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Identification and Characterization of HU Protein from *Mycoplasma gallisepticum*

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A hypothetical ORF of *Mycoplasma gallisepticum* with a putative 99-amino-acid product (ORF99) was noted previously in the upstream region from the type II topoisomerase gene. The amino acid sequence shows weak homology with the *Escherichia coli* histone-like protein HU. To identify and characterize the protein product of ORF99, we prepared mouse antiserum against recombinant GST-ORF99 fusion protein. The antiserum reacted with an 11-kDa peptide in the crude cell extract of *M. gallisepticum*, indicating that this protein is an ORF99 product. ORF99 protein binds to DNA, although its binding affinity is weaker than that of *E. coli* HU. When ORF99 was cloned in a plasmid and expressed in *E. coli* cells depleted of HU, Mu phage growth was strongly promoted in the cells, showing the presence of HU activity. The effect of IHF mutation was suppressed when a high level of ORF99 protein was expressed in an *E. coli* mutant deficient in IHF.

***Key Words: Mycoplasma gallisepticum;* histone-like protein; ORF99; HU protein.**

HU is a small, basic, histone-like protein widely found in prokaryotes. HU is a major component of nuclear proteins and plays an important role in the formation of higher-order DNA structure and in the regulation of DNA replication, transcription and recombination (2). HU generally exists as a homodimer, although it consists of two distinct HU subunits in *E. coli*, *Salmonella typhimurium* and *Serratia marcescens* (3-5).

Mycoplasmas are small, cell-wall-lacking, parasitic bacteria usually found in the mucosal surface of host organisms (6). They have the smallest genome size among known self-replicating organisms, and are believed to be a suitable model for defining the minimum genetic constitution of self-replicating cells (7).

It is not clear at this point whether HU protein exist in Mycoplasmas. However, recent complete sequencing of two Mycoplasma genomes [*M. genitalium* and *M. pneumoniae* (8, 9)] noted HU gene candidates in these genomes [as ORFs with unknown function; MG353 and G12_orf109 (10, 11)]. In addition, a computer search for databases showed two more candidates for HU gene in another Mycoplasma species. (12). These are hypothetical ORFs of *M. gallisepticum* located in the upstream region from the typeII topoisomerase gene [ORF99; GenBank accession No. L35044, see (1)] and *M. capricolum* [clone MC352; GenBank accession No. Z33259, see (13)].

We are interested in knowing whether these ORFs are functional genes and these products have HU-like activities. To clarify this problem, we focused on the ORF99 of *M. gallisepticum*, an avian pathogen that causes severe respiratory disease. In this study, we searched for ORF99 product using antiserum raised against GST-ORF99 fusion protein. We also expressed ORF99 in *E. coli* and characterized *in vivo* function.

MATERIALS AND METHODS

Bacterial strains and culture. *M. gallisepticum* PG-31 strain (14) was cultured in PPLO medium [2.1% PPLO Broth (Difco), 0.25% glucose, 0.002% phenol red, 10% horse serum (Gibco Lab.), 200 unit/ml penicillin G] at 37°C. *E. coli* strains were cultured in LB medium (10 g of Bacto tryptone, 5 g of yeast extract, 5 g of NaCl and 0.1% Glucose /liter) or LBCaMg (10 g of Bacto tryptone, 5 g of yeast extract and 10 g of NaCl /liter containing 1 mM $CaCl_2$ and 2.5 mM $MgSO_4$) (15). *E. coli* strains; YK1100 is a *trpC*9941 derivative of W3110 (16); YK1151 is a Mucts62 lysogen of YK1100; YK1344 is a HU deficient (*hupA*16 *hupB*11) (16) derivative of YK1151; YK2920 (17) and YK5663 are Δ82[*himA*]::Tn10 Δ3[*hip*]::cat and *zih*35::Tn*10* deriva-

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Abbreviations used: A, absorbance; Ap, ampicillin; CBB, Coomassie brilliant blue; GST, glutathione-S-transferase; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IPTG, isopropyl-β-D- thiogalactopyranoside; ORF, open reading frame; PA, polyacrylamide; PCR, polymerase chain reaction; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; wt, wild type.

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tive of YK1100, respectively. Mucts62 is a temperature-inducible repressor mutant of wt Mu phage (18).

