in the literature, we found that the public data show that there are BCG Pasteur substrains with a DU1 (data [15]) and others without a DU1 (data [12, 20]). Similarly, experimental analysis of our in-house Pasteur strains (1721, 1173 ATCC 35734) showed absence of a DU1 (Fig. 2d). Additionally, a DU1-China was detected in some data sources [15, 16], but not in others [12], which is likely explained by the use of two different substrains of BCG that are both named BCG China [8]. DU1-Birkhaug
was consistently detected in all reported sequencing data of that BCG strain.

Characterization of a derivative of BCG Danish 1331, the sapM KO

Using the same genome analysis methodology, we determined the complete genome assembly for a KO mutant in the SapM secreted acid phosphatase. Since the sapM gene is located in the DU2, the sapM locus is present twice in WT cells. The assembly for the sapM KO strain did not contain a DU2 repeat, as the KO engineering entirely out-recombined one of the copies of the DU2 to form a single sapM KO locus (Fig. 4a). The absence of the DU2 was unequivocally validated by performing a copy-number analysis of multiple genes in and surrounding the DU2 (Fig. 4b). Furthermore, we detected one SNP compared to the parental BCG Danish WT strain, a missense SNP in BCG_3966 or BCGDan_4053 (encoding a conserved hypothetical protein), which was validated by Sanger

![Figure 2](image_url)

**Fig. 2** Organization of the BCG Danish 1331 (07/270) genome, focusing on the DU1 and DU2. a Circular representation of the BCG Danish chromosome. The scale is shown in megabases on the outer black circle. Moving inward, the next two circles show forward (dark blue) and reverse (yellow) strand CDS (coding sequence). The next circle shows 3 rRNAs (5S, 16S and 23S; orange), 45 tRNAs (black), 1 tmRNA (ssrA; green) and 1 ncRNA (rnpB; dark green). The subsequent circle shows DU2-III (dark blue), DU1-Danish (purple) and RD (light blue, names of RD in black) that are typical for BCG Danish. The two inner circles represent G + C content and GC skew. b Organization of the two tandem duplications in BCG Danish and confirmation by PCR. The DU2 is made up by two repeats (R1 and R2), as well as the DU1-Danish (R3 and R4). Used primer pairs (1–8) to validate their organization are indicated. c Visual representation of the oriC with position and size of DU1-China, -Danish, -Pasteur and -Birkhaug. The table indicates which substrains have the DU1. d Copy-number analysis of genes (indicated in grey in subfigure c) in and surrounding the DU1 region for Pasteur 1173 ATCC 35734, Pasteur 1721 and Danish 1331 NIBSC 07/270. The represented data are averages (± SD) of four technical replicates.
Fig. 3 (See legend on next page.)
sequencing (Additional file 1: Table S2 and S3). The single DU2 sapM KO is a useful chassis for further vaccine engineering, as another target gene for improving BCG vaccine efficacy (sigH ([22])) is novo haploid in this strain, facilitating its future knockout to generate a sapM/sigH double knockout.

Discussion

All BCG strains originate from a common ancestor [23], but since then, they have incorporated many gene deletions and evolved gene amplifications (DU1 and DU2), that differentiate the different BCG strains from each other. Several studies on BCG vaccine strains have mapped these genomic changes using a variety of comparative genomic techniques, starting from subtractive genomic hybridization [24] to whole genome sequencing [7, 8, 25], enabling the deciphering of a genealogy of the BCG strains. The study of Abdallah and others used short-read Illumina sequencing data for 14 of the most widely used BCG strains in combination with a large-indel detection pipeline to identify a number of previously unknown deletions and insertions [8]. Most genetic signatures identified for BCG Danish by that study were also found in the complete long read/short read hybrid genome assembly that we generated for BCG Danish 1331. However, some RDs could not be found. We hypothesize that inherent repeat structures in these regions triggered the undue assignment of these regions as RD in the short-read Illumina sequencing dataset. Unequivocal assembly of repeat-containing sequences, clearly requires long sequencing reads, as generated for example by PacBio SMRT sequencing in this study.

