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Construction, characterization and preclinical evaluation of MTBVAC, the first live-attenuated *M. tuberculosis*-based vaccine to enter clinical trials

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34 **ABSTRACT**

35

36 The development of a new tuberculosis vaccine is an urgent need due to the failure of the
37 current vaccine, BCG, to protect against the respiratory form of the disease. MTBVAC is an
38 attenuated *Mycobacterium tuberculosis* vaccine candidate genetically engineered to fulfil the
39 Geneva consensus requirements to enter human clinical trials. We selected a *M.*
40 *tuberculosis* clinical isolate to generate two independent deletions without antibiotic-
41 resistance markers in the genes *phoP*, coding for a transcription factor key for the regulation
42 of *M. tuberculosis* virulence, and *fadD26*, essential for the synthesis of the complex lipids
43 phthiocerol dimycocerosates (DIM), one of the major mycobacterial virulence factors. The
44 resultant strain MTBVAC exhibits safety and biodistribution profiles similar to BCG and
45 confers superior protection in preclinical studies. These features have enabled MTBVAC to
46 be the first live attenuated *M. tuberculosis* vaccine to enter clinical evaluation.

47 **1. Introduction**

48

49 The only vaccine against tuberculosis (TB) in use today, Bacille Calmette-Guerin (BCG), is a
50 live attenuated strain of *Mycobacterium bovis* effective in reducing the rate of severe forms of
51 TB (meningitis and miliary TB) in children, but is inconsistent in preventing spread of
52 pulmonary TB, the most common form of the disease in adolescents and adults, which fuels
53 the continuing epidemic [1, 2]. Developed a century ago by repeated subculture, the principal
54 genetic basis for BCG attenuation is the loss of RD1 region, which encodes the machinery
55 required to synthesize and export the major T-cell antigen/virulence factor ESAT-6/CFP-10
56 [3, 4]. Subsequent worldwide distribution of BCG and repeated subculture in non-
57 standardized conditions has led to emergence of a number of daughter BCG sub-strains,
58 which comprise natural mutants of well-recognized virulence factors, suggesting that some
59 sub-strains may be more attenuated and otherwise less immunogenic than others [3, 5-7].
60 New vaccines able to prevent respiratory forms of TB will have a tremendous impact in
61 preventing transmission and control of the disease [8-10].

62

63 We have previously described the construction of the SO2 strain by insertion of a kanamycin-
64 resistance cassette in the *phoP* gene of a *M. tuberculosis* clinical isolate [11]. PhoP is a key
65 transcriptional regulator, which controls approximately 2% of *M. tuberculosis* coding capacity,
66 including the synthesis of the immunomodulatory trehalose-derived lipids, diacyl- (DAT) and
67 polyacyl-trehaloses (PAT), and the secretion of the virulence factor ESAT-6 [12-15]. Ten
68 years of rigorous preclinical testing of SO2 as a vaccine candidate has provided robust data
69 for its high degree of safety and improved immunogenicity and protective efficacy compared
70 to BCG in relevant animal models of TB, from mice to non-human primates [16-19]. Despite
71 the promising results, the establishment of the Geneva consensus for new live mycobacterial
72 vaccines, demanding the presence of two stable independent mutations without antibiotic-
73 resistance markers for *M. tuberculosis*-based candidates, in addition to a safety and efficacy

74 profile at least comparable to BCG in the relevant animal models, rendered SO2 unsuitable
75 for entry into clinical trials [20, 21].
76
77 Here we describe the construction and extensive preclinical characterization of MTBVAC, the
78 first live-attenuated vaccine based on a *M. tuberculosis* human isolate that entered first-in-
79 human clinical evaluation in January 2013. This vaccine is based on the prototype SO2 and
80 is genetically engineered to fulfil the Geneva consensus requirements for progressing new
81 live mycobacterial vaccines into Phase 1 clinical trials, requiring two non-reverting unmarked
82 independent mutations [20]. MTBVAC contains two stable deletions in the *phoP* and *fadD26*
83 genes without antibiotic-resistance markers. FadD26 is essential for the synthesis of
84 phthiocerol dimycocerosates (DIM), a family of surface lipids involved in *M. tuberculosis*
85 virulence [22, 23]. MTBVAC is safe in all preclinical studies and confers superior protection in
86 mice compared to the reference licensed strain BCG Danish 1331 used in the clinic.

2. Materials and Methods

2.1. MTBVAC construction and culture conditions

M. tuberculosis Mt103, the parental strain in this study, was isolated from an immunocompentent TB patient [24]. MTBVAC vaccine candidate was constructed following standard mycobacterial genetic-engineering protocols [25-27] (for a detailed description of the process see supplementary methods).

Mycobacterial strains were grown at 37°C in Middlebrook 7H9 broth (Difco) supplemented with albumin, dextrose and catalase (ADC) (Difco) and 0.05% (v/v) Tween-80, or on solid Middlebrook 7H10 or 7H11 (Difco) supplemented with ADC (Difco). When required, kanamycin (20 μg/ml) was added to the media. All chemicals were purchased from Sigma-Aldrich except where indicated.

2.2. *Protein isolation and western-blot analysis*

Whole-cell proteins. Mycobacteria from early log-phase liquid cultures of Mt103 or MTBVAC were pelleted by centrifugation, washed and resuspended in phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors. Cell suspensions were disrupted by sonication.

Secreted proteins. M. tuberculosis strains were grown in 7H9 supplemented with dextrose (2 g/l) and supernatants were separated by centrifugation. Secreted proteins were precipitated by incubation with 10% (v/v) trichloroacetic acid for 30 min on ice and then centrifuged at 4ºC for 30 min. Pelleted proteins were rinsed with cold acetone and resuspended in 150 mM Tris-HCl pH=8.

Western blot. Both protein preparations were sterilized using a 0.22-μm low protein-binding filter (Pall) and quantified using the RC DC protein assay (BioRad). 5 μg of protein were loaded per well and separated by SDS-PAGE. Immunodetection was carried out using primary monoclonal mouse antibodies anti-ESAT6 or anti-GroEL (Abcam), followed by

114 incubation with an anti-mouse secondary antibody and developed with a chemiluminiscent
115 substrate (Immobilon^{TM} western, Millipore).
116
117 *2.3. Complex lipids extraction and thin-layer chromatography analysis*
118 Mt103 and MTBVAC strains were grown in 16 ml of liquid medium for 10 days (exponential
119 phase). Radiolabelling of methyl-branched fatty acids was performed by incubating the
120 cultures with 7 μCi of ^{14}C-propionate (specific activity 55 mCi/mmol; MP Biomedicals) for 24h
121 at 37°C with continuous agitation and mycobacterial lipids were then extracted as previously
122 described [28]. Lipid profiles were analyzed by spotting equivalent amounts of crude extracts
123 (resuspended in $CHCl_3$) on silica gel G60 plates (Merck), which were then run in various
124 solvent systems ($CHCl_3/CH_3OH/H_2O$ 60:16:2 (v/v) for DAT; $CHCl_3/CH_3OH$ 99:1 (v/v) for PAT;
125 and petroleum ether/diethyl ether 9:1 (v/v) for DIM). Radiolabeled lipids were visualized with
126 a Typhoon PhosphorImager (Amersham Biosciences).
127
128 *2.4. Animal studies*
129 All the animals were kept under controlled conditions and observed for any sign of disease.
130 Experimental work was conducted in agreement with European and national directives for
131 protection of experimental animals and with approval from the competent local ethical
132 committees.
133
134 *Mouse studies*
135 For immunogenicity studies, BALB/c mice (Charles River) were mock-treated or
136 subcutaneously inoculated with $5x10^5$ colony forming units (CFU) of reconstituted lyophilized
137 MTBVAC, prototype SO2 or BCG Danish 1331. At 7, 28 and 60 days post-inoculation,
138 splenocytes were collected from 4 animals per group and stimulated overnight with purified
139 protein derivative (PPD) [16]. Intracellular staining of IFNγ was performed using BD
140 Cytofix/Cytoperm Fixation/Permeabilization kit following manufacturer instructions.

