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1 ***Mycobacterium avium* subsp. *paratuberculosis*-specific *mpt* operon expressed**  
2 **in *M. bovis* BCG as vaccine candidate**

3

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24 **Abstract**

25 *Mycobacterium (M.) avium* subspecies *paratuberculosis* is the etiological agent of  
26 Paratuberculosis (Johne's disease) in ruminants. Vaccination against  
27 Paratuberculosis with an attenuated live vaccine has been shown to prevent or  
28 reduce disease symptoms but also to have severe side effects. In contrast, the  
29 tuberculosis vaccine strain *M. bovis* BCG is considered safe and the efficacy of  
30 vaccination with *M. bovis* BCG transformants carrying foreign antigens has been  
31 shown in several studies. The *mpt* genes of *M. avium* subsp. *paratuberculosis* are  
32 part of a putative pathogenicity island and have been described as possible virulence  
33 determinants. In this study we show that the *mpt* genes are transcribed on a single  
34 polycistronic mRNA in *M. avium* subsp. *paratuberculosis*. We cloned the entire *mpt*  
35 operon, transformed it into *M. bovis* BCG Pasteur using the integrative vector  
36 pMV306 and show transcription and expression of the *mpt* genes in the *M. bovis*  
37 BCG transformant. In a challenge experiment with Balb/c mice we demonstrate that  
38 immunization with *M. bovis* BCG expressing the *M. avium* subsp. *paratuberculosis*-  
39 derived *mpt* operon significantly reduces amplification of *M. avium* subsp.  
40 *paratuberculosis* in liver and spleen of the host in comparison to both the mock-  
41 immunized animals as well as the *M. bovis* BCG-immunized control. These findings  
42 imply that immunization with *M. bovis* BCG transformants may constitute a new  
43 strategy in the development of an efficacious and safe vaccine against  
44 paratuberculosis.

## 45 1. Introduction

46 Paratuberculosis (Johne's disease) is a progressive, incurable enteritis of  
47 ruminants caused by *Mycobacterium (M.) avium* subsp. *paratuberculosis*; it is  
48 responsible for severe economic losses in the livestock industry (Kreeger, 1991,  
49 Harris and Barletta, 2001) and has been associated with human Crohn's Disease  
50 (Feller et al. 2007). Vaccination reduces clinical symptoms and shedding of the  
51 pathogen but can cause adverse reactions and interference with both  
52 paratuberculosis serology and bovine tuberculosis skin testing (Patterson et al.,  
53 1988, Kohler et al., 2001).

54 In contrast, *M. bovis* BCG being the most frequently used vaccine worldwide is  
55 considered safe for humans and the environment (Bloom and Fine, 1994, Dietrich et  
56 al., 2003). It has been used only experimentally in animals (Buddle et al., 2005 and  
57 Lesellier et al., 2006), and the potential of recombinant *M. bovis* BCG Pasteur (rBCG)  
58 as a vaccine against bacterial and viral infections has been elucidated exemplary in  
59 several studies (Pym et al., 2003, Stover et al., 1993, Dennehy et al., 2007). .

60 The *M. avium* subsp. *paratuberculosis*-specific *mpt*-genes are encoded on a  
61 putative 38kb pathogenicity island and the MptD-protein was found to be surface-  
62 exposed and to be expressed during infection (Stratmann et al., 2004). Additionally it  
63 has been indicated as a possible virulence determinant in a recent study (Shin et al.,  
64 2006).

65 In the study presented here we cloned the *M. avium* subsp. *paratuberculosis*  
66 *mpt* genes, elucidated their genetic organization, constructed an *M. bovis* BCG  
67 transformant carrying the *mpt* operon on an integrative vector on the genome,  
68 investigated recombinant *mpt* gene expression, and examined the potential of this  
69 strain as a vaccine candidate against paratuberculosis in a mouse infection model.