In 2001, Bedwell and others identified two sub-strains admixed in a Copenhagen commercial preparation of the BCG vaccine (a.k.a. BCG Danish 1331) [10]. These two genetic populations differed in the senX3-regX3 region, having 2 or 3 repeats of 77 bp. We documented only one version for the senX3-regX3 region, with 3 repeats of 77 bp for the BCG Danish 1331 WHO reference reagent strain. In contrast, Magdalena et al. reported the presence of 2 repeats for a M. bovis BCG Danish vaccine strain provided by M. Lagranderie (Institut Pasteur, Paris, France) [26]. These data indicate that different substrains of BCG Danish are in circulation, and that this region probably is genetically drifting. Extensive genomic characterization of the WHO reference reagent for BCG Danish (as provided by this study) will facilitate the identity assurance of the genomic integrity of new lots of the BCG Danish vaccine.

Similarly, we document the presence of a DU1-like duplication in this WHO reference BCG strain (DU1-Danish), that has never been reported on before, as the DU1 was thought to be exclusively restricted to BCG Pasteur [11, 23]. Furthermore, we showed that not all BCG Pasteur strains contain the DU1-Pasteur, based on experimental analysis of in-house Pasteur strains and based on reanalysis of publicly available sequencing data. In addition, we detected a DU1-China in one of the two different substrains of BCG that are both named BCG China [8]. Seemingly the oriC is prone for duplication, as DU1-like duplications were observed for BCG Pasteur, BCG Birkhaug, BCG China and BCG Danish. The genealogy of BCG strains is thus further complicated by the genomic instability of the oriC during in vitro cultivation (Fig. 5, Additional file 2: Table S8). A DU1-like duplication has also been identified in a ‘non-vaccine’ strain; in a clinical isolate (3281), identified as BCG, a 7-kb region that covered six genes and crossed the oriC was repeated three times [27], further indicating that this region is prone to (possibly reversible) duplication. Together, these data underline the importance of the genomic characterization of the BCG vaccine strains, including their dynamic duplications. Furthermore, they demand for the specification of the exact origin of the BCG strain(s) used in studies on this vaccine and the determination of the presence of the RD documented for that strain. The implementation of copy number analysis via qPCR as described here, could allow for easy discrimination whether a certain strain contains a DU1-like duplication or not, instead of requiring next-generation sequencing (more expensive) and bioinformatics analyses (requires expert knowledge).

Until now, no driving factor for the DU1 has been identified, as the DU1 in BCG Pasteur contains 31 genes and none of these genes are expected to give an obvious in vitro growth advantage upon duplication [13]. Perhaps, this could now be elucidated by examining the
Fig. 4 BCG Danish 1331 sapM KO has lost the DU2 to form the sapM KO locus. a Illustration of the outrecombination of the DU2 duplicated genomic region in the formation of the BCG Danish 1331 sapM KO from BCG Danish 1331 WT, containing two sapM loci, due to the presence of the sapM locus in the DU2. b Genomic organization of the sapM region for BCG Danish WT and sapM KO. The organization of the DU2 is indicated. †: truncated sapM. c Copy-number analysis of selected genes (indicated in grey in subfigure b) in and surrounding the DU2 via qPCR on gDNA for BCG Danish 1331 WT and sapM KO. The represented data are averages (± SD) of four technical replicates.
gene functions of the genes common to all DU1-like duplications. Seven genes are duplicated in all DU1 (DU1-Pasteur, -Birkhaug, -China and -Danish and the DU1-like triplication identified in the clinical isolate BCG 3281), namely BCG_3979c, BCG_3980c, rnpA, rpmH, dnaA, dnaN and recF (Table 1). It remains however difficult to speculate about the impact of two copies of oriC (dnaA-dnaN region) on the biology of BCG strains [13]. Bacteria carefully regulate the activity of the initiator protein DnaA and its interactions with the oriC to assure correct timing of the chromosome duplication [30]. Therefore, one has assumed that multiple copies of the oriC are deleterious, as they can provoke uncoordinated replication [13, 31]. It remains however difficult to speculate about the impact of two copies of oriC (dnaA-dnaN region) on the biology of BCG strains [13]. Bacteria carefully regulate the activity of the initiator protein DnaA and its interactions with the oriC to assure correct timing of the chromosome duplication [30]. Therefore, one has assumed that multiple copies of the oriC are deleterious, as they can provoke uncoordinated replication [13, 31]. It is known that M. smegmatis transformants with two functional DnaA gene copies cannot be obtained [31], as observed in both B. subtilis [32] and S. lividans [33]. However, such an inhibitory effect was not observed when a complete dnaA gene was transformed to M. smegmatis [34], although Salazar and others questioned whether the construct did not acquire a point mutation or small deletion that inactivated dnaA [31]. Until now, no sequence differences were observed between the different copies of the dnaA-dnaN region, suggesting that both copies of the origin are functional in vivo. It has been speculated that BCG 3281 (containing 3 copies of the dnaA-dnaN region) would likely be capable of enduring greater gene expression burdens in replication [27]. Indeed, as DnaA and oriC are so closely genetically linked, duplication of this genomic region is not necessarily the same as just increasing the gene copy number or overexpressing DnaA. It could be envisioned that selection for rapid growth on rich medium may favor or tolerate more rapid genomic replication initiation, but also that this selective advantage may collapse in the face of e.g. nutrient limitation or prolonged stationary phase cultivation. Possibly this is at the heart of the observed unpredictable behavior of this genomic duplication. Confirmation of this hypothesis awaits experimental confirmation.