141 For biodistribution studies, groups of male or female BALB/c mice (Charles River) received
142 an intradermal injection of $5x10^5$ CFU of reconstituted lyophilized MTBVAC or BCG Danish
143 1331. At weeks 1, 2, 4, 8, 16 and 24 post-inoculation, 4 mice per group were randomly
144 selected and sacrificed for enumeration of viable bacteria in inguinal and axillary lymph
145 nodes, spleen, liver, lungs, kidneys, testis, ovaries and brain. Urine and stool samples were
146 collected at each time point. Organs were homogenized and viable bacteria were counted by
147 plating onto selective 7H11-ADC supplemented with antibiotics to avoid contamination.
148 For protection studies, groups of 8 C57BL/6 mice (Janvier) were mock-treated or
149 subcutaneously vaccinated with $5x10^5$ CFU of reconstituted lyophilized MTBVAC or BCG
150 Danish 1331. 8 weeks post-vaccination, mice were intranasally challenged with 100 CFU of
151 virulent *M. tuberculosis* H37Rv. 4 weeks later, mice were humanely sacrificed and CFU
152 quantified in lungs and spleen.
153 For safety studies, groups of 12 CB-17/Icr Ico SCID mice (Charles River) received a single
154 subcutaneous administration of $2.5x10^7$ CFU (equivalent to 50 times the dose recommended
155 for BCG in humans, $5x10^5$) of vaccine strains MTBVAC, BCG Pasteur or BCG Danish 1331;
156 a group was inoculated with 10^5 CFU of parental *M. tuberculosis* Mt103 as the virulence
157 control. The endpoint of the experiment was defined as survival up to 13 weeks post-
158 inoculation and then animals were humanely euthanized and bacterial load in lungs and
159 spleen was quantified.
160
161 *Guinea pig studies*
162 For protection studies, groups of 8 Dunkin-Hartley guinea pigs (Harlan) were mock-treated or
163 subcutaneously vaccinated in the nape of the neck with $5x10^3$, $5x10^4$ and $5x10^5$ CFU of
164 reconstituted lyophilized MTBVAC, or $5x10^4$ CFU of SO2 or BCG Danish 1331. 12 weeks
165 post-vaccination, animals were subjected to an aerosol challenge of 10-50 CFU per lung of
166 *M. tuberculosis* H37Rv (NCTC 7416) [29], using a Henderson apparatus [30]. Bacterial
167 burden in lungs and spleen at 4 weeks post-challenge was quantified. The severity of the

168 microscopic lesions in lungs and spleen was also evaluated by a subjective histopathology
169 scoring matrix [29].
170 For shedding and excretion experiments, groups of 10 Dunkin-Hartley guinea pigs (5 males
171 and 5 females) (Harlan), were intradermally vaccinated with $5x10^5$ CFU of reconstituted
172 lyophilized MTBVAC or BCG Danish 1331. Animals were observed over a period of 7 weeks
173 post-vaccination during which samples (injection site swab, urine and stool) were collected at
174 the selected time points and plated for the detection of possible vaccine shedding or
175 excretion. Injection site swabs were collected at 0, 3, 7, 14, 21, 28, 35, 42 and 49 days post-
176 inoculation. In addition, swabs of the vaccine site were collected immediately on observation
177 of a site 'opening' event. Urine and stool samples were collected at 2, 7, 21 and 49 days
178 post-inoculation.
179 For safety studies, groups of 8 Dunkin-Hartley guinea pigs (Harlan) were subcutaneously
180 vaccinated with $2.5x10^7$ CFU (equivalent to 50 times the dose recommended for BCG in
181 humans, $5x10^5$) of reconstituted lyophilized MTBVAC or BCG Danish 1331. Animals were
182 observed daily over a period of 42 days post-administration and body weights of all animals
183 were recorded weekly. At the end of the experiment, animals were euthanized and examined
184 for any signs of TB lesions in lungs, spleen, liver and lymph nodes. Any potential lesion was
185 recovered and submitted for culture and/or fixed and submitted for histological assessment.

186 **3. Results**

187

188 *3.1. From SO2 to MTBVAC: generation of two unmarked deletions in phoP and fadD*26
189 *genes to fulfil the Geneva consensus*

190 Considering that attenuation by two non-reverting independent mutations without antibiotic-
191 resistance markers is required to fulfil the Geneva consensus criteria, we sought to construct
192 an SO2-based vaccine that accomplished these criteria for progressing this vaccine
193 candidate into clinical evaluation [20]. We followed a stepwise approach to genetically
194 engineer two stable deletions in *phoP* and *fadD26* genes in the SO2 strain, with subsequent
195 elimination of antibiotic-resistance markers, generating a novel vaccine candidate that was
196 named MTBVAC (Fig. 1A). No significant differences in growth behaviour were observed
197 between MTBVAC and SO2 in axenic culture (Fig. S3).
198 To confirm the biochemical phenotype of MTBVAC, the lipid content of the cell wall envelope
199 was analysed by thin-layer chromatography. This analysis confirmed that, due to *fadD26*
200 deletion, the outermost layer of MTBVAC is devoid of DIM [22] (Fig. 1B), and that *phoP*
201 inactivation renders MTBVAC unable to synthesize trehalose-derived lipids DAT and PAT
202 [12] (Fig. 1C). In addition to these characteristic lipid deficiencies, the deletion of *phoP* in
203 MTBVAC reduces the amount of intracellular ESAT-6 and prevents the secretion of this
204 major virulence factor [14] (Fig. 1D).
205 Once the *in vitro* phenotype provided by the *phoP* and *fadD26* deletions was corroborated,
206 MTBVAC was subjected to an extensive preclinical characterization to support its progress to
207 clinical evaluation.

208

209 *3.2. MTBVAC has vaccine properties comparable to SO2*

210 To corroborate that the phenotypic equivalence of MTBVAC and SO2 translates to functional
211 comparability, bridging studies for vaccine efficacy in guinea pigs and immunogenicity in
212 mice were conducted.

213 First, mouse immunogenicity studies, using the clinical dose and route of administration,
214 showed comparable results for MTBVAC and SO2 as measured by the percentage of splenic
215 IFNγ-producing $CD4^+$ cells, following stimulation with *M. tuberculosis* PPD, at different time
216 points post-vaccination (Fig. S4).
217 Second, in the guinea-pig short-term protection experiment, MTBVAC conferred statistically
218 equivalent protection compared to SO2 both in lungs (Fig. 2A) and spleen (Fig. 2B). Similar
219 protection was provided by all the tested doses of MTBVAC. A comparable protective
220 efficacy was obtained with BCG (data not shown), as previously described for SO2 [16].
221

222 *3.3. MTBVAC is as safe as the licensed vaccine BCG Danish 1331*

223 To support entry into clinical trials in Europe, a battery of preclinical studies of MTBVAC
224 freeze-dried preparation, produced in compliance with Good Manufacturing Practices (GMP),
225 was conducted in mice and guinea pigs, meeting Regulatory requirements in Spain (country
226 of GMP manufacture) and in Switzerland (country of Phase 1 trial) in accordance with the
227 European Pharmacopoeia monograph [31] and the WHO Recommendations to Assure the
228 Quality, Safety and Efficacy of BCG freeze-dried vaccines for human use [32]. As BCG
229 Danish 1331 is the only licensed TB vaccine in Europe, it was used as the reference
230 comparator in the preclinical characterization of MTBVAC freeze-dried product.
231 MTBVAC showed a comparable safety profile to BCG Danish 1331 in the survival
232 experiment using immunocompromised SCID mice inoculated with 50 times the
233 recommended human dose for BCG (Table 1). All the SCID mice inoculated with the vaccine
234 strains survived to the end of the experiment. Equivalent bacterial loads both in lungs (Fig.
235 3A) and spleen (Fig. 3B) were observed for MTBVAC and BCG Danish 1331. In contrast,
236 mice in the Mt103 group died by week six post-inoculation and a significantly higher bacterial
237 burden was observed. In the case of the guinea pig study, none of the animals inoculated
238 with 50 times the BCG dose recommended for humans died or showed signs of TB in-life or
239 at autopsy (table 1).