## 70 2. Materials and Methods

71

### 72 2.1 Bacterial strains, plasmids, primers and peptide.

73 The bacterial strains, plasmids, primers, and the peptide used in this study are  
74 listed in Tables 1 and 2.

75

### 76 2.2 Media and growth conditions

77 *Mycobacterium avium* subsp. *paratuberculosis* was cultured either on  
78 Middlebrook 7H10 Agar (MB agar; Difco Laboratories Detroit, Michigan)  
79 supplemented with 1 x OADC (10 x OADC is 0.85 % (w/v) NaCl, 5 % (w/v) bovine  
80 serum albumin fraction V, 2 % (w/v) dextrose, 0.003 % (w/v) catalase and 0.06 %  
81 (v/v) oleic acid (all Roth, Karlsruhe, Germany), 0.5 % (v/v) glycerol, and 2  $\mu\text{g ml}^{-1}$   
82 mycobactin J (Synbiotics, Lyon, France) or in Middlebrook 7H9 Broth supplemented  
83 with 1 x OADC, 2.5 % (v/v) glycerol, 0.4 % (v/v) Tween<sup>®</sup>80, and 2  $\mu\text{g ml}^{-1}$  mycobactin  
84 *J. M. bovis* BCG was either grown on Middlebrook 7H10 Agar supplemented with 0.5  
85 % (v/v) glycerol, 1 x ADS (10 x ADS is 5 % bovine serum albumin fraction V, 2 %  
86 dextrose, and 0.81 % NaCl), or in Middlebrook 7H9 broth supplemented with 1 x  
87 ADS, 0.5 % glycerol (v/v), and 0.05 % (v/v) Tween<sup>®</sup>80. Cultures of *M. bovis* BCG  
88 transformants were additionally supplemented with 50  $\mu\text{g ml}^{-1}$  hygromycin B (Roche,  
89 Mannheim, Germany). Mycobacteria were either cultivated on agar plates sealed in  
90 plastic bags or in tightly closed glass bottles with slight stirring at 37 °C. *E. coli*  
91 transformants were cultivated in Luria-Bertani medium supplemented with  
92 hygromycin B (50  $\mu\text{g ml}^{-1}$ ) at 37 °C.

93

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95

96 2.3 Cloning of the *mpt* operon and construction of a *M. bovis* BCG transformant

97 A genomic library of *M. avium subsp. paratuberculosis* was generated by  
98 partial Sau3AI digest and ligation in vector pGH433. Two clones each carrying a part  
99 of the *mpt* operon (pGH1 and pGH7) were identified by DNA Dot Blot. The *mpt*-gene  
100 cluster was assembled using the BglII site in the *mpt*-gene cluster. A BglII/NheI  
101 fragment of pGH7 was ligated into BglII/SphI restricted pGH1 using a NheI/SphI  
102 linker thereby generating plasmid pMP401. Plasmid pMP401 contained the entire  
103 *mpt*-gene cluster including the the putative promoter region on an NheI fragment.  
104 Plasmid pMP1102 was constructed by transferring the 8,383 bp NheI fragment from  
105 plasmid pMP401 into the integrative *Escherichia coli*-mycobacteria shuttle-vector  
106 pMV306 (carrying a hygromycin resistance gene as selectable marker) restricted with  
107 XbaI. *M. bovis* BCG Pasteur was transformed with plasmid pMP1102 by  
108 electroporation (Pavelka and Jacobs, 1999). Transformants were grown on  
109 Middlebrook 7H10 Agar with the required supplements and 50 µg ml<sup>-1</sup> hygromycin B  
110 at 37 °C for approximately 4 weeks. Successful transformation was confirmed by  
111 PCR and Southern Blot analyses, the stability of transformants was repeatedly  
112 confirmed by PCR after reisolation from mice (data not shown).

113

114 2.4 RNA isolation and RT PCR

115 For RNA preparation mycobacterial cultures with an optical density of 1 at  
116 600 nm (OD<sub>600</sub>=1) were harvested by centrifugation (7,000 x g, 15 min). RNA was  
117 isolated using the FastRNA<sup>®</sup> Blue Kit (Qbiogene, Heidelberg, Germany), using a  
118 FastPrep<sup>®</sup> instrument (Qbiogene). RNA was purified using the RNeasy Mini Kit  
119 (Qiagen, Hilden, Germany). For subsequent RT-PCR applications RNA was treated  
120 with the TurboDNA-free<sup>™</sup> Kit (Ambion, Austin, USA). Reverse Transcription was  
121 performed with SuperScript<sup>™</sup>II Reverse Transcriptase (Invitrogen, Karlsruhe,

122 Germany) using 5 µg of RNA and 5 pmol of the respective gene-specific reverse  
123 primer in each reaction (Table 2). Reactions without reverse transcriptase served as  
124 controls. When necessary, a positive control utilizing 16s RNA-specific primer o16rev  
125 was used. The cDNA generated served as template for subsequent PCR reactions  
126 using the primer pairs listed in Table 2.