Preparation of GST-ORF99 fusion protein and specific antiserum. The nucleotide sequence of ORF99 was amplified from genomic DNA of *M. gallisepticum* PG-31 strain by PCR, using the following primers; 5′GGGGATCCTCATGGCAAAAATCAAATCA3′ and 5′AAAGCTTTTCGTTGTAATTTGATAC3′. The amplified region was sequenced and it completely matched that reported for *M. gallisepticum* A5659 strain (1) (data not shown). After blunt-ending with T4 polymerase followed by *Bam*HI treatment, the amplified ORF99 DNA was ligated into the *Bam*HI-*Sma*I site of pGEX-3X expression vector (Pharmacia Biotech). GST-ORF99 fusion protein, a 37 kDa polypeptide specifically induced in the presence of 0.4 mM IPTG in *E. coli* JM83 strain (19) harboring the constructed plasmid, was purified by GST-affinity chromatography (Pharmacia Biotech). One hundred μg of purified fusion protein mixed with Freund's complete adjuvant (Difco) was used to immunize mice. Booster immunization was done with the same amount of protein mixed with Freund's incomplete adjuvant (Difco) at 3 weeks after the first immunization. Antiserum was prepared 3 weeks after the booster injection, and specific reactivity to GST-ORF99 peptide was confirmed by Western blot analysis (data not shown).

Fractionation of DNA-binding proteins. DNA-cellulose column chromatography was based on the method of Goshima *et al.* (20). *M. gallisepticum* PG-31 was cultured in 250 ml of PPLO medium at 37°C to late-log phase. Cells were harvested by centrifugation and washed twice with PBS. The pellet was suspended in 5 ml of buffer I (25 mM HEPES pH 7.6/1 mM 2-mercaptoethanol/1 M KCl) and sonicated. Then 10 μg of DNaseI and 50 μl of 1 M $MgCl_2$ were added to the sonicated homogenate, and the whole was put on ice for 1 hr. The homogenate was centrifuged at 100,000xg for 40 min and the supernatant was dialyzed against buffer II (25 mM HEPES pH 7.6/ 5 mM Na_2·EDTA/1 mM 2-mercaptoethanol/50 mM NaCl) (cleared lysate), and applied to a double-stranded calf thymus DNA-cellulose (SIGMA) column (1 cm^3) equilibrated with buffer II. Column was washed out with 10 ml of buffer II and the bound proteins were eluted stepwise with 2 ml each of 0.1 M, 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M NaCl in buffer II. Proteins were concentrated by TCA precipitation from a 1 ml aliquot and were electrophoresed (0.1% SDS-15% PA gel). After electrophoresis, proteins were visualized by CBB staining or Western blotting.

Construction of ORF99-cloned plasmids. A 543 bp DNA fragment containing ORF99 (300 bp) and flanking regions (149 bp upstream and 94 bp downstream) was amplified by PCR from *M. gallisepticum* PG-31 genomic DNA using primers 5′GATCAACCGACTTCACCATC3′ and 5′AAAGCTTTTCGTTGTAATTTGATA3′. The amplified fragment was blunt-ended with T4 polymerase and inserted into the *Hinc*II site of pCL1920 plasmid, a derivative of pSC101 (21). Two plasmids, pCLMG1 and pCLMG2, having the insert in opposite orientations, were selected (see Fig. 3A). The cloned ORF99 regions of the two plasmids were sequenced and the results were identical with the previously reported sequence for *M. gallisepticum* A5659 strain (data not shown).

Construction of high expression plasmid pCLMG1L was illustrated in Fig. 4A. In this plasmid, ORF99 is expressed using the promoter and SD sequence of *lacZ*. High-level expression of ORF99 in the *E. coli* JM83[pCLMG1L] cell was confirmed by Western blot analysis compared with JM83[pCLMG1] in the absence and the presence of 0.5 mM IPTG (see Fig. 4B).

Thermal induction of Mucts62. Mucts62 lysogens harboring ORF99-cloned plasmids were cultured at 30°C to A_{550} = 0.4 in LB containing 50 μg of Sp/ml with or without 0.4 mM IPTG. Heat-treatment was done at 42°C for 25 min followed by shaking for 120 min at 37°C. After centrifugation, the supernatant was treated with chloroform. Mu phage titer of the supernatant was measured by plaque formation on YK1100 lawns at 37°C on LBCaMg plates containing 1% agar with 0.5% top agar.