240 Vaccine biodistribution in mice was mainly in a localization restricted to lymphoid organs,
241 especially lymph nodes where a peak of colonies was observed between two and four weeks
242 post-vaccination, followed by progressive clearance thereafter (Fig. 4). In addition, MTBVAC
243 and BCG Danish 1331 could not be detected in urine and stool (table 1). In guinea pigs,
244 viable MTBVAC or BCG were uniquely found in the site of vaccination only immediately after
245 administration. In the case of BCG, some "opening" events in the site of vaccination were
246 observed (table 1).
247
248 *3.4. MTBVAC induces improved protection in mice*
249 Having established the comparable safety and biodistribution profile of MTBVAC and BCG
250 Danish 1331 clinical lots, we conducted a preclinical protection experiment in mice to
251 compare the efficacy of the two vaccines (table 1). Following a two-month vaccination by the
252 clinical route and dose of administration, we compared the efficacy of MTBVAC and BCG
253 Danish 1331 at one month post-challenge with virulent *M. tuberculosis* H37Rv (the reference
254 laboratory strain) by the natural respiratory route of infection (Fig. 5). Even though both
255 vaccines conferred significant protection compared to saline controls, a significantly higher
256 reduction in bacterial burden was observed in MTBVAC group compared to BCG, both in
257 lungs (Fig. 5A) and spleen (Fig 5B).

258 **4. Discussion**

259

260 One of the main limitations presented with BCG is the large variability in protective efficacy
261 afforded in clinic ranging from 0% to 80% [1, 2]. Thus, a better vaccine that can induce
262 superior protection over BCG and which could last into adolescence and adulthood against
263 pulmonary disease would have a tremendous impact on TB control programs [10]. MTBVAC
264 is the first *M. tuberculosis*-based vaccine candidate to fulfil the Geneva consensus
265 requirements for progressing new live tuberculosis vaccines to clinical trials [20]. Two
266 unmarked deletions have been engineered in the genes *phoP* and *fadD26* to render the final
267 vaccine construct phenotypically comparable to the vaccine prototype SO2 (Fig. 1).

268

269 MTBVAC is a derivative of a clinical isolate of *M. tuberculosis*, a classical approach to human
270 vaccinology. Most of the whole cell live vaccines used in human immunization schedules,
271 except for small pox, TB and rotavirus infections, are based on the attenuated pathogen from
272 human origin [33]. MTBVAC is a derivative of a clinical isolate of *M. tuberculosis*, a classical
273 approach to human vaccinology. The scientific rationale behind an *M. tuberculosis*-based
274 vaccine that could replace BCG is that the latter is derived from the bovine pathogen *M.*
275 *bovis*, an animal adapted close relative of *M. tuberculosis* which lost a series of genes in its
276 genome in the process of its co-evolution with the immune system of its natural host. In
277 addition, when compared to *M. tuberculosis* clinical isolates, more than one hundred genes
278 are absent in BCG genome [3, 34]. These *M. tuberculosis*-restricted genes must be
279 important in the successful interaction with the human immune system. Therefore, a vaccine
280 based on a human pathogen should be more effective at inducing more specific protective
281 immunity against TB in the clinic. To demonstrate this rationale it is imperative to go to
282 human efficacy trials, provided that the current animal models for TB are exhausted.
283 Remarkably, all the current TB vaccine strategies under clinical evaluation are based on
284 BCG [35, 36]. Consequently, the use of a vaccine based on the human pathogen as
285 MTBVAC is a novel strategy.

Results obtained in immunogenicity (Fig. S4) and protective efficacy (Fig. 2) bridging experiments provide evidence that MTBVAC is functionally comparable to its prototype SO2 and, therefore, data generated in preclinical studies with SO2 were accepted by the Swiss Regulatory Authorities as valid to support MTBVAC Phase 1 clinical evaluation. SO2 proved to be safe in guinea pigs and was more attenuated than BCG Pasteur in severe combined immunodeficiency (SCID) mice [16, 18]. In addition, SO2 conferred better protection than BCG Danish 1331 in a high-dose challenge long-term protection model in guinea pigs [16, 29]. SO2 also showed improved reduction of lung bacillary burden in rhesus macaques when compared to BCG [19]. Finally, immunogenicity data in mice showed that SO2 was able to induce a higher differentiation of antigen-specific $CD4^+$ T cells into central memory T cells, which correlated with longer protective efficacy in this model [37]. This latter result is especially important because some authors hypothesize that this inconsistent efficacy conferred by BCG may concern insufficient induction of long-lived memory T-cell responses [38].

5. Conclusions

Data shown provide evidence that MTBVAC is functionally and phenotypically comparable to its prototype SO2. The results of these studies fulfil the first and second Geneva consensus safety requirements for entry into clinical trials of live attenuated *M. tuberculosis* vaccines [20, 21]. The absence of front-line lipids, lack of ESAT-6 secretion and down-expression of the PhoP regulon, essential for virulence and pathogenesis of *M. tuberculosis*, may explain the satisfactory safety profile of MTBVAC.

Altogether, the improved protection levels against TB disease achieved by prototype SO2 and MTBVAC in mice, guinea pigs and non-human primates and the rigorous preclinical

314 safety and biodistribution data presented in this work have satisfied the Regulatory
315 Authorities and enabled MTBVAC to be the first *M. tuberculosis* vaccine candidate to enter
316 human clinical evaluation, positioning MTBVAC as a reliable vaccine for human use with
317 potential to replace BCG.
318
319
320 **Competing interests:** AA, JG, BG and CM are co-inventors on a composition of matter
321 patent “tuberculosis vaccine” filled by the University of Zaragoza. AP, CF and EP are
322 employees of Biofabri, the exclusive licensee for MTBVAC. There are no other conflicts of
323 interest.
324
325
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340

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439

440 **Figure legends**

441

442 **Figure 1. From SO2 to MTBVAC: step-by-step construction (A) and biochemical**
443 **characterization of MTBVAC (B-D). (A)** Deletions in *fadD26* and *phoP* genes were
444 genetically engineered in SO2 strain. The final strain MTBVAC is a DIM-deficient *phoP*
445 mutant which provides better assurance of genetic stability and has no antibiotic-resistance
446 markers, fulfilling the Geneva consensus requirements. *phoP* and *fadD26* genes are
447 represented as grey arrows, white rectangles illustrate antibiotic-resistance markers and
448 black arrow-heads depicts *res* sites. Vertical discontinuous lines indicate the position of
449 restriction sites used for strain construction and horizontal discontinuous lines depict DNA
450 regions that are not to scale. **(B-C)** Thin-layer chromatography analysis showing that
451 MTBVAC is devoid of cell-wall lipids DIM **(B)** and DAT/PAT **(C)**, as direct consequence of
452 *fadD26* and *phoP* deletions, respectively. Compounds of interest are indicated by arrow-
453 heads. **(D)** The absence of ESAT-6 secretion, characteristic of *M. tuberculosis phoP*
454 mutants, was tested by Western blot.

455

456 **Figure 2. Bridging efficacy studies of MTBVAC and SO2 in Guinea Pigs.** Guinea pigs
457 received a subcutaneous administration of the indicated doses of MTBVAC (white, grey and
458 black diamonds) or SO2 (black circles), or not vaccinated (white squares), followed by an
459 aerosol challenge with virulent H37Rv. At 4 weeks post-challenge, bacterial burden was
460 assessed in lungs **(A)** and spleen **(B)**. Comparable CFU reduction was observed with SO2
461 and the three tested doses of MTBVAC. Data are expressed as Mean ± SEM and compared
462 by non-parametric Mann-Whitney test. ***, $P < 0.001$.