To demonstrate how the genome analysis methodology, developed in this study, contributes to full characterization of improved BCG-derived engineered vaccines, we applied it to a KO for the SapM secreted acid phosphatase, located in the analytically challenging
long duplication region DU2 [11]. Our BCG genome analysis workflow unequivocally demonstrated that the KO engineering had inadvertently out-recombined one of the copies of this DU2 and had furthermore given rise to a single SNP. The out-recombination of the DU2 will most probably not have a dramatic impact on the phenotype of the sapM KO, as all the genes are still present as a single copy. One could perhaps expect slower growth of the sapM KO in glycerol-containing media, as the DU2 probably arose due to inadvertent selection for increased growth rate on glycerol [11]. GlpD2, encoding glycerol-3-phosphate dehydrogenase, is one of the three genes present in all DU2 versions and higher levels of glpD2 probably gave a growth advantage to strains with duplications [11]. We did not observe a decreased growth rate in the Middlebrook 7H9 standard medium for the sapM KO. Perhaps, the growth advantage attributed to the DU2 would only be apparent in Calmette’s glycerol-containing medium, traditionally used to subculture the BCG strains before the introduction of a frozen seed-lot system in 1956 [37]. The effect of the SNP in BCG_3966 (or Rv3909) is hard to estimate. The mutated gene encodes for a conserved hypothetical protein of 802 amino acids and is predicted to be an outer membrane protein [38]. The missense SNP converts the asparagine (located at the end of the protein) in the WT to a threonine in the sapM KO (pAsn737Thr). However, as the gene has been found to be essential for in vitro growth of M. tb H37Rv [39, 40], we suspect that the protein function is retained. Such unexpected genomic alterations may be more common than thought in engineered live attenuated TB vaccines, but may have so far gone largely unnoticed due to lack of a complete reference genome and/or suitable genome analysis methodology.

The implementation of both short (Illumina) and long (PacBio) sequencing reads in one genome analysis methodology allowed for the straightforward generation of completely assembled genomes of BCG strains. These included the decomposition of the analytically challenging long duplication regions DU1 and DU2, thanks to the inclusion of long sequencing reads, whereas one formerly needed many additional experimentation (Table 2). Furthermore, the generated genome assemblies were highly polished at base level, due to the incorporation of reliable Illumina sequencing reads (single-pass error rate of 0.1%), in addition to the more error-prone PacBio sequencing reads (single-pass error rate of 10–15%) [41, 42]. This methodology is thus currently the most cost-effective strategy that allows to create high-quality BCG genomes, solely based on next-generation sequencing strategies.

| Gene/feature M. tb H37Rv (BCG Pasteur) | Product/Function | Functional category |
|----------------------------------------|------------------|---------------------|
| Rv3921c (BCG3979c) | Probable conserved transmembrane protein | cell wall and cell processes |
| Rv3922c (BCG3980c) | Possible hemolysin | virulence, detoxification, adaptation |
| rmpA | Ribonuclease P protein component RnpA or RNaseP. RNaseP catalyzes the removal of the 5’-leader sequence from PRE-trNA to produce the mature 5’ terminus. It can also cleave other RNA substrates such as 4.5S RNA. The protein component plays an auxiliary but essential role in vivo by binding to the 5’-leader sequence and broadening the substrate specificity of the ribozyme. | information pathways |
| rpmH | 50S ribosomal protein L34 RpmH. Involved in translation mechanism. This protein is one of the early assembly proteins of the 50S ribosomal subunit. | information pathways |
| dnaA | Chromosomal replication initiator protein DnaA. Plays an important role in the initiation and regulation of chromosomal replication. Binds to the oriC; it binds specifically dsDNA at a 9 bp consensus (DnaA box): 5′-TTATC(C/A)A(C/A)AA3′. DnaA binds the oriC, ATP and ADP, acidic phospholipids and exhibited weak ATPase activity. | information pathways |
| oriC | Sequence in the genome at which replication is initiated. Contains non-overlapping MtrA- and DnaA-binding boxes. Is located in the dnaA-dnaN genomic region [35]. | – |
| dnaN | DNA polymerase III (β-chain) DnaN (DNA nucleotidyltransferase). DNA polymerase III is a complex, multichain enzyme responsible for most of the replicative synthesis in bacteria. This DNA polymerase also exhibits 3’ to 5’ exonuclease activity. The β-chain is required for initiation of replication. Once it is clamped onto DNA, it slides freely (bidirectionally and ATP-independently) along duplex DNA. | information pathways |
| recF | DNA replication and repair protein RecF (ssDNA binding protein) is involved in DNA metabolism and recombination; it is required for DNA replication and normal SOS inducibility. Binds preferentially to linear ssDNA. It also seems to bind ATP. | information pathways |

