Manuscript Title: Development of oriC-plasmids for use in *Mycoplasma hyorhinis*

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Electronic Supplementary File: Figures and Legends

**Supplemental Fig.S1** Hemolytic activity of *M. hyorhinis*. Mouse RBCs was incubated with supernatant of wild-type *M. hyorhinis* (2-fold dilution) and PBS (control). The hemolytic activity was observed. The image was cropped and full-length image is included in the **Supplementary Fig.S11**.

**Supplemental Fig.S2** Detection of the *pGEMT-LoriC* and *pGEMT-MoriC* plasmids in a single clone sub-culture passages. DNA was extracted on the third passages from control untransformed culture (C), cells transformed with the *pGEMT-LoriC* plasmid (P1) and cells transformed with the *pGEMT-MoriC* plasmid (P2). The presence of these plasmids in the transformants was detected by *tetM*-specific PCR (using P9 primers, Table-1) amplifying an about 339-bp of *tetM*. The gel image was cropped and full-length gel is included in the **Supplementary Fig.S12**.
**Supplemental Fig. S3** Detection of the pGEMT-LoriC plasmid following integration by single cross over at the oriC site of *M. hyorhinis*. Analysis of the DNA extracted from pGEMT-LoriC transformants by *tetM* specific PCR (using **P9** primers, Table-1), had detected the *tetM* (about 339-bp) and further confirmed the presence of the integrated pGEMT-LoriC plasmid. M = DNA molecular marker, C = control untransformed culture and the lanes (1-4) represent number of tetracycline resistant colonies tested. The gel image was cropped and full-length gels and blots are included in the Supplementary Fig. S.13.

**Supplemental Fig. S4.** Investigation of the possible integration of pGEMT-MoriC plasmid containing Mini-oriC (MoriC) at the oriC-region of *M. hyorhinis*. Following integration of the plasmid at the *oriC* region, a predicted 2122-bp PCR product could be detected with specific integration primers (**P10**, Table-1). We got no PCR product indicating the absence of plasmid integration at this region by single cross-over. Lanes (1-10) indicate number of tetracycline resistant colonies investigated. The gel image was cropped and full-length gel image was included in the Supplementary Fig. S.14.
Supplemental Fig.S5 Detection of the tetM insertion at the hemolysin site using Mini-oriC-HT2 plasmid. The presence of tetM was investigated with tetM specific PCR (using P9 primers, Table-1 and the 3072-bp PCR product that was amplified with hlyC flanking primers P11, Table-1 as a template). M = DNA molecular marker, C+ = Mini-oriC-HT2 positive control (untransformed plasmid), C = negative control (untransformed culture) and the lanes (1-4) represent number of colonies tested. The gel image was cropped and full-length gels and blots are included in the Supplementary Fig.S14.

Supplemental Fig.S6 Investigation of a single cross-over event between any arm of hlyC leading to integration of the full Mini-oriC-HT1 plasmid. The PCR using single-cross primer-F (P12-F) and single-cross primer-R (P12-R) to detect the predicted product following integration of full Mini-oriC-HT1 plasmid at the right arm of hlyC by single cross-over, had failed to detect a band of about 1017-bp, indicating the insertion of tetM at the hemolysin site is due to a double cross-over event. Lanes (1-8) indicate number of tetracycline resistant colonies investigated. The gel image was cropped and full-length gel is included in the Supplementary Fig.S15.
Supplemental Fig. S7 Alignment of the DNA sequences of hlyC in different M. hyorhinis strains. The alignment of the DNA sequences of hlyC of the six M. hyorhinis strains: \textsuperscript{a} = HUB-1 (Accession CP002170 Region: 304679...305920), \textsuperscript{b} = DBS 1050 (Accession CP006849 Region: 438049..439291), \textsuperscript{c} = GDL-1 (Accession CP003231 Region: 438066..439308), \textsuperscript{d} = MCLD (Accession CP002669 Region: 170809..172050), \textsuperscript{e} = MDBK-IPV (Accession CP016817 Region: 437951..439192) and \textsuperscript{f} = SK76 (Accession CP003914.1 Region: 305879-307120) by Clustal V method showed that only one base different in hlyC sequence in two strains (DBS 1050 and GDL-1).

Supplementary Fig. S8. Full-length gels images of Fig. 2

(A) tetK

(B) [gel bands labeled]

(C) [gel bands labeled]

(D) [gel bands labeled]

(E) [gel bands labeled]

(F) [gel bands labeled]
Supplementary Fig. S9. Full-length gel image of Fig. 4

Supplementary Fig. S10. Full-length gel image of Fig. 6
Supplementary Fig. S11. Full-length image of Fig. S1

Supplementary Fig. S12. Full-length image of Fig. S2
Supplementary Fig. S13. Full-length image of Fig. S3

Supplementary Fig. S14. Full-length gel image of Fig. S4
Supplementary Fig. S15. Full-length gel image of Fig. S5

Supplementary Fig. S16. Full-length gel image of Fig. S6