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2 Differential transcription of the major antigenic protein 1 multigene family of
3 *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks
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1 **Abstract**

2

3 The rickettsial pathogen *Ehrlichia ruminantium* causes heartwater in ruminants and is transmitted by
4 ticks of the genus *Amblyomma*. The *map1* gene, encoding the major surface protein MAP1, is a
5 member of a multigene family containing 16 paralogs. In order to investigate differential transcription
6 of genes of the *map1* multigene family *in vivo* in unfed and feeding ticks, RNA was extracted from
7 midguts and salivary glands of *E. ruminantium*-infected adult female *Amblyomma variegatum* ticks and
8 analysed by RT-PCR using MAP1 paralog-specific primers. In unfed ticks, only transcripts from the
9 *map1-1* gene were observed in midguts and no transcripts were detected in salivary glands. In feeding
10 ticks, *map1-1* transcripts were more abundant in midguts whereas high levels of *map1* transcripts were
11 observed in salivary glands. Our results show that differential transcription of genes of the *E.*
12 *ruminantium map1* cluster occurs *in vivo* in different tissues of infected ticks before and during
13 transmission feeding, indicating that this multigene family may be involved in functions of biological
14 relevance in different stages of the life cycle of *E. ruminantium*.

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16 **Keywords:** *Ehrlichia ruminantium*; MAP1; gene expression; multigene families; *Amblyomma*
17 *variegatum*.

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1 Introduction

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3 The rickettsial pathogen *Ehrlichia ruminantium* is the causative agent of heartwater or cowdriosis. The
4 disease affects domestic and wild ruminants and occurs throughout sub-Saharan Africa and also on
5 some islands in the Caribbean (Uilenberg, 1983). The organism is transstadially transmitted by ticks of
6 the genus *Amblyomma* (Bezuidenhout, 1987).

7 Multigene families encoding for outer membrane proteins have been identified in the genus *Ehrlichia*
8 and designated P28 (OMP-1) in *Ehrlichia chaffeensis*, P30 in *Ehrlichia canis* and the major antigenic
9 protein 1 (MAP1) family in *E. ruminantium* (Ohashi et al., 1998a, 1998b; Sulsona et al., 1999). These
10 genes are thought to be responsible for adaptation to the environment in the tick and involved in
11 transmission to the mammalian host (Felek et al., 2003; Unver et al., 2002).

12 Recently, the entire *map1* cluster of the Welgevonden isolate of *E. ruminantium* was characterised and
13 shown to contain 16 genes located upstream of a *secA* gene (van Heerden et al., 2004). All 16 paralogs
14 were transcriptionally active when *E. ruminantium* was grown in bovine endothelial cells, whereas
15 between 4 and 11 paralogs were found to be transcribed in *E. ruminantium*-infected tick cell lines
16 (Bekker et al., 2005; van Heerden et al., 2004) and transcripts of only two out of three paralogs were
17 detected in unfed infected adult *Amblyomma variegatum* ticks (Bekker et al., 2002). In the present
18 study, we analysed transcription of the entire *map1* cluster in midguts and salivary glands of *E.*
19 *ruminantium*-infected *A. variegatum* ticks and assessed levels of transcription of *map1* genes in relation
20 to the numbers of organisms present in different tissues of unfed and feeding ticks.

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1 **Materials and methods**

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3 **Laboratory propagation of ticks.**

4 *A. variegatum* were obtained from the Centre de Coopération internationale en Recherche agronomique
5 pour le Développement (CIRAD-EMVT), Pointe-à-Pitre, Guadeloupe, and established as an *E.*
6 *ruminantium*-free laboratory colony at Utrecht University. Larvae and nymphs were fed on rabbits,
7 and adults were fed on sheep. The engorged, moulting and unfed stages were maintained at 27 °C, 95
8 % relative humidity (Heyne et al., 1987).