*Table 1 Genes (and genome feature) common to all DU1-like duplications (DU1-Pasteur, -Birhaug, -China and -Danish and the DU1-like triplication identified in the clinical isolate BCG 3281)*
Table 2 List of M. bovis BCG strains for which high per-bp coverage complete genomes are available

| Strain               | Method                                                                 | type DU2 | DU2 resolved | Accession numbers | Reference | Year of publication |
|----------------------|------------------------------------------------------------------------|----------|--------------|-------------------|-----------|---------------------|
| BCG Pasteur 1173P2   | Sanger sequencing (ABI 3700) of a pUC19, two pMAQ1b, a M13 and a cloned shotgun library, earlier analysis of BAC clones had already identified the DU1 and DU2 [13] | IV       | yes          | PRAEA18059, AM408590 | [11]      | 2007                |
| BCG Tokyo 172        | SOLID (Agencourt Bioscience Corporation) sequencing of a Pagen vector library (about 4 kb and 800 bp inserts) and a fosmid insert library (about 40 kb) | I        | yes (2x)     | PRIDA31211, AP010918 | [9]       | 2009                |
| BCG Moreau RDJ       | Sanger (ABI 3730) sequencing of 2 pBluescript libraries, gap closure via PCR | I        | no           | PRJA70285, AM412059 | [43]      | 2011                |
| BCG Tice ATCC 35743  | Illumina genome sequencing, multiplex PCR and primer walking          | IV       | no           | PRJNA43839, CP009494 | [7]       | 2011                |
| BCG Mexico 1931      | Roche 454 pyrosequencing, Sanger sequencing of fos-end sequences of 250 fosmids, sequencing of three fosmids and 110 PCR end reads | IV       | yes          | PRJNA45811, CP002095 | [44]      | 2011                |
| BCG Korea 1168P      | Roche 454 (GS-FLX) and Illumina (HiSeq) sequencing, gap closure via PCR and primer walking | IV       | yes (2x)     | PRJNA170028, CP0093900 | [45]      | 2013                |
| BCG Russia 368       | Roche 454 (GS Junior) sequencing on a shotgun and a 3 kb paired-end library, gap closure via PCR and Sanger sequencing | I        | yes (2x)     | PRJNA256163, CP009243 | [46, 47]  | 2014                |
| BCG 3281 (clinical isolate) | Roche 454 (GS-FLX) and Illumina (HiSeq2500) sequencing, gap closure via PCR | III      | yes          | PRJNA251957, CP008744 | [27]      | 2015                |
| BCG Russia BCG-1     | Roche 454 (GS-FLX) sequencing of a shotgun library, Illumina sequencing of a mate-pair library | I        | no           | PRJNA306822, CP013741 | [48]      | 2016                |
| BCG Danish 1331 (07/270) | PacBio (RSII) (long read) sequencing (235x coverage) and Illumina (HiSeq) (short read) sequencing | III      | yes          | PRJNA494982, this study CP093850 | [49]      | 2019                |

For each strain, we indicated the used method to create the assembled genome, the type of DU2 present in the strain, whether the DU2 was resolved in the genome assembly, the BioProject and genome accession number, the reference to the study in which genome assembly method was published and the year of publication. In the ‘method’ description, we have put labor/capital-intensive aspects in bold, illustrating that our approach using solely massive parallel sequencing, is the only one that provides both high per-bp accuracy (allowing for SNP calling) and complete resolution of the assembly across large repeat regions.