463

464 **Figure 3. MTBVAC safety in SCID mice.** SCID mice received a single subcutaneous
465 administration of MTBVAC (black), BCG Danish 1331 (dark grey) or BCG Pasteur (light grey)
466 equivalent to 50 times the dose recommended for BCG in humans. A group inoculated with
467 one single dose of virulent Mt103 was used as control (white). CFU in lungs **(A)** and spleen

468 **(B)** were determined at the endpoint of the experiment (13 weeks), except for Mt103-infected
469 animals which died by week 6. All the animals inoculated with the vaccine strains survived
470 until the end of the experiment and presented comparable bacterial burden. Data are
471 expressed as Mean ± SEM and compared using unpaired Student's *t* test. ***, $P < 0.001$.
472
473 **Figure 4. MTBVAC biodistribution profile.** BALB/c mice were intradermally inoculated with
474 MTBVAC (black diamonds) or BCG Danish 1331 (white circles) and bacterial load in lymph
475 nodes was determined. A similar clearance profile was obtained for both strains. Data are
476 expressed as Mean ± SEM and compared using unpaired Student's *t* test. *, $P < 0.05$; **, $P <$
477 0.01.
478
479 **Figure 5. Protective efficacy of MTBVAC in mice.** C57BL/6 mice received a subcutaneous
480 administration of MTBVAC (black diamonds) or BCG Danish 1331 (white circles), or were not
481 vaccinated (white squares), followed by an intranasal challenge with virulent H37Rv. At 4
482 weeks post-challenge, bacterial burden was assessed in lungs **(A)** and spleen **(B)**.
483 Significant CFU reduction was observed in MTBVAC vaccinated animals compared to BCG.
484 Data are expressed as Mean ± SEM and compared using unpaired Student's *t* test. *, $P <$
485 0.05; **, $P < 0.01$; ***, $P < 0.001$.
486
487 **Supplementary figure 1. Verification of *fadD26* deletion and subsequent elimination of**
488 **the antibiotic-resistance marker. (A, C)** Schematic representation of *fadD26* genomic
489 region in Mt103 **(A, C)**, SO2Δ*fadD26*::*hyg* **(A)** and SO2Δ*fadD26* **(C)**. Location of specific
490 primers is represented above by arrow-heads and approximated expected sizes for the PCR
491 products are indicated below. **(B, D)** PCR analysis of the strains using the primer
492 combinations indicated in left panels (A, C).
493
494 **Supplementary figure 2. Verification of *phoP* deletion and subsequent elimination of**
495 **the antibiotic-resistance marker. (A, C)** Schematic representation of *phoP* genomic region

496 in Mt103 **(A, C)**, SO2Δ*fadD26*Δ*phoP*::*hyg* **(A)** and MTBVAC **(C).** Location of specific primers

497 is represented above by arrow-heads and approximated expected sizes for the PCR

498 products are indicated below. **(B, D)** PCR analysis of the strains using the primer

499 combinations indicated in left panels (A, C).

500

501 **Supplementary figure 3. *In vitro* growth characterization of MTBVAC. (A)** Growth

502 kinetics of Mt103 (squares), SO2 (circles) and MTBVAC (diamonds) was studied in 7H9

503 liquid medium by measuring the optical density at 600 nm (OD_{600nm}) (filled symbols) and CFU

504 counts (open symbols). **(B)** Colony morphology of the strains on solid 7H10 medium. No

505 major differences in the growth rate of the three strains were observed in liquid broth, while

506 colonies were smaller for both vaccine candidates on solid medium.

507

508 **Supplementary figure 4. Bridging immunogenicity study of MTBVAC and SO2.** BALB/c

509 mice were inoculated with MTBVAC (black), SO2 (dark grey) or BCG Danish 1331 (light

510 grey), or not vaccinated (white), and immunogenicity was measured as the percentage of

511 $IFN\gamma^+$ $CD4^+$ splenocytes. MTBVAC and SO2 induce equivalent T-cell responses at all the

512 studied post-inoculation time-points. Data are expressed as Mean ± SEM.

513 **Supplementary methods**

514

515 *1. Construction of plasmids*

516 *E. coli* strains, used for cloning processes, were grown at 37°C in LB broth or agar plates.
517 Media were supplemented with ampicillin (100 μg/ml), gentamicin (Gm) (15 μg/ml),
518 kanamycin (Km) (20 μg/ml) or hygromycin (Hyg) (50 μg/ml), when necessary. The same
519 strategy was used for the construction of the plasmids for *fadD26* (pAZ5) and *phoP* (pAZ18)
520 inactivation (table S1). DNA fragments containing the *fadD26* or *phoP* genes were amplified
521 from *M. tuberculosis* H37Rv genomic DNA using primers fadD26F and fadD26R or phoPF
522 and phoPR [12] (table S1), respectively, and cloned in pGEM-T Easy (Promega). An *EcoR*V-
523 *BamH*I fragment from pWM27 [25], containing a *res*-Ω*hyg*-*res* cassette, was inserted
524 between either *BamH*I and *EcoR*V sites in *fadD26* (1,511-bp deletion) or *EcoR*V and *Bcl*I in
525 *phoP* (94-bp deletion). The fragments containing the deleted *fadD26* or *phoP* genes, and
526 their flanking regions, were then released by digestion with *Xho*I and subsequently inserted
527 in pJQ200-*xylE* [26] (table S1), a mycobacterial suicide plasmid containing the counter-
528 selectable marker *sacB* and the reporter gene *xylE*.
529 A resolvase expression plasmid carrying a Km-resistance cassette, pAZ20 (table S1), was
530 constructed by inserting the *BsaB*I-*Sph*I fragment from plasmid pPR23 [27] containing the
531 *sacB* gene and a Gm-resistance cassette, between *Sph*I and *Sfo*I sites in pCG124 [25].

532

533 *2 Construction of MTBVAC vaccine candidate*

534 Except where indicated, mycobacterial strains were grown at 37°C in Middlebrook 7H9 broth
535 (Difco) supplemented with ADC (Difco) and 0.05% Tween-80, or on solid Middlebrook 7H10
536 (Difco) supplemented with OADC (ADC plus oleic acid) (Difco). When required, Km, Hyg (20
537 μg/ml), Gm (10 μg/ml) or 2% (w/v) sucrose (Suc) were added to the media.
538 SO2 strain was electrotransformed with pAZ5 (table S1) and allelic exchange events were
539 selected in two steps. Transformants were selected on Hyg-containing plates and single

540 colonies were assayed for Suc sensitivity. In the second step, two Hyg^R Suc^S colonies were
541 propagated in liquid medium to allow a second recombination event to occur, and serial
542 dilutions were plated on Suc-containing plates to select bacteria that had lost the *sacB* gene.
543 To discard false-positives due to mutations in *sacB* gene, a XylE activity assay (by spreading
544 a catechol solution) was performed. Hyg^R Suc^R clones were tested by PCR using various
545 combinations of primers located either in the *fadD26* deleted region (fadD26I1, fadD26I2;
546 table 1) or hybridizing in the *res* site (res1, res2; table S1) [25] and outside the *fadD26*-
547 flanking regions cloned in pAZ5 (fadD26up, fadD26down; table S1 & Fig. S1A). One clone
548 giving the pattern corresponding to allelic exchange was retained to continue the
549 construction and named SO2Δ*fadD26*::*hyg* (Fig 1A & Fig. S1B). Then, to eliminate the Hyg-
550 resistance marker, pWM19 [25] (table S1) was introduced in SO2Δ*fadD26*::*hyg* strain and
551 transformants were selected at 30°C on Gm-containing plates. Two transformants were
552 grown until saturation in liquid broth at 30°C to allow resolvase expression, and serial
553 dilutions were plated on Suc-supplemented plates and incubated at 39°C forcing loss of
554 pWM19. Several clones were tested for Hyg and Gm sensitivity and by PCR using primers
555 fadD26F and fadD26R (table S1 & Fig S1C). Sequencing of the PCR product obtained from
556 the selected clone SO2Δ*fadD26* confirmed the presence of one copy of *res* site as “scar” of
557 the resolution process (Fig 1A & Fig. S1D).
558 Next step was the replacement of the Km-resistance cassette inserted in *phoP* gene in SO2
559 strain by a deletion containing a *res*-flanked cassette. SO2Δ*fadD26* was electrotransformed
560 with pAZ18 (table S1) and allelic exchange events were selected as described above to
561 obtain the strain SO2Δ*fadD26*Δ*phoP*::*hyg* (Fig. 1A) and PCR analysis was performed using
562 various combinations of specific primers (res1, res2, phoPF, phoPR; table S1 & Fig S2A-B)
563 to confirm homologous recombination. Finally, plasmid pAZ20 (table S1) was introduced in
564 SO2Δ*fadD26*Δ*phoP*::*hyg* to generate the *phoP* unmarked mutant. Transformants were
565 selected on Km-containing plates, grown until saturation in liquid medium and plated on Suc-
566 supplemented plates. Clones obtained were tested by PCR using primers phoRF and phoPR

567 (table S1 & Fig. S2C) and one clone giving the appropriate PCR product was selected and
568 named MTBVAC (Fig. 1A & Fig. S2D).