9

10 **Experimental infection of sheep and ticks.**

11 Naïve female Texelaar sheep, aged between 6 and 8 months, were maintained at Utrecht University
12 under the regulations of the Animal Ethics Committee (Dierexperimentencommissie) of the University.
13 The sheep were monitored daily for changes in rectal temperature and clinical signs of disease, and
14 serum samples were collected weekly. Sheep # 3464 was inoculated intravenously with 2 ml of
15 cryopreserved sheep blood infected with the Gardel isolate of *E. ruminantium* (Uilenberg et al., 1985)
16 (designated stabilate CR366 at Utrecht University). Uninfected *A. variegatum* nymphs were applied in
17 cloth patches onto the back of the infected sheep on days 7, 8, 9 and 10 after inoculation and allowed to
18 acquisition-feed during the febrile reaction of the animal. Engorged nymphs were collected and
19 allowed to moult and harden at 27 °C, 95 % relative humidity for 6-8 weeks. Approximately 50 each of
20 the resultant adult males and females were transmission-fed on the back of a second susceptible sheep #
21 3456. Following attachment, five females were detached daily on days 1-4 of feeding. Midguts and
22 salivary gland pairs from five unfed ticks and from five ticks harvested on each day of feeding were
23 dissected out and pooled according to tissue and feeding day. Tissues from uninfected control ticks,
24 both unfed and fed on an uninfected sheep for 1-4 days, were processed similarly.

25

1 **Growth and harvest of *E. ruminantium* from *in vitro* cell cultures.**

2 The Gardel isolate of *E. ruminantium* was cultured in bovine umbilical cord endothelial cells (BUE) as
3 described previously (Jongejan et al., 1991). Bacterial growth was monitored by microscopic
4 examination of Giemsa-stained cytocentrifuge smears. When *E. ruminantium*-infected BUE cultures
5 showed about 90 % of cytolysis due to infection, the remaining adherent cells were scraped from the
6 bottom of the culture flasks and the cultures centrifuged at 15000 g for 20 min at 4 °C. The resultant
7 pellet containing both infected cells and free *E. ruminantium* organisms was resuspended in 1 ml of Tri
8 reagent (Sigma) and frozen at -70 °C until used for DNA and RNA extraction.

9
10 **DNA and RNA isolation.**

11 The midgut and salivary gland pools dissected from the unfed ticks and ticks harvested daily after 1 to
12 4 days of feeding either on sheep #3456 (*E. ruminantium*-infected ticks), or on the uninfected control
13 sheep (uninfected control ticks), were snap-frozen in liquid nitrogen and ground into powder. DNA
14 and RNA from the resultant homogenised tissues were simultaneously extracted using the TRI reagent
15 protocol (Sigma) and cleaned using the columns from QIAamp extraction kits (Qiagen) according to
16 the manufacturer's instructions. DNA and RNA were eluted from columns with 100 µl of elution
17 buffer (10 mM Tris/HCl; 0.5 mM EDTA; pH 9.0) or 50 µl of RNase-free water respectively. Total
18 DNA and RNA from *E. ruminantium*-infected endothelial cell cultures were extracted as described
19 above and 10-fold dilution series ($10^0 - 10^{-3}$) were prepared from these original DNA and RNA stocks
20 diluted in RNase-free water.

21
22 **Real-time PCR.**

23 The *map1-1* gene (GenBank accession no. AY652746) was selected as the target to quantify bacterial
24 DNA in tick tissues and in the 10-fold DNA dilution series from *E. ruminantium*-infected *in vitro*
25 cultures. Primers specific for the *map1-1* gene (forward primer F3: 5'-TAC GGT AAA GAC TCT

1 CCA ACT AAA-3'; reverse primer R1: 5'-TAT GCA GCT TCA ATC TCT ACT-3') were used to
2 amplify a fragment of 182 bp from test DNA samples and from a positive control plasmid, PBAD/Myc-
3 His (Invitrogen), containing the *map1-1* gene. The PCR reaction mixture used was as follows: 12.5 µl
4 of SYBR Green supermix (Bio-Rad), 1 µl of forward primer, 1 µl of reverse primer (0.4 µmol l⁻¹ final
5 concentration) and 5 µl of template, in a 25 µl PCR reaction. The cycling programme consisted of: 95
6 °C for 15 minutes; and cycles of denaturing, 95 °C for 30 seconds; annealing, 55 °C for 30 seconds;
7 and extension, 72 °C for 30 seconds, repeated 40 times. Ct values were determined and melting graphs
8 analysed accordingly using the iCycler software.