127

## 128 2.5 Peptide aMptD-mediated capture PCR

129 Expression of recombinant protein on the surface of the *M. bovis* BCG  
130 transformants was investigated applying the peptide aMptD-mediated capture PCR  
131 (Stratmann et al., 2004) with some modifications. Briefly, suspensions of  
132  $10^5$  CFU ml<sup>-1</sup> of *M. bovis* BCG transformed with pMP1102 and *M. bovis* BCG  
133 transformed with pMV306 (negative control) in 1 ml homogenized milk were  
134 incubated with aMptD-coupled paramagnetic beads (10 µg; Chemicell, Berlin,  
135 Germany). In order to prevent unspecific binding a 100- and 1000-fold excess of *M.*  
136 *avium* subsp *avium* was added. The mixtures were incubated with slight agitation  
137 overnight at 4°C. Washing of the beads and release of mycobacterial DNA was  
138 performed as described (Stratmann et al., 2006), and PCR reactions were performed  
139 using primers specific for mycobacteria from the *M. tuberculosis*-complex (Rodriguez  
140 et al., 1995).

141

## 142 2.6 Vaccination and challenge trial

143 Female Balb/cAnNCrl mice, three to four weeks of age, were obtained from  
144 Charles River WIGA (Deutschland) GmbH (Sulzfeld, Germany) and housed in  
145 standard plastic cages with free access to water and standard mouse chow. Animals  
146 were cared for according to the principles outlined by the European Convention for  
147 the Protection of Vertebrate Animals Used for Experimental and Other Scientific

148 Purposes (European Treaty Series no. 123 [http://conventions.coe.int/](http://conventions.coe.int/treaty/EN/V3menutraits.asp)  
149 [treaty/EN/V3menutraits.asp](http://conventions.coe.int/treaty/EN/V3menutraits.asp); permit no. 33-42502-04/817). Mice were randomly  
150 assigned to three groups of eight mice each. The time scheme for the vaccination  
151 trial and immunization and challenge doses were adopted with minor modifications  
152 from previous studies (Dennehy and Williamson, 2005, Huntley et al., 2005,). Briefly,  
153 bacteria were washed three times with phosphate-buffered saline (PBS; 150 mM  
154 NaCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 9 mM Na<sub>2</sub>HPO<sub>4</sub>, and 2.5 mM KCl [pH 7.2]); all mice were  
155 immunized intraperitoneally with 100 µl PBS (group 1), *M. bovis* BCG pMV306  
156 transformants (1 x 10<sup>7</sup> colony forming units [cfu]; group 2), and *bovis* BCG pMP1102  
157 transformants (1 x 10<sup>7</sup> cfu; group 3). Thirty days later animals were infected i.p. with  
158 5 x 10<sup>7</sup> cfu of *M. avium* subsp. *paratuberculosis* strain 6783 in 100 µl PBS. Four  
159 months after infection mice were sacrificed by cervical dislocation and weighed.  
160 Spleens and livers were removed aseptically and also weighed. To determine the cfu  
161 counts 100 mg of liver tissue (containing pieces from all liver lobes) and  
162 approximately 50 mg of spleen (the spleen was cut lengthwise) were homogenized  
163 with glass beads (3 mm in diameter, 6 beads per tube) in 5 volumes of saline in a  
164 Fast Prep<sup>®</sup> instrument (Qbiogene) for thirty seconds at intensity setting 4.0, and  
165 serial 10-fold dilutions were plated on supplemented MB agar. Statistical significance  
166 of different cfu counts obtained for each immunization group was determined using  
167 the Wilcoxon signed-rank test.

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174 **3. Results**

175

176 3.1 Transcriptional organization of the *mpt*-genes

177 To elucidate the transcriptional organization of the *mpt*-genes in *M. avium*  
178 subsp. *paratuberculosis* the regions between the predicted *mpt*-genes (Stratmann et  
179 al., 2004) were amplified by RT-PCR. PCR products were obtained for all intergenic  
180 regions, indicating that the *mpt*-genes are transcribed on a single polycistronic mRNA  
181 (Fig. 1).

182

183 3.2 Expression of the *mpt* operon in *M. bovis* BCG

184 In order to determine transcription of the *mpt* operon RT-PCR reactions were  
185 performed using total RNA from the *M. bovis* BCG pMP1102 and *M. bovis* BCG  
186 pMV306 transformants. Transcripts for *mptAB* to *mptD* were present only in *M. bovis*  
187 BCG pMP1102 transformants. 16sRNA transcripts were equally detected in all  
188 samples (Fig. 2 A).