569 **Table 1. Summary of the preclinical studies conducted with final MTBVAC product to**
570 **support entry into clinical trials**
571

Study	Description	Animal species	Duration (weeks)	Laboratory	Results
Safety 50 human doses	Survival; CFU in lungs and spleen	SCID mice	13	Germans Trias I Pujol	Survival & CFU recovered equivalent to BCG
	Survival; histopathology	Guinea pigs	6	Biofabri	Survival & absence of TB lesions
Biodistribution, Excretion & Shedding	CFU in lungs, liver, kidneys, spleen, local lymph nodes, brain and gonads	BALB/c mice	22-24	University of Zaragoza	Biodistribution equivalent to BCG
	Excretion in urine and stool				Absence of excretion
	Excretion in urine and stool & Shedding at vaccination site	Guinea pigs	7	PHE	Absence of excretion
Protection	CFU in lungs and spleen	C57BL/6 mice	4 post-vaccination	University of Zaragoza	Protection superior to BCG

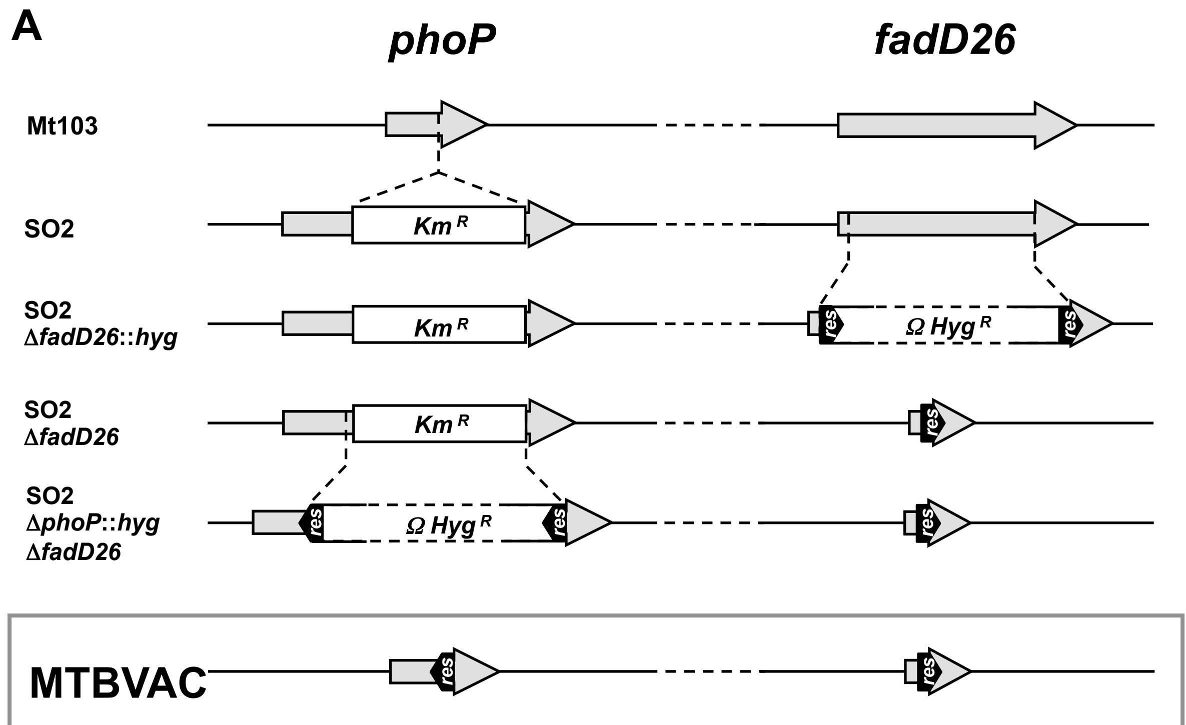
572

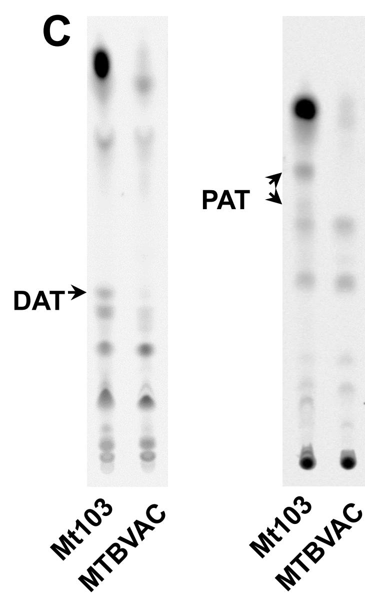
573 **Table S1. Bacterial strains, plasmids and primers used in this study**

Mycobacterial strains	Description	Reference
Mt103	Parental *M. tuberculosis* clinical isolate	[24]
SO2	*phoP* insertion mutant in Mt103 (Km^R)	[11]
MTBVAC	Double unmarked *phoP* and *fadD26* deletion mutant in Mt103 (constructed from SO2)	This work
BCG Pasteur	Laboratory reference BCG strain	Our laboratory collection
BCG Danish 1331	Only licensed BCG vaccine in Europe	Our laboratory collection
Plasmids	**Description**	**Reference**
pJQ200-*xylE*	Mycobacterial suicide plasmid carrying the counterselectable marker *sacB* and the reporter gene *xylE* (gm^R)	[26]
pWM27	Plasmid harboring the *res*-Ω*hyg*^{R}-*res* cassette (hyg^R)	[25]
pAZ5	pJQ200-*xylE* derivative containing a deleted copy of *M. tuberculosis fadD26* gene (hyg^R, gm^R)	This work
pAZ18	pJQ200-*xylE* derivative containing a deleted copy of *M. tuberculosis phoP* gene (hyg^R, gm^R)	This work
pWM19	Mycobacterial thermo-sensitive plasmid for resolvase $\gamma\delta$ expression, harboring the counterselectable marker *sacB* (hyg^R, gm^R)	[25]
pCG124	Mycobacterial plasmid for resolvase $\gamma\delta$ expression (km^R)	[25]
pPR23	Mycobacterial thermo-sensitive plasmid carrying the counterselectable marker *sacB* (hyg^R, gm^R)	[27]
pAZ20	Mycobacterial plasmid for resolvase $\gamma\delta$ expression, harboring the counter selectable marker *sacB* (km^R, gm^R)	This work
Primers	**Nucleotide sequence**	**Reference**
fadD26F	5'-CTCGAGTTCTCTATCCGTGTATTC-3'	This work
fadD26R	5'-CTCGAGGTTGGTCTTGACAG-3'	This work
phoPF	5'-AATCTAGATCAAGCATCAGCCGAGGTAC-3'	[12]
phoPR	5'-AATCTAGAGATCACCCGAACGTAGAACC-3'	[12]
fadD26I1	5'-CACGAATGTCATTGCCAATG-3'	This work
fadD26I2	5'-GCTTGAGCATGACCTCTTCG-3'	This work
fadD26up	5'-CAACGCAAGACGACATGG-3'	This work
fadD26down	5'-GCACCGTCTTGATGAAGC-3'	This work
res1	5'-CTAGAGCAACCGTCCGAAATATTATAA-3'	[25]
res2	5'-GATCTCATAAAAATGTATCCTAAATCAAATATC-3'	[25]
phoRF	5'- AATCTAGAGGGCAAGGGCAACAAGGAAC-3'	This work