9

10 **cDNA synthesis and paralog-specific PCR.**

11 Reverse transcriptase PCR (RT-PCR) was carried out on RNA from *E. ruminantium*-infected and
12 uninfected ticks and from the 10-fold RNA dilution series extracted from *in vitro* cultures. Five
13 microlitres of DNase-treated RNA was used to prepare 20 µl of cDNA using 100 ng of random
14 hexamer primers of the SuperScriptTM first-strand synthesis system (Invitrogen) according to the
15 manufacturer's instructions. Control samples were prepared by omitting reverse transcriptase from the
16 reaction. Two microlitres of cDNA was used subsequently as template in a PCR reaction containing:
17 1X Taq PCR buffer (Promega), 3 mM MgCl₂, 1.25 U of Taq polymerase (Promega), 400 µM of each
18 deoxynucleoside triphosphate and 10 pmol of each primer in a 25 µl reaction. The sequences of the
19 paralog-specific primers used were those described previously (van Heerden et al., 2004). Reactions
20 were carried out in an iCycler (Bio-Rad) using the following programme: 2 min at 94 °C followed by
21 40 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 1 min at 72 °C and a final elongation step of 7 min at 72
22 °C. Positive controls incorporated genomic DNA and cDNA from *E. ruminantium*-infected endothelial
23 cell cultures. PCR products were visualised by running the samples on agarose gels and staining with
24 ethidium bromide.

25

1 **Results**

2

3 **Experimental infection of sheep and ticks.**

4 Sheep # 3464, infected by injection with *E. ruminantium* blood stabilate CR366, reacted with fever 13
5 days post inoculation and treatment with 10 % engemycin was started on the third consecutive day of
6 fever; however the sheep died on day 17 post inoculation. Sheep # 3456, infected by tick bite,
7 developed fever 13 days after tick application and was found dead on day 14. Heartwater was
8 confirmed in both sheep *post mortem* by observation of *E. ruminantium* colonies in Giemsa-stained
9 brain smears. Pools of dissected midguts and salivary glands from five unfed female ticks and five
10 female ticks harvested daily after 1 to 4 days of feeding were submitted to simultaneous DNA and
11 RNA extraction. To assess if the samples were infected with *E. ruminantium*, a PCR was run on
12 genomic DNA using two different combinations of primers specific to the *map1-1* gene. Amplicons of
13 the corresponding expected sizes were observed in all samples of midguts and salivary glands from
14 unfed and feeding ticks indicating that all the pools analysed were positive for *E. ruminantium*
15 infection (data not shown). No products were obtained from uninfected ticks using the same PCR
16 conditions (data not shown), confirming specificity of the primers.

17

18 **Real-time PCR.**

19 In order to quantify the number of bacteria present in the samples, real-time PCR was performed on
20 DNA extracted from pools of midguts and salivary glands as well as from *E. ruminantium*-infected
21 endothelial cell cultures. Quantitative measurements were based on a standard plot using a 10-fold
22 dilution series of a plasmid encoding the *map1-1* gene of *E. ruminantium*. Since *map1-1* is known to be
23 a single copy gene (Collins et al., 2005), it allows a direct estimation of the number of *E. ruminantium*
24 organisms per sample. Standards were diluted in water, as amplification of plasmid standards diluted
25 in carrier DNA (DNA from uninfected ticks) did not interfere with the sensitivity of the test (data not