Conclusions
Our data highlight the importance of characterizing our BCG vaccine strains, as more variability exists among these strains than was thought. The availability of the complete reference genome for BCG Danish 1331 as well as the associated genome analysis workflow, now permits full genomic characterization of (engineered) TB vaccine strains, which should contribute to more consistent manufacturing of this highly cost-effective vaccine that protects the world’s newborns from disseminated TB, and that is used as a basic chassis for improved TB vaccine design.

Methods
Mycobacterial strains, gDNA and reference genomes
The strains used include the M. bovis BCG Danish 1331 sub-strain (14 WHO Reference Reagent, 07/270, National Institute for Standards and Control (NIBSC), Hertfordshire), the BCG Pasteur 1173 strain (ATCC#35734™, ATCC, Manassas), the streptomycin-resistant BCG Pasteur 1721 strain [49] (RpsL: K43R; a gift of Dr. P. Sander, Institute for Medical Microbiology, Zürich). From the Danish 1331 strain, a sapM knockout (KO) strain was constructed (detailed procedure of the strain construction can be found in Additional file 1: Methods). Strains were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.05% Tween-80 and Middlebrook OADC (Becton Dickinson). Preparation of genomic DNA (gDNA) from mycobacterial strains was performed as previously described [50]. As reference genomes, M. tb H37Rv (NC_000962.3 [51]), M. bovis AF2122.97 (NC_002945.4 [52]) and BCG Pasteur 1173P2 (NC_008769.1 [53]) were used.

Whole genome sequencing of BCG Danish 1331 WT and sapM KO strain
For PacBio SMRT sequencing, the gDNA was sheared using a Megaruptor device (large hydrospore, Megaruptor, Diagenode, shearing size 35 kb), used for PacBio SMRT library preparation (SMRTbell Temp Prep Kit 1.0, Pacific Biosciences). Size selection was done on a BluePippin device (0.75% DF marker S1 high-pass 15-20 kb, Sage Science). The prepared samples were sequenced on a PacBio RSII instrument (DNA/Polymerase Binding Kit P6 v2, DNA Sequencing Kit 4.0 v2, Pacific Biosciences), the mean read length was 13.7 kb. One SMRT-cell was used for the KO sample (229x coverage) and 2 SMRT-cells were run for the WT sample (140x and 95x coverage). For Illumina sequencing, libraries were prepared with the Nextera DNA Library Preparation kit and
sequenced on an Illumina MiSeq instrument (MiSeq Reagent Kit v2 Nano, PE250 (paired end 250 bp), 500 Mb), with an average of 55-56x coverage per genome.

**Genome assembly and analysis**

Illumina reads were quality-filtered and adapter sequences were trimmed (Trimmomatic v0.36 [54]), after which overlapping paired-end reads were merged into single reads (BBMerge v3.69 [55]). PacBio read sequences were corrected using the high quality Illumina reads (Lordec v0.6 [56]). The unmerged and merged Illumina reads were assembled into a draft assembly (SPAdes v3.9.0 [57]). The draft assembly was scaffolded using the corrected PacBio reads (SSPACE-LongRead v3.0 [58]). Finally, gaps in the scaffold were closed (GapFiller v1.10 [59]) and the assembly was improved (Pilon v1.20 [60]), both using the trimmed Illumina reads.

The exact sequence of the DU1 region was based on a second round of local de novo assembly (SPAdes v3.9.0 [57]) using soft-clipped Illumina reads surrounding the draft DU1 region where the Illumina read coverage is more than two times higher than the background coverage. The DU2 repeat was resolved by comparing the SPAdes assembly with the assembly from HINE (v201705) [61], where the R1 and R2 regions have been separated. The junction sequences of DU1 and DU2 were further confirmed by aligning uniquely mapped PacBio reads and the results were always consistent with PCR and Sanger sequencing.

Annotation was done by combining an automatic gene prediction program with heuristic models (GeneMark.hmm [62]) and the existing M. bovis BCG Pasteur and M. tb reference [51] gene models (GMAP [63] and TBLASTN [64]) along with UniProt database [65] (BLASTP [64]). Non-coding RNA were predicted (tRNAScan-SE [66] and Infernal [67]). The assigned annotations were manually checked (Artemis [68] and CLC Main Workbench 8 [69], e.g. correct start codon), by comparative analysis with the 3 reference genomes for M. tb [51], M. bovis [52] and M. bovis BCG Pasteur [53], as listed above. Inconsistencies in the annotation and/or assembly were analyzed in detail and/or verified by PCR and Sanger Sequencing.