574

Figure(s)





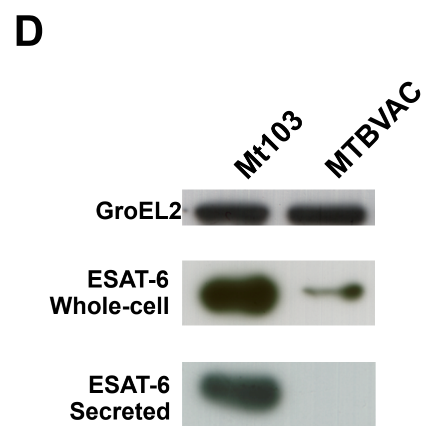
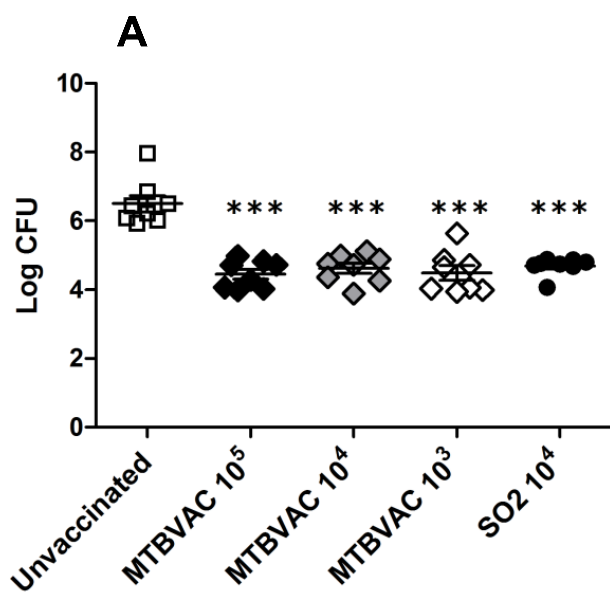


Figure 1

Arbues *et al*



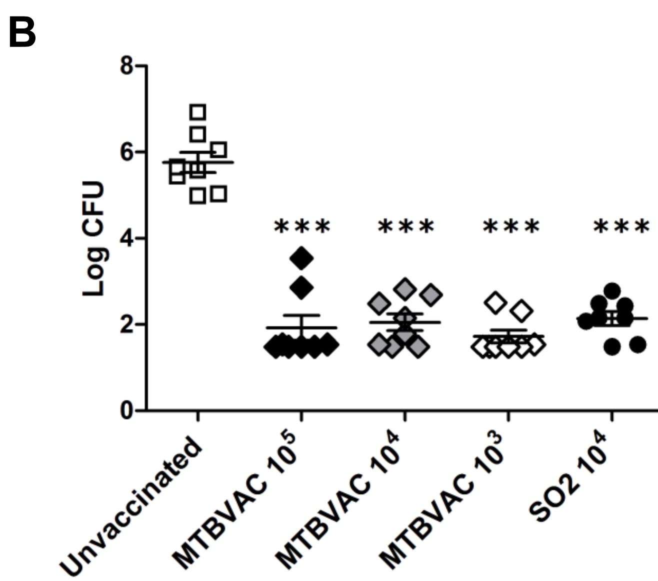


Figure 2

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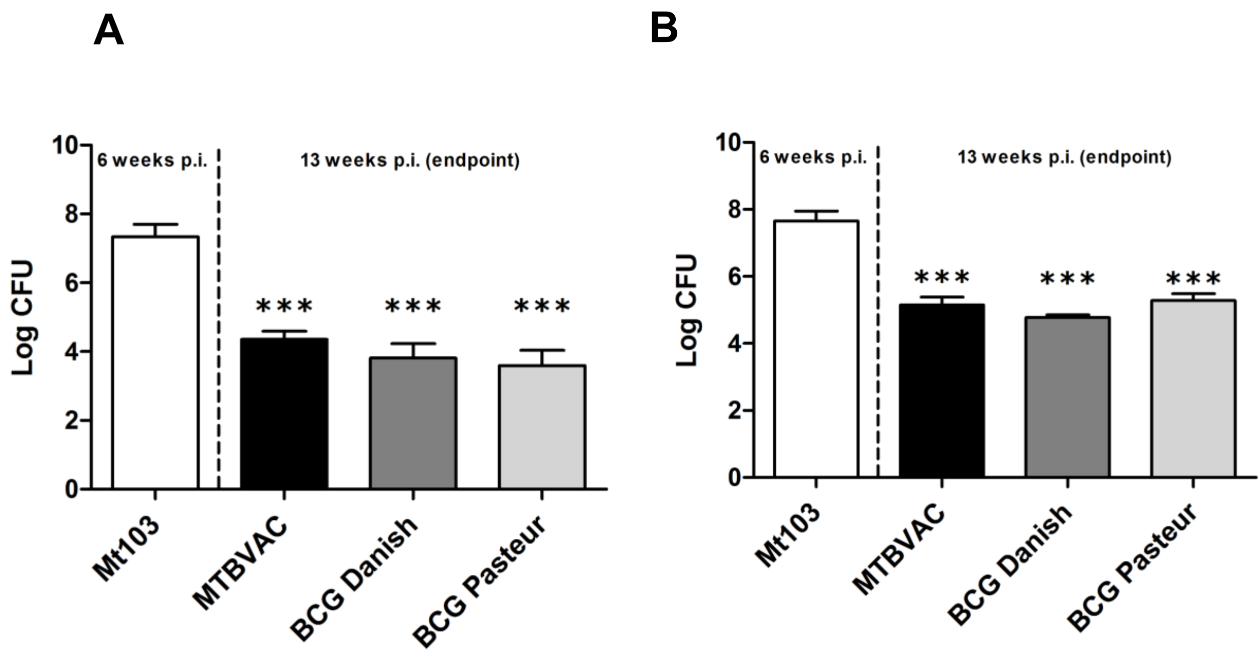

Figure 3

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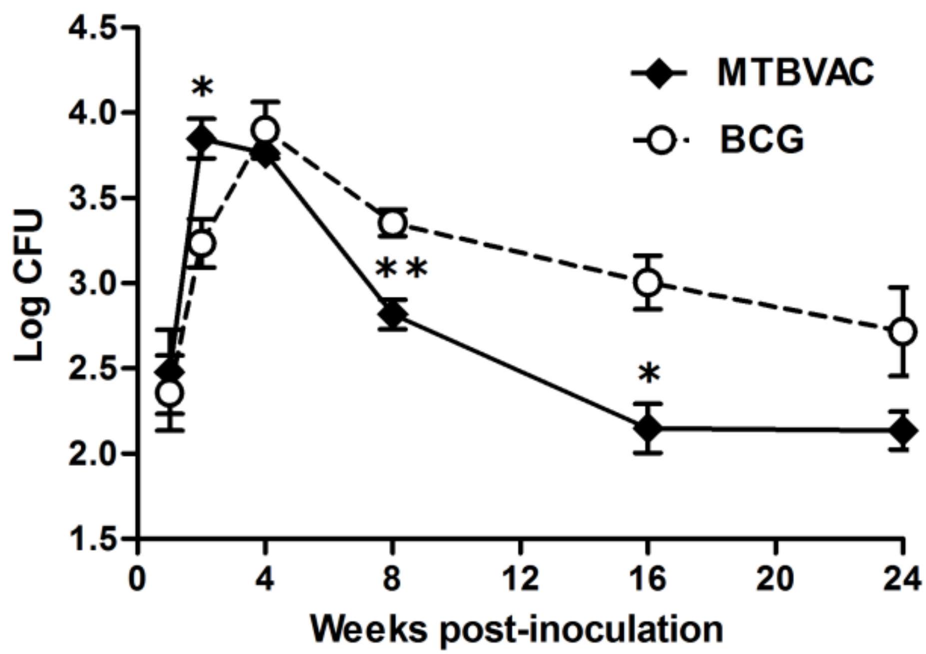