1 shown). A linear correlation was found within the range of 10^2 - 10^6 plasmid copies per PCR reaction
2 and comparable standard curve slopes were obtained from two independent experiments. An identical
3 melting temperature of 77.5 °C was obtained for the standard plasmids and all samples analysed
4 containing *E. ruminantium* template DNA. There was no interference in the PCR reaction from non-
5 specific products or primer dimers (data not shown). The results are shown in Fig. 1a and are expressed
6 as the number of bacteria per tick (equivalent to number of bacteria per 20 µl of DNA suspension). *E.*
7 *ruminantium* was present in both midguts and salivary glands of unfed infected ticks (day 0). The
8 numbers of bacteria in midguts increased 7-fold from 1.2×10^6 per tick in unfed ticks to 9.3×10^6 per
9 tick after three days of feeding. The bacterial load in salivary glands increased forty-fold from $1.2 \times$
10 10^5 per tick in unfed ticks to 5.3×10^6 per tick after three days of feeding. In salivary glands a fourfold
11 decrease in numbers of bacteria was observed after day 3. In the serial dilutions of DNA from *E.*
12 *ruminantium*-infected endothelial cells, the number of bacteria per 20 µl of DNA suspension ranged
13 from 10^7 bacteria in the undiluted sample to 10^4 bacteria in the 10^{-3} dilution.

15 **Transcriptional analysis.**

16 RT-PCR was performed to study the transcriptional activity of the entire *map1* cluster *in vivo* in ticks
17 and to assess possible differences in transcription of paralogs of the *map1* cluster between midguts and
18 salivary glands of unfed and feeding ticks and between different days of feeding.

19 Typical transcriptional profiles of the *E. ruminantium map1* cluster in ticks are shown in Fig. 2. In
20 unfed ticks, only amplicons from the *map1-1* gene were detected in midguts (Fig. 2c, 2d). In contrast
21 in feeding ticks (day 4 in Fig. 2e, 2f), PCR products were detected in midguts and salivary glands for
22 both the *map1* (685 bp) and *map1-1* (550 bp) genes. Additional bands, which did not correspond to the
23 expected molecular mass, were seen when using *map1-4*, *1-7*, *1-9* and *1-10* gene-specific primers (Fig.
24 2). Sequencing of all amplicons demonstrated that only the transcripts from the *map1* and *map1-1*
25 gene-specific primers were *E. ruminantium*-specific products. The expected PCR products were

1 obtained from *E. ruminantium* positive control DNA and cDNA samples using each primer pair,
2 confirming the ability of the primers to amplify the target sequences (Fig. 2a, 2b). No transcripts of
3 any of the *map1* paralogs were detected in uninfected tick tissue or in infected tick tissue without
4 reverse transcriptase, indicating the absence of contamination of genomic DNA in the RNA preparation
5 (data not shown).

6 Transcription of *map1* and *map1-1* in different tissues and on different days of feeding was compared
7 with numbers of bacteria (Fig. 1a and b). Transcripts of the *map1-1* gene were found in midguts of
8 unfed and feeding ticks, whereas transcripts of the *map1* gene were detected in midguts of ticks after 4
9 days of feeding. The amount of the amplified *map1-1* gene product increased as feeding progressed
10 despite a reduction of the number of bacteria on day 4. In contrast, in salivary glands no transcripts
11 were found in unfed ticks, whereas in feeding ticks transcripts of the *map1* and *map1-1* genes were
12 detected on days 3 and 4 of feeding, with the *map1* transcripts being predominant. The highest signal
13 generated from the amplified *map1* gene product was observed on day 4 despite a significant reduction
14 in the number of bacteria on that day. These results suggest that there is differential transcription of
15 *map1* and *map1-1* genes depending on the stage of feeding of the tick and the location of the bacteria
16 within the tick. The amount of RNA extracted from the tick tissues was insufficient for quantitative
17 RT-PCR analysis so, for comparison purposes, transcriptional analysis of the *map1* and *map1-1* genes
18 was carried out on the dilution series prepared from the RNA extracted from the *E. ruminantium*-
19 infected endothelial cells. Transcripts of the *map1* gene were detected in all the dilutions, which had
20 contained RNA from between 10^7 and 10^4 bacteria, whereas transcripts of the *map1-1* gene were
21 detected only in the 10^0 and 10^{-1} dilutions of RNA extracted from 10^7 and 10^6 bacteria respectively; the
22 numbers of bacteria determined by real-time PCR in the tick tissues fell within this range. For both
23 genes the intensity of the bands diminished as the number of bacteria decreased (Fig. 3).