189 To further investigate the expression of *mpt*-genes in the *M. bovis* BCG transformant  
190 a peptide aMptD-mediated capture PCR (Stratmann et al., 2006) was performed.  
191 After overnight capture, only *M. bovis* BCG pMP1102 transformants were detected  
192 by PCR analysis thereby showing translation of the *mpt*-genes and surface  
193 expression in the *M. bovis* BCG transformant (Fig. 2 B).

194

195 3.3 Immunization and challenge in Balb/c mice

196 Initially, persistence and growth of *M. avium* subsp. *paratuberculosis* strain  
197 6783 upon intraperitoneal infection ( $5 \times 10^7$  cfu per mouse) in mice was confirmed; the  
198 strain was reisolated from spleens and livers of infected mice four months after

199 infection. In contrast *M. bovis* BCG could only be reisolated from spleens of infected  
200 mice (data not shown).

201 In the challenge trial animals were sacrificed four months after challenge, and  
202 persistence and growth of *M. avium* subsp. *paratuberculosis* was determined by  
203 quantifying the number of cfu in liver and spleen (Fig. 3). The mock-immunized  
204 animals showed high levels of bacterial infection of  $1.83 \times 10^6$  cfu/g (liver) and  
205  $9.36 \times 10^5$  cfu/g (spleen) confirming the virulence of *M. avium* subsp.  
206 *paratuberculosis* strain 6783 in Balb/c mice when delivered via the intraperitoneal  
207 route. Immunization with the vector control (*M. bovis* BCG pMV306 transformants)  
208 reduced the number of *M. avium* subsp. *paratuberculosis* at reisolation significantly to  
209  $5.5 \times 10^4$  cfu/g in liver ( $p= 0,012$ ) and  $3.68 \times 10^4$  cfu/g in spleen ( $p=0,012$ ).  
210 Vaccination with the *M. bovis* BCG pMP1102 transformant further reduced the  
211 bacterial load to  $8.25 \times 10^3$  cfu/g in liver ( $p=0.036$ ) and  $6.03 \times 10^3$  cfu/g in spleen  
212 ( $p=0.025$ ) in comparison to the vector control. Both transformants were reisolated  
213 from the spleens of all respective animals 120 days after immunization and  
214 differentiated from *M. avium* subsp. *paratuberculosis* by their hygromycin resistance,  
215 their faster and mycobactin J-independent growth with distinct colony morphology  
216 and confirmed by PCR. The consistent presence of the integrative plasmid pMP1102  
217 in *M. bovis* BCG was also determined by PCR with *mptD*-specific primers oABC5 and  
218 oABC6 (Table 1; data not shown).

219

220

#### 221 **4. Discussion**

222

223 *Mycobacterium bovis* BCG is the live vaccine that has been used most  
224 frequently worldwide without causing significant side effects and, therefore, is

225 considered safe. More recently, it has also been used to vaccinate badgers against  
226 *M. bovis* lung infection and, thereby, reduce the wildlife reservoir (Lesellier et al.,  
227 2006). In cattle, *M. bovis* BCG has been used experimentally to protect from bovine  
228 tuberculosis (Buddle et al., 2005). A use in commercial cattle populations so far is  
229 prevented by the inability to discriminate between *M. bovis*-infected and *M. bovis*  
230 BCG-vaccinated animals. However, recent work by Hewison and coworkers offered a  
231 possibility to resolve this problem (Hewinson et al., 2006). Thus, the group identified  
232 skin testing-active immunogenic antigens present in *M. bovis* but not in *M. bovis* BCG  
233 which, therefore, would be suitable as negative marker antigens to differentiate BCG-  
234 immunized from *M. bovis*-infected animals (DIVA principle; van Oirschot, 2001).  
235 Another possible drawback of using *M. bovis* BCG as a vector vaccine is the  
236 presence of a protective *M. bovis* immune response in animals to be vaccinated  
237 which might cause a premature inactivation of the live vaccine strain and, thereby,  
238 prevent a protective immune response against the recombinant antigen. Since these  
239 drawbacks can be overcome or do not apply in most countries with a severe  
240 paratuberculosis problem and since *M. bovis* BCG transformants have been shown  
241 to be highly efficacious as vaccine carriers (Stover et al., 1991, Stover et al., 1993,  
242 Nasser and Kaufmann, 2005, Dennehy et al., 2007) we decided to use it in an  
243 investigation of the protective efficacy of the *M. avium* subsp. *paratuberculosis*-  
244 specific Mpt proteins. Since the transcriptional and regulatory organization of the *mpt*  
245 genes was not known we first confirmed that the *mpt*-genes are cotranscribed as an  
246 operon. In order to prevent detrimental effects and ascertain a correct localization  
247 and folding of the Mpt proteins we used the integrative vector pMV306hyg not  
248 carrying a foreign promoter. We confirmed transcription of the *mpt*-operon, and the  
249 correct localization and folding was supported by the peptide aMptD-mediated  
250 capture of *M. bovis* BCG pMP1102 transformants. Also, stability of the transformants