Figure 4

Arbues *et al*

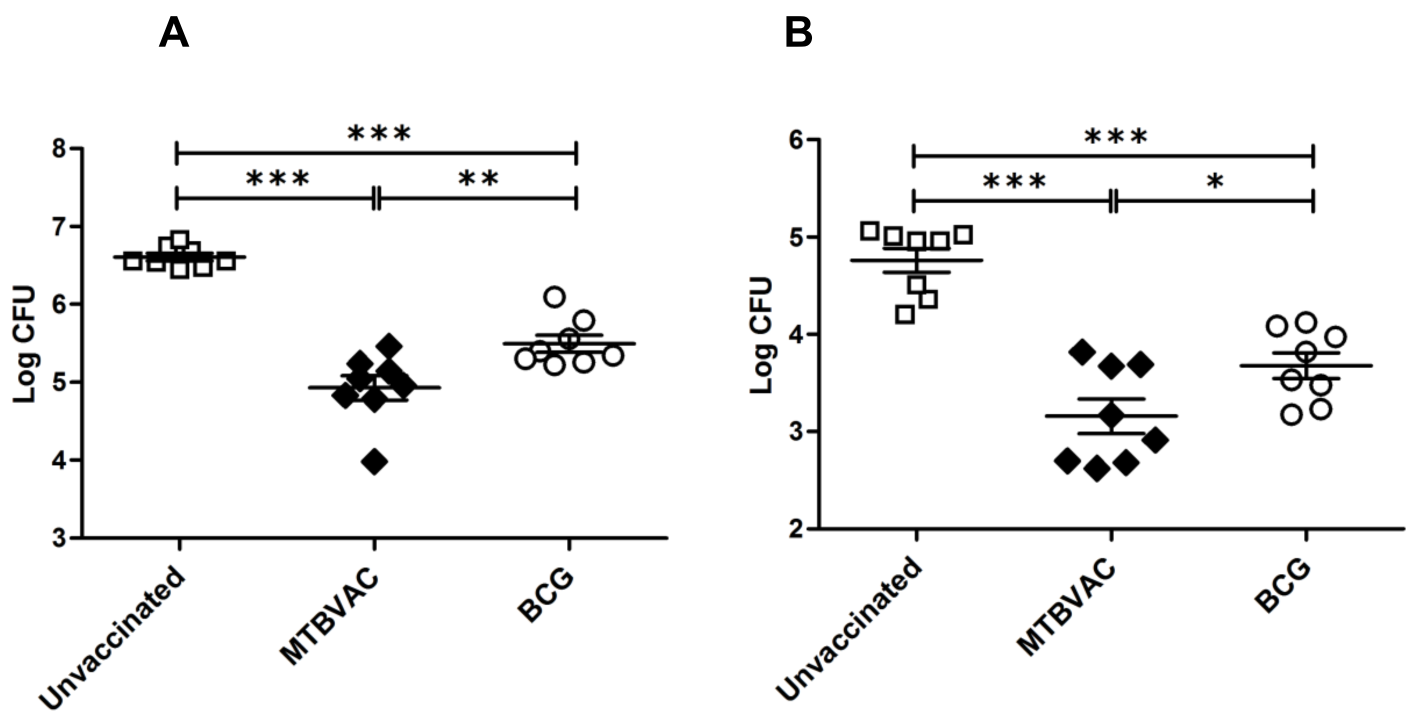

Figure 5

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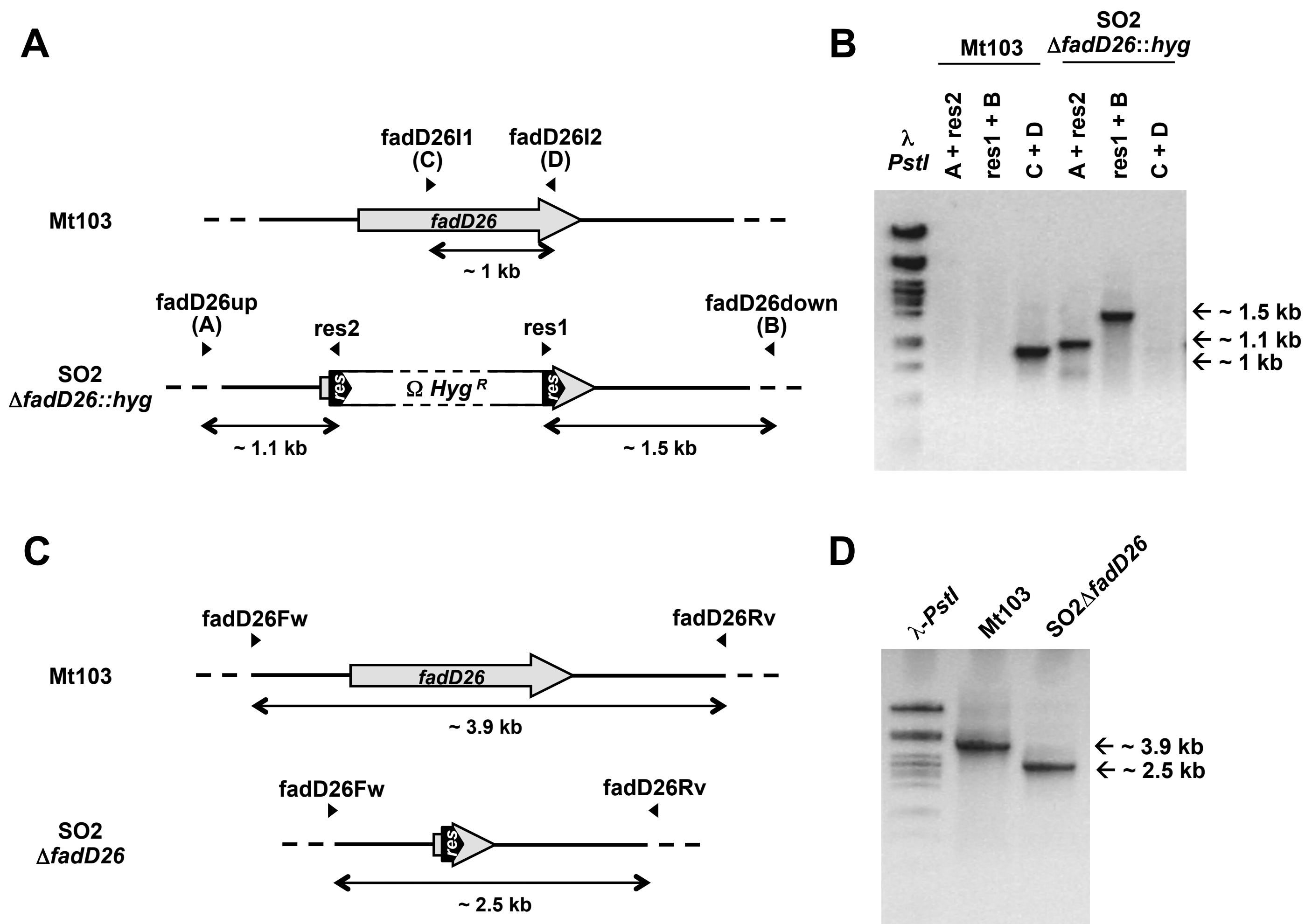