24

25

1 Discussion

2

3 In this study we analysed the transcriptional activity of the *map1* multigene family in different tissues
4 of *E. ruminantium*-infected ticks and found differences in transcription between midguts and salivary
5 glands of both unfed and feeding ticks.

6 Out of 16 paralogs examined, only transcripts from two genes, *map1* and *map1-1*, were detected in
7 ticks. This was in agreement with a preliminary study (Bekker et al., 2002) in which transcripts of
8 *map1* and *map1-1*, but not *map1-2*, were detected in whole unfed adult *A. variegatum* ticks infected
9 with *E. ruminantium* (Senegal). In the closely-related pathogens *E. canis* and *E. chaffeensis*,
10 transcription of only one paralog each of the orthologous multigene families was detected in vector
11 ticks (Unver et al., 2001; 2002); in both cases the transcribed gene was the ortholog of *map1-1*. In the
12 present study, *map1-1* transcripts were detected in unfed ticks only in midguts, despite the presence of
13 *E. ruminantium* organisms in the salivary glands before the start of feeding as determined by real-time
14 PCR. During tick feeding, transcripts of the *map1* paralog were more abundant in salivary glands than
15 in midguts, although the number of bacteria was always substantially higher in midguts than in salivary
16 glands. Moreover, the number of bacteria in both tissues showed a peak on day 3 while levels of *map1-*
17 *1* and *map1* transcripts increased over the time course of tick feeding in midguts and salivary glands
18 respectively. In contrast, in the ten-fold dilution series prepared from *E. ruminantium* grown in
19 endothelial cells, transcripts of the *map1* and *map1-1* genes decreased as the number of bacteria
20 decreased. These observations indicate that the transcription pattern observed in infected ticks reflected
21 real differences in the levels of mRNA rather than in the numbers of bacteria, and that differential
22 regulation of these genes occurs in ticks, related to the tissue and the stage of feeding. Our results are
23 similar to those reported for differential transcription of members of the *p44* multigene family of
24 *Anaplasma phagocytophilum* during tick transmission (IJdo et al., 2002). Several *p44* transcripts were

1 detected in salivary glands of transmission-feeding nymphs during engorgement but not in unfed
2 nymphs, suggesting that tick engorgement may induce transcription of *p44* genes.

3 The development of *E. ruminantium* in ticks and the route of transmission to the mammalian host are
4 incompletely understood. Electron microscopy studies detected colonies of *E. ruminantium* in sections
5 of midgut epithelial cells of unfed infected nymphal *Amblyomma hebraeum* (infected as larvae), as well
6 as in those collected on days 1-4 of feeding, while in salivary glands colonies of bacteria were only
7 found in nymphs that had fed for 4 days (Kocan et al., 1987a). *E. ruminantium* colonies were detected
8 in midguts of adult *A. hebraeum* on days 1-3 of transmission-feeding, but not thereafter and not in the
9 corresponding salivary glands (Hart et al., 1991), and in midguts of moulting nymphal and newly
10 moulted adult *A. variegatum* (Kocan et al., 1987b). An *E. ruminantium*-specific DNA probe has been
11 used to detect *E. ruminantium* infection in midguts and salivary glands of unfed adult *A. hebraeum*
12 (Yunker et al., 1993) and midguts of unfed adult *A. variegatum* incubated for 24 hours at 37°C
13 (Waghela et al., 1991). To our knowledge, the present study is the first report of quantification of *E.*
14 *ruminantium* in midguts and salivary glands of unfed and feeding ticks. Our results from real-time PCR
15 confirmed that *E. ruminantium* was already present in both midguts and salivary glands of infected
16 ticks before the start of the transmission feeding and that the number of bacteria increased substantially
17 after 3 days of feeding in both tissues. The midguts appeared to be the main tissue location for
18 colonisation and replication of *E. ruminantium* as the number of bacteria always remained higher in
19 midguts than in salivary glands, consistent with the relative ease of detection by previous workers of
20 colonies in midguts compared to salivary glands (Cowdry, 1925; Kocan et al., 1987a; Hart et al., 1991).