A probabilistic variant analysis was performed by mapping the BBmerged Illumina reads to the BCG Pasteur reference genome (BWA-MEM [70]) and calling variants by GATK UnifiedGenotyper [71] (Count ≥10 & Variant Probability >0.9), whereafter variant annotations and functional effect prediction were carried out with SnpEff and SnpSift [72]. The orthologous relationships between M. tb, M. bovis BCG Pasteur and BCG Danish WT and sapM KO were investigated, the proteins of strains (M. tb H37Rv [51], BCG Pasteur 1173P2 [53], BCG Danish WT and sapM KO (this study)) were searched using all-against-all with BLASTP [64], after which the result was analyzed by TribeMCL [73] and i-ADHoRe 3.0 [74] based on the genome synteny information (Additional file 3: Table S9).

To validate the detection of the DU1, the DU1 duplication region was reanalyzed in published genome data [12, 15–20]. Probes on tiling array or Illumina short sequencing reads were mapped to the M. tb reference strain [48] (BWA-MEM [70]). The tiling array data were directly compared by the intensity ratio between H37Rv and the sampled strains (ratio = strain / H37Rv). A ratio larger than one was considered as a duplication in the sampled strain. The DU1 duplications in the Illumina data were detected by cn.mops [75]. In brief, cn.mops first took all aligned BAM files (BWA-MEM) and normalized the mappable read counts to make it compatible across all samples in the comparison. A mixture of Poisson model was then used to compare read counts for each genomic position (bin size 500 bp) across all samples. A mixture of Poisson model will not be affected by read count variations along the chromosomes caused by technical or biological noise, since a separate model is constructed at each position. Using a Bayesian approach, read counts and the noise across samples were decomposed by an expectation maximization algorithm into integer copy numbers (with confidence intervals).

In Fig. 1 a graphical overview of the performed genome analysis pipeline is given. All presented next-generation sequencing data were integrated in an online genome browser (JBrowse) [76].

**PCR analysis, gel electrophoresis and sanger sequencing**

PCR (GoTaq‘Green, Promega) was performed on gDNA using primers listed in Additional file 1: Table S1 and S4. PCR products were run on a 1.2% agarose gel, stained with Midori Green and visualized under ultraviolet light. To confirm the single nucleotide polymorphisms (SNPs), regions of interest were amplified (Phusion High-Fidelity DNA Polymerase, NEB) from gDNA with primers listed in Additional file 1: Table S5. The resulting PCR products were purified (AMPure XP beads) and Sanger sequenced with (a) nested primer(s) (Additional file 1: Table S1 and S5).

**Copy number profiling via qPCR**

Real-time quantitative PCR was done on a LightCycler 480 (Roche Diagnostics) using the SensiFast SYBR-NoRox kit (Bioline) in quadruplicate for each gDNA sample using primers listed in Additional file 1: Table S6. Determination of the average relative quantities was performed using the qbasePLUS software (Biogazelle). All results were normalized using the reference genes 16S rRNA, mnuG and mptpB.
Additional files

Additional file 1: Supplementary Methods, Figures S1–S3 and Tables S1–S7. (DOCX 513 kb)

Additional file 2: Table S8. Distribution of regions of difference, deletions and tandem duplications (DU1 and DU2) in the different BCG strains compared to M. bovis AF2122_97 (NC_002945.4). (XLSX 14 kb)

Additional file 3: Table S9. Ortholog of mycobacterial genes between M. tb H37Rv, M. bovis BCG Pasteur, M. bovis BCG Danish WT and sapM KO. (XLSX 880 kb)

Abbreviations

BCG: Bacillus Calmette-Guérin; CDS: Coding sequence; gDNA: Genomic DNA; KO: Knockout; M. bovis: Mycobacterium bovis; M. tb: Mycobacterium tuberculosis; NIBSC: National Institute for Biological Standards and Control; RD: Region of difference; SNP: Single nucleotide polymorphism; TB: Tuberculosis; WHO: World Health Organization; WT: Wild type

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Authors’ contributions

KB designed and performed the experiments, analyzed the data and co-wrote the manuscript. JYO performed the bioinformatics analysis. PKZ assisted in the data analysis. GM assisted in performing the experiments. EP performed the RT-qPCR analysis. FT and AH constructed pCoa1757apM700 and transformed BCG Danish 1331 with this plasmid. NF assisted in experimental design and interpretation and co-wrote the manuscript. YCL performed the bioinformatics analysis, assisted in experimental design and interpretation and co-wrote the manuscript. All authors read an approved the final manuscript.

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Additional files

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