Figure S1

Arbues *et al*

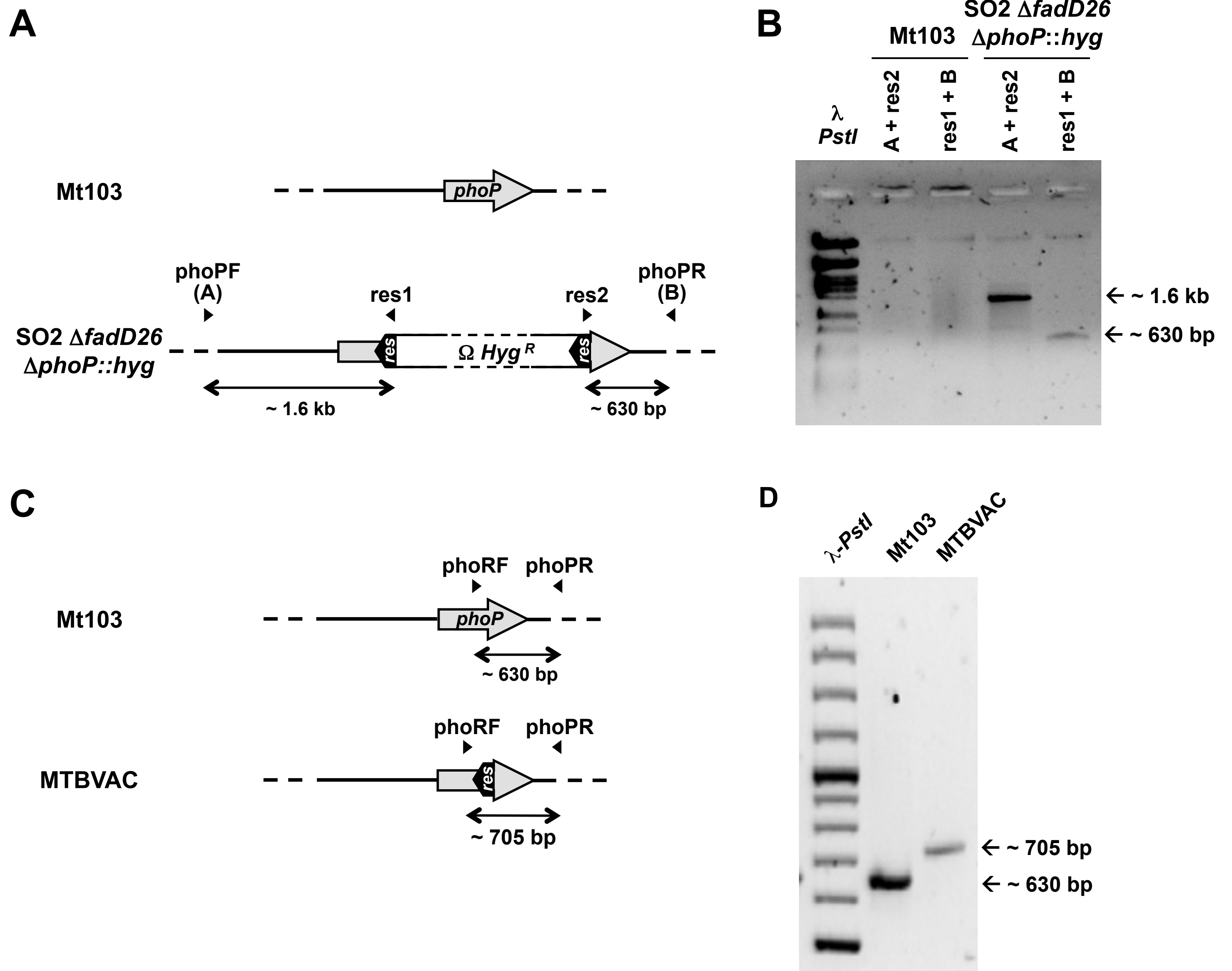
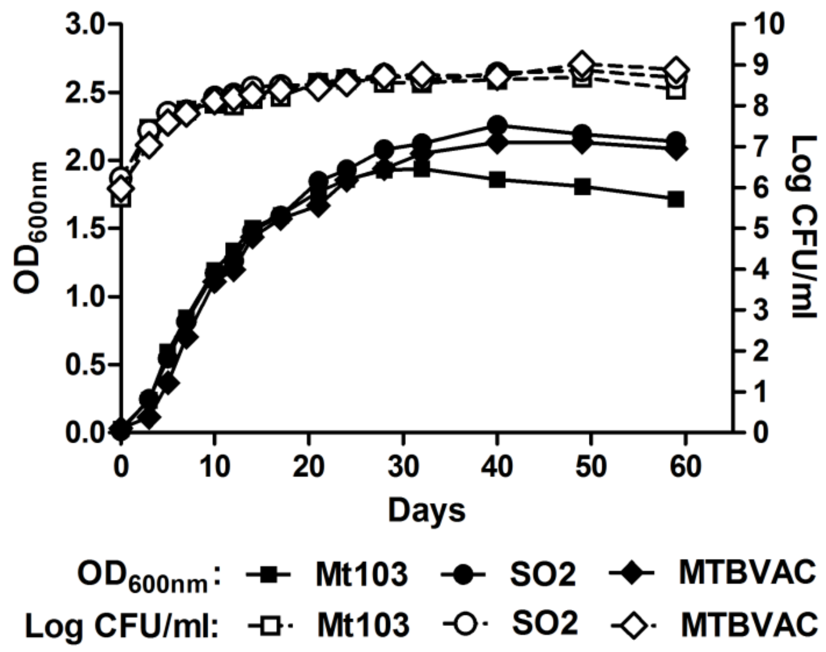


Figure S2

Arbues *et al*

A



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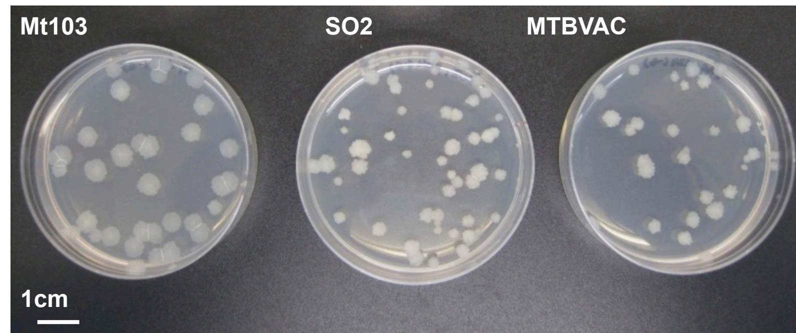

Figure S3

Arbues *et al*

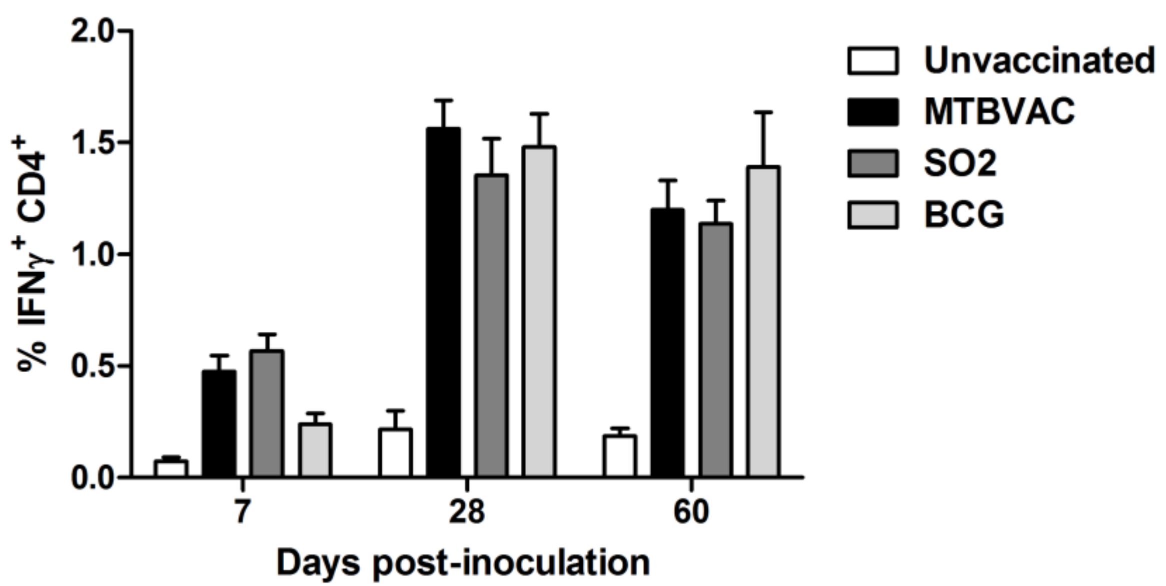

Figure S4

Arbues *et al*