21 The presence of mammal-infective *E. ruminantium* in tick saliva has not been reliably demonstrated
22 (Bezuidenhout, 1981), but our results suggest that transmission of organisms replicating in the salivary
23 glands may be the principal route of infection for *E. ruminantium* as there was a dramatic decrease in
24 the number of bacteria in salivary glands, but not in midguts, between days 3 and 4. This observation

1 is consistent with the finding that *E. ruminantium* requires a period of about 75 hours of feeding prior
2 to transmission by adult ticks (Bezuidenhout, 1987).

3 A marked increase in the infectivity of *E. ruminantium*-infected ticks during feeding has been reported
4 (Bezuidenhout, 1981), which may reflect replication leading to a higher number of organisms and/or
5 developmental maturation into infective stages. Reticulated bodies and, to a lesser extent, electron-
6 dense forms, which could be considered to resemble respectively the non-infectious forms and the
7 infective stages (elementary bodies) of the bacteria occurring in mammalian cell cultures (Jongejan et
8 al., 1991), have been detected in both midguts and salivary glands of partially fed infected *A. hebraeum*
9 ticks by electron microscopy (Kocan et al., 1987a). The differentiation of *E. ruminantium* to
10 mammalian-infective forms in the tick appears to be a complex process in which the differential
11 transcription of genes coding for outer membrane proteins of *E. ruminantium* in unfed and feeding ticks
12 as demonstrated here for two *map1* gene family paralogs may be involved. However it will be
13 necessary to determine whether the differential transcription of *map1* paralogs observed *in vitro*
14 (Bekker et al., 2005) and *in vivo* is reflected at the protein level.

15

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17

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1 **Figure legends**

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3 **Figure 1: Numbers of *E. ruminantium* organisms and transcription of *map1* and *map1-1* genes in**
4 **midguts and salivary glands of unfed ticks (day 0) and ticks fed for 3 and 4 days.** DNA and RNA
5 were simultaneously extracted from pools of 5 midguts and 5 salivary gland pairs per feeding day. a.
6 Bacterial numbers were determined by real-time PCR and are presented as mean per tick \pm standard
7 deviation. The mean values were calculated from three readings obtained from two different
8 experiments on the same set of samples for each time point. b. The panels show ethidium bromide-
9 stained RT-PCR products of *E. ruminantium map1* and *map1-1* paralogs from midguts and salivary
10 glands of unfed and feeding ticks, allowing comparison between amount of RT-PCR products and the
11 number of bacteria present on each day in the respective tissue.

12

13 **Figure 2: Transcriptional profiles of the *E. ruminantium map1* cluster in unfed and feeding ticks.**
14 The panels show PCR products from genomic DNA (a) and cDNA (b) amplified with *map1* cluster
15 primers for [left to right] *map1-14* to *map1+1*; UN is an unknown gene (van Heerden et al., 2004); (c,
16 d) RT-PCR products from *E. ruminantium*-infected midguts and salivary glands respectively of unfed
17 ticks and (e, f) from midguts and salivary glands respectively of ticks that had fed for 4 days. M:
18 molecular marker, the band that corresponds to 500 bp is indicated in panels a and b. NT: no template.
19 Nucleotide base pair numbers of specific amplified products corresponding to *map1-1* (550 bp) and
20 *map1* (685 bp) are indicated on the left and right panels respectively. Gels were stained with ethidium
21 bromide.

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1 **Figure 3: Transcription of *map1* and *map1-1* genes in *E. ruminantium* -infected endothelial cell**
2 **cultures.** DNA and RNA were simultaneously extracted from *E. ruminantium* (Gardel)-infected
3 endothelial cells. Numbers of bacteria, quantified by real-time PCR in a serial dilution of DNA are
4 shown at the top of both panels. mRNA generated by the respective number of bacteria, was converted
5 to cDNA and amplified using specific primers for the *map1* (left panel) and *map1-1* (right panel) genes.
6 RT-PCR products were visualised on agarose gels stained with ethidium bromide.