251 in the host was confirmed by the finding that *M. bovis* BCG pMP1102 transformants  
252 tested after four months in mice without any selective pressure still contained the *mpt*  
253 operon.

254 The protective efficacy achieved through immunization with *M. bovis* BCG  
255 alone is most likely due to cross-protection between mycobacterial species as it has  
256 been reported previously (Collins, 1971, Young et al., 2007) and is considered to be  
257 a major hindrance in vaccinating against human tuberculosis in developing countries  
258 (Brandt et al., 2002). The effect is also in accordance with results of Rosseels and  
259 co-workers (2006) showing that the protective efficacy against *M. avium* subsp.  
260 *paratuberculosis* infection in mice was similar for a commercial *M. bovis* BCG vaccine  
261 and  $\gamma$ -irradiated *M. avium* subsp. *paratuberculosis* ATCC 19698 (Rosseels et al.,  
262 2006). These findings of Rosseels et al. made it possible to forgo an additional  
263 control group immunized with either a commercially available paratuberculosis  
264 vaccine or inactivated *M. avium* subsp. *paratuberculosis* in this study for animal  
265 welfare reasons. The improved protective efficacy observed upon immunization of  
266 mice with the *M. bovis* BCG pMP1102 transformant in comparison to *M. bovis* BCG  
267 alone strongly suggests that proteins encoded on the *mpt* operon are responsible for  
268 this difference. The protective efficacy of the *M. bovis* BCG pMP1102 transformant is  
269 supported by the fact that it is comparable to the effect achieved in an immunization  
270 trial in mice with an expression library of *M. avium* subsp. *paratuberculosis* (Huntley  
271 et al., 2005).

272 In the future, it remains to be determined whether vaccination with the *M.*  
273 *bovis* pMP1102 transformant would also be efficacious in cattle not only preventing  
274 clinical symptoms but also decreasing fecal shedding as described by Koets et. al  
275 (2006) for an Hsp70-based subunit vaccine. In addition, a definition of the protective  
276 Mpt antigen could lead to an improvement of vaccination efficacy as it might facilitate

277 a selectively increased expression in rBCG which has been suggested to correlate  
278 with immunogenicity (Himmelrich et al., 2000).

279

280

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282

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287

288

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391 Table 1. Strains and plasmids used in this study  
 392  
 393

Strain or plasmid	Characteristics <sup>1</sup>	Source and/or reference
<b>Strains</b>		
<i>M. avium</i> subsp. <i>paratuberculosis</i> 6783	Clinical isolate, DSM44135	University of Veterinary Medicine Hannover, Germany
<i>M. bovis</i> BCG Pasteur	Vaccine strain	Statens Serum Institut, Copenhagen, Denmark
<i>Escherichia coli</i> HB101	K-12 derivative, <i>supE44</i> , <i>hsd20</i> , <i>r<sub>B</sub>M<sub>B</sub></i> , <i>recA13</i> , <i>ara-14</i> , <i>proA2</i> , <i>lacY1</i> , <i>galK2</i> , <i>rpsL20</i> , <i>xyl-5</i> , <i>mtl-1</i>	(Boyer and Roulland-Dussoix, 1969)
<b>Plasmids</b>		
pGH433 <i>lacI</i>	<i>Escherichia coli</i> vector carrying an ampicillin resistance gene, the <i>lacI</i> gene, and a tac promoter	Advanced Vectors Inc., Hopkins, USA
pMV306hyg	<i>Escherichia coli</i> – mycobacteria shuttle vector carrying a hygromycin resistance gene as selectable marker and an <i>att</i> -site for integration into the mycobacterial genome	W.R. Jacobs, Albert Einstein College of Medicine, New York, USA
pGH1	Sau3AI fragment from <i>M. avium</i> subsp. <i>paratuberculosis</i> in pGH433 <i>lacI</i> including part of the <i>mpt</i> operon (GenBank pos. no. 4152686 to 4159895)	this work
pGH7	Sau3AI fragment from <i>M. avium</i> subsp. <i>paratuberculosis</i> in pGH433 <i>lacI</i> including part of the <i>mpt</i> operon (GenBank pos. no. 4151394 to 4156122)	this work
pMP401	BglII/NheI fragment of pGH7 ligated into SphI/BglII restricted pGH1 using a NheI/SphI linker assembling the entire <i>mpt</i> -gene cluster by using the internal BglII site (Genbank pos. no. 4154112)	this work
pMP1102	8,383 bp NheI fragment of pMP401 containing the <i>mpt</i> -gene cluster including the preceding potential Fur-box (GenBank pos. no. 4151394 to 4159777) ligated into XbaI restricted pMV306hyg	this work