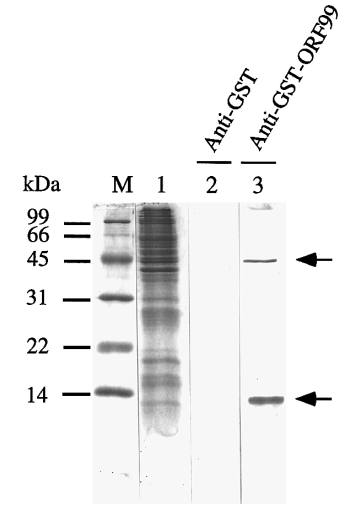

FIG. 1. Western blot analysis of whole cell lysate of *M. gallisepticum* with anti-GST-ORF99 antiserum. *M. gallisepticum* PG-31 strain were cultured in PPLO medium at 37°C to mid-log phase. Cells were harvested by centrifugation and suspended in SDS-PAGE sample buffer (125 mM Tris·HCl pH 6.8/2% SDS/10% glycerol/5% 2-mercaptoethanol). Equal aliquots of samples (lanes 1 to 3) and size marker (lane M) were electrophoresed (0.1% SDS-15% PA gel) and proteins were either visualized by CBB staining (lanes M and 1) or transferred electrophoretically to nitrocellulose membrane (lanes 2 and 3). The blots were treated with mouse anti-GST-ORF99 antiserum (lane 3) and anti-GST antiserum (lane 2) (each 1/200 dilution) and cross-reactive material was detected by the alkaline phosphatase-conjugated anti-mouse-Ig (Promega) method. Proteins detected are indicated by arrows.

P1-mediated transduction. The *himA*::Tn*10* and *zih*35::Tn*10* were introduced into cells by $P1_{kc}$-mediated transduction using the method described by Miller (22). *E. coli* YK1100 harboring ORF99-cloned plasmid was cultured in LB containing 50 μg of Sp/ml at 37°C, and infected for 30 min with an equal amount of $P1_{kc}$ propagated on YK2920 or YK5663. After incubation at 37°C for 2 h in LB containing 1% Na_3·citrate, transductants were selected on LB agar (1.5%) plates containing 12 μg of Tc/ml.

RESULTS AND DISCUSSION

Identification of ORF99 product in the cell extract of M. gallisepticum. To confirm whether ORF99 sequence is a functional gene of *M. gallisepticum*, Western blot analysis was done for whole cell lysate of *M. gallisepticum* using antiserum against GST-ORF99 fusion protein. Figure 1 shows that the antiserum against GST-ORF99 fusion protein reacted with 11 kDa and 45 kDa proteins of *M. gallisepticum* (lane 3). On the other hand, antiserum against GST did not react with *M. gallisepticum* proteins (lane 2). We, therefore, concluded that the 11 kDa protein is ORF99 product because its size was estimated to be 11 kDa, as judged from the ORF sequence. We did not attempt to characterize further the 45 kDa protein, since we could not find 45 kDa protein in the cleared lysate (see Fig. 2B, lane 1).

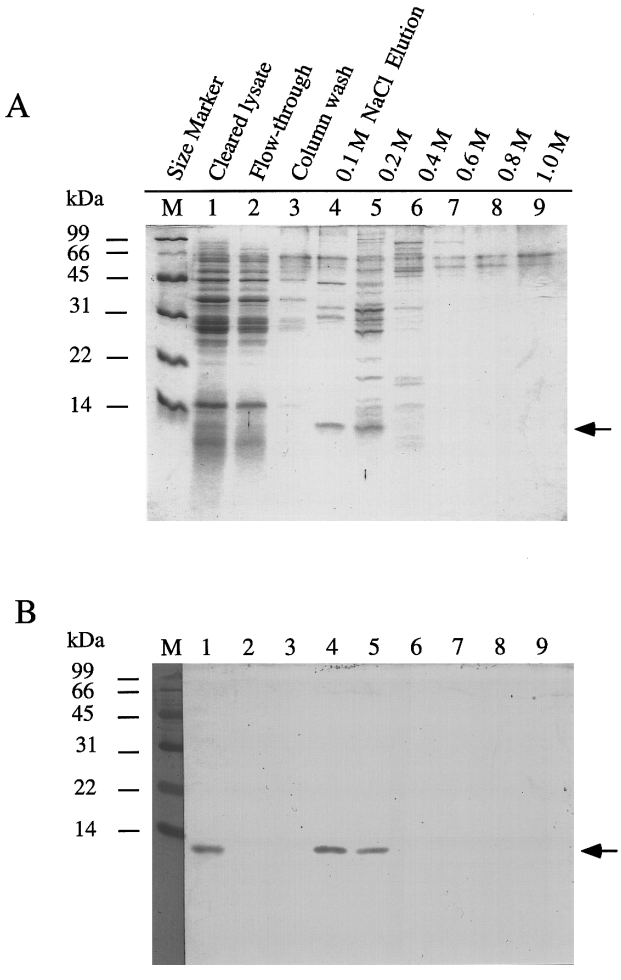

FIG. 2. Electrophoresis of fractionated DNA-binding proteins of *M. gallisepticum*. Cell lysate was prepared as described in Materials and Methods. (A) Stained with CBB. Size markers (lane M), cleared lysate of *M. gallisepticum* (lane 1), flow-through fraction from the column (lane 2), column washout (lane 3) and each eluted protein fractions (lanes 4, 5, 6, 7, 8 and 9) were shown. (B) Western blot analysis with anti-GST-ORF99 antiserum. Detection of cross-reactive materials was as described in the legend to Fig. 1. The lanes correspond to those of panel A. Lane M (size markers) was stained with CBB after transfer to the membrane.