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Fig. 1

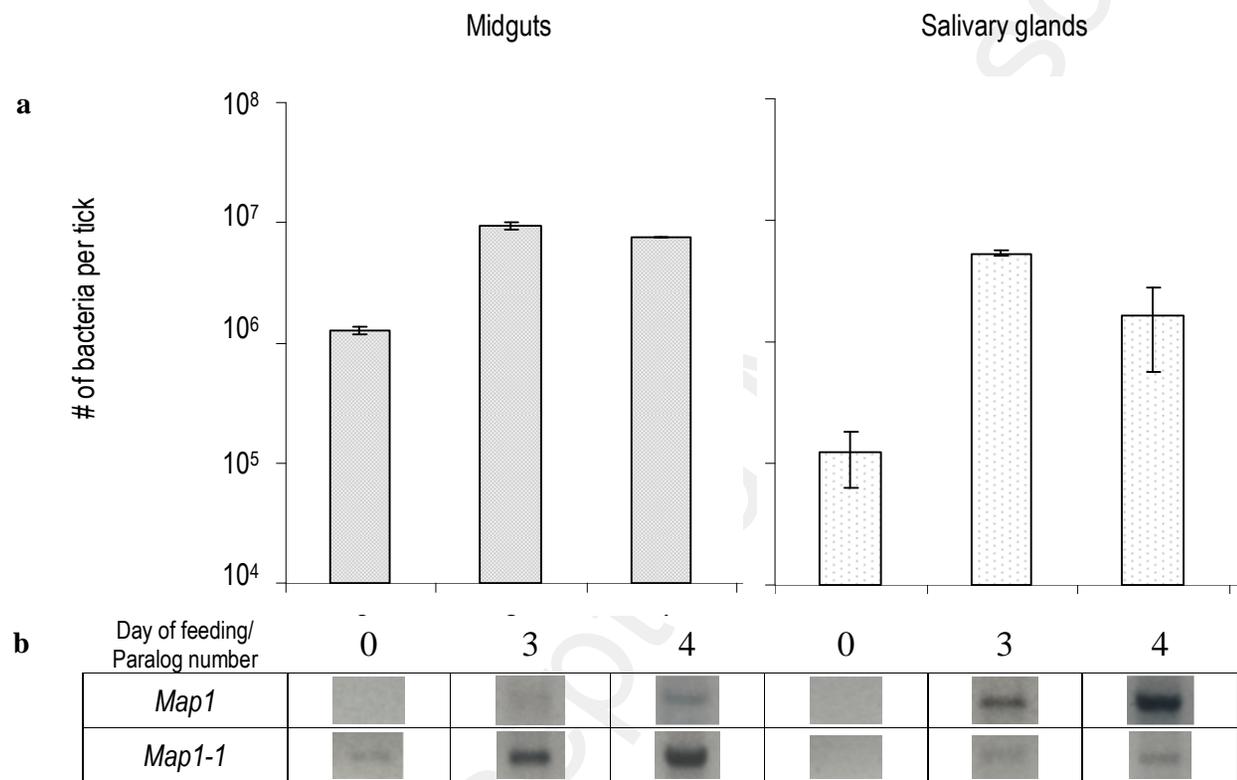


Fig. 2