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<sup>1</sup>) The position numbers given relate to GenBank accession no. NC 002944

396 Table 2. Primers and peptide used in this study  
 397

Primer or peptide	Description or sequence (5' to 3') <sup>1)</sup>	Source and/or reference
<b>Primers</b>		
oMptAfw	CATTCCATCGTGGAAC ( <i>mptA</i> derived forward primer situated at position 1708-1723)	this work
oMptBrev	CACTGCATGAACTCTG ( <i>mptB</i> derived reverse primer situated at position 1868-1883)	this work
oMptBfw	CGAACAAGGCACCCATTTTCG ( <i>mptB</i> derived forward primer situated at position 2961-2980)	this work
oMptCrev	CGTATCAACCGGCTTGTGGA ( <i>mptB</i> derived reverse primer situated at position 3217-3236)	this work
oMptCfw	CCGATCAGATACTGGTGCTC ( <i>mptA</i> derived forward primer situated at position 4681-4700)	this work
oMptDrev	CACGTTGATCGCGAACACAG ( <i>mptB</i> derived reverse primer situated at position 4956-4975)	this work
oMptDfw	CTAATCGCATTTCGACCTGTC ( <i>mptA</i> derived forward primer situated at position 5375-5394)	this work
oMptErev	GTAGTAGGTAAGCGACTGC ( <i>mptB</i> derived reverse primer situated at position 5675-5692)	this work
oMptEfw	CTTATAGCGTCGAGCCTTC ( <i>mptA</i> derived forward primer situated at position 6004-6022)	this work
oMptFrev	CGTATGTGCAAGTCCACTC ( <i>mptB</i> derived reverse primer situated at position 6244-6262)	this work
16sfwd	CCTACGGGAGGCAGCAGT	(Granger et al., 2004)
16srev	CGGGCCCCCGTCAAT	
JB21	TCGTCCGCTGATGCAAGTGC	(Rodriguez et al., 1995)
JB22	CGTCCGCTGACCTCAAGAAG	
oABC5	TAG CTC GAC GAC CAA GTC CA	(Stratmann et al., 2006)
oABC6	TCA GCC CGA ATA CCA CCG TG	
<b>Peptide</b>		
aMptD	GKNHHHQHHRPQ	(Stratmann et al., 2006)

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<sup>1)</sup> The position numbers given relate to GenBank accession no. AF419325

400 **Figure Legends**

401

402 Fig. 1 Transcriptional organization of the *mpt* operon in *M. avium* subsp.  
 403 *paratuberculosis* determined by amplification of the transcribed regions between the  
 404 predicted individual open reading frames *mptAB* (A), *mptB* and *mptC* (B), *mptC* and  
 405 *mptD* (C), *mptD* and *mptE* (D), *mptE* and *mptF* (E). Lanes 1 - 3 correspond to  
 406 dilutions of the respective cDNAs with (1) undiluted, (2) 1:10 dilution, (3) 1:100  
 407 dilution. Lanes 4 - 6 show the respective dilutions without Reverse Transcriptase;  
 408 Lane 7 shows the positive control (chromosomal DNA of *M. avium* subsp.  
 409 *paratuberculosis* 6783), lane 8 is the negative control (water).

410

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413 Fig. 2 Expression of *mpt*-genes in *M. bovis* BCG transformants. (A) Transcription of  
 414 *mptABCD* in *M. bovis* BCG pMP1102 (lanes 1, 3, 5, 7) and pMV306 (lanes 2, 4, 6, 8)  
 415 transformants; cDNA is amplified with primers oMptBfw and oMptCrev (lanes 1, 2),  
 416 oMptCfw and oMptDrev (lanes 3, 4), o16Sfw and o16Srev (lanes 5, 6). Lanes 7 and  
 417 8 contain the negative controls (RNA without reverse transcriptase amplified with  
 418 o16Sfw and o16Srev). The numbers to the left indicate the size of the PCR products  
 419 in bp. (B) Peptide aMptD-mediated capture PCR of *M. bovis* BCG pMP1102 (lane 1,  
 420 2) and pMV306 (lanes 3, 4) transformants; milk was spiked with  $10^5$  cfu *M. bovis*  
 421 BCG transformants and a 100-fold (lanes 1, 3) or 1000-fold (lanes 2, 4) excess of *M.*  
 422 *avium* subsp. *avium*; PCR was performed with primers JB21 and JB22 with total  
 423 lysates of *M. bovis* BCG pMP1102 (lane 5) and pMV306 (lane 6) transformants  
 424 serving as controls.

425

426 Fig. 3 Reisolation of *Mycobacterium avium* subsp. *paratuberculosis* from livers and  
427 spleens of rBCG immunized Balb/c mice. Mice were sacrificed four months after  
428 intraperitoneal infection with *M. avium* subsp. *paratuberculosis*. The number of cfu  
429 was determined in individual organ homogenates by plating; the cfu per gram organ  
430 was calculated and depicted on a logarithmic scale. Arithmetic means are indicated  
431 by horizontal bars; asterisks indicate statistical significance ( $p < 0.05$ ) in the Wilcoxon  
432 signed-rank test.

Figure 1

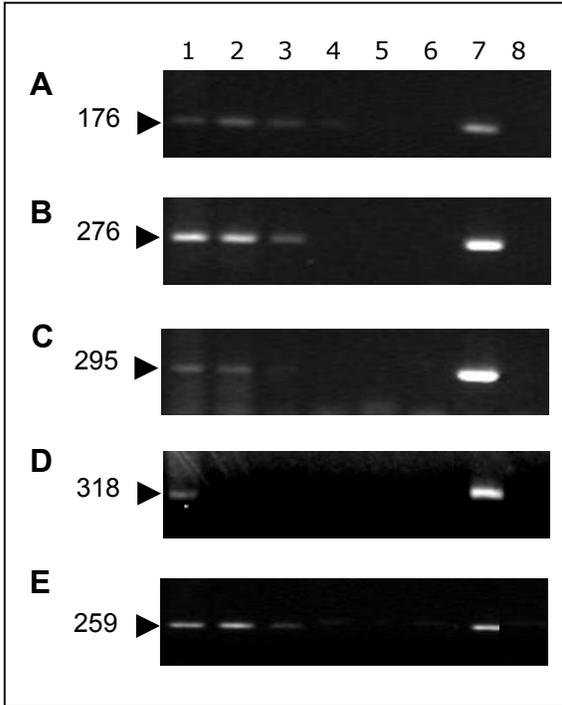


Figure 2

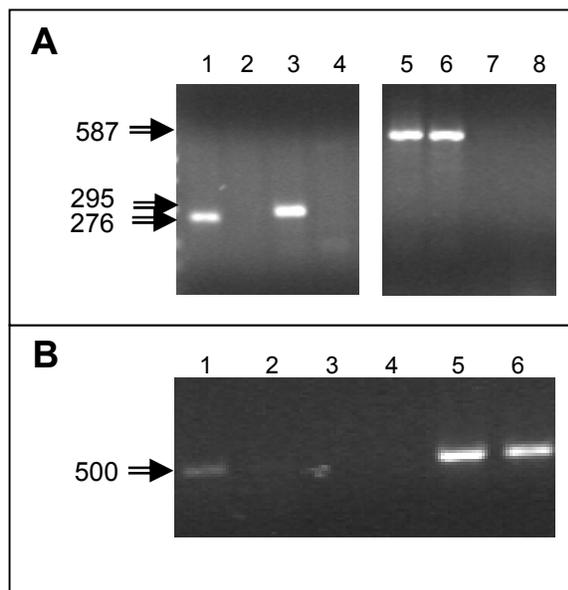


Figure 3