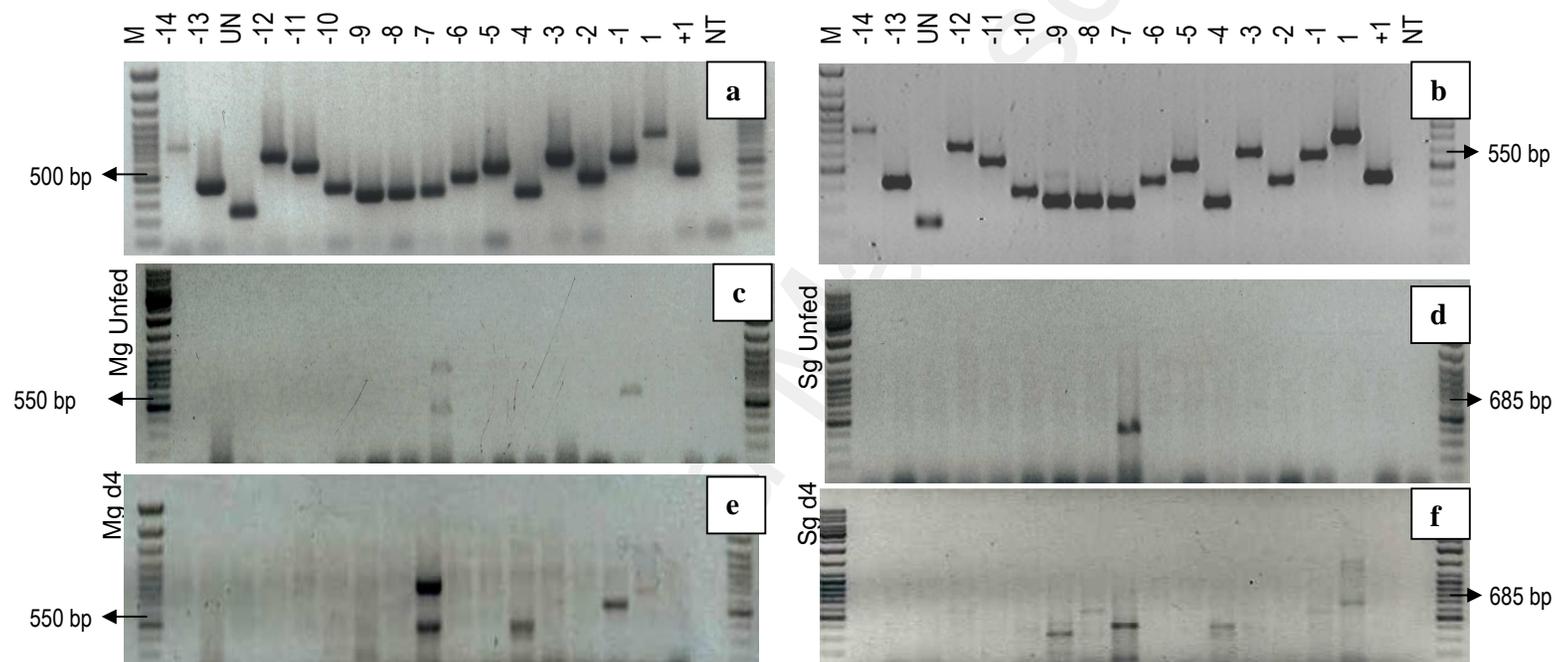
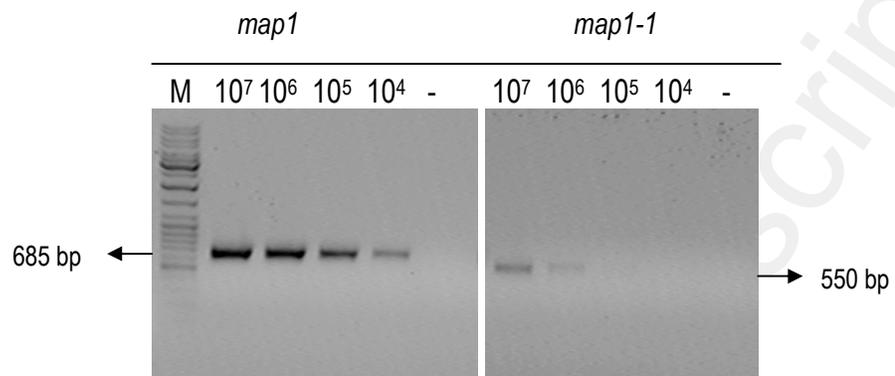


Fig. 3



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