ORF99 contains second AUG codon in its sequence. If translation started from this internal AUG codon, a 9.3 kDa protein (84 amino acids) should be produced. It is, however, not likely that translation starts from this AUG codon, because no protein with the expected size could be detected.

DNA binding activity of ORF99 protein. We then examined the DNA binding properties of the ORF99 protein. Cellular proteins of *M. gallisepticum* were fractionated by DNA-cellulose column chromatography and analyzed by SDS-PAGE (Fig. 2A). We found that the 11 kDa DNA-binding protein was eluted from the column with 0.1 M to 0.2 M NaCl (Fig. 2A, lanes 4 and 5). Western blotting with anti-GST-ORF99 antiserum clearly showed that this 11 kDa protein is ORF99 product (Fig. 2B, lanes 4 and 5). In this DNA-cellulose column chromatography system, *E. coli* HU is usually eluted with 0.3 M to 0.4 M NaCl (20, 23 and our unpublished data). It was, therefore, suggested that the binding affinity of ORF99 protein to DNA is weaker than that of *E. coli* HU. It is of interest whether the weaker affinity of ORF99 protein to DNA was caused evolutionarily by an adaptation to the low GC content (about 32%) (7) of *M. gallisepticum* genome. Further analysis of DNA binding property of HU-like proteins of Mycoplasma species will answer this question.

Stimulation of Mu phage growth by ORF99 protein. It is known that the growth of bacteriophage Mu in

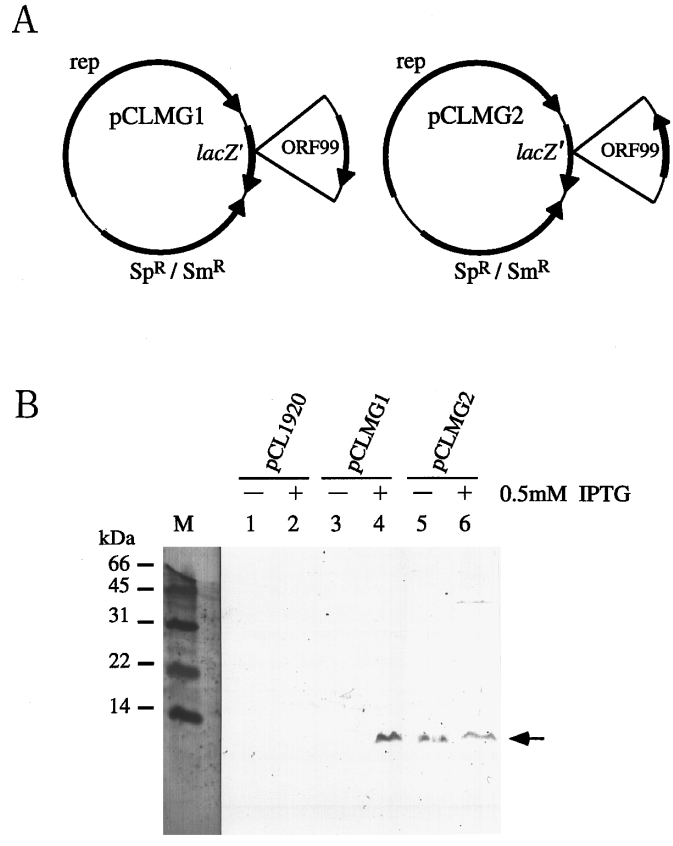

FIG. 3. Expression of ORF99 in *E. coli* cells. (A) Structure of ORF99 -cloned plasmids. The location and direction of the pSC101 replicon (rep), *lacZ'* gene and Sp^R/Sm^R gene are shown by the arrow-headed thick lines on the circle. The orientation of ORF99 sequences are shown as a pop-out arc. (B) Detection of ORF99 protein. *E. coli* JM83 harboring pCL1920, pCLMG1 and pCLMG2 were cultured overnight in 5 ml of LB containing 50 μg of Sp/ml at 37°C with or without 0.5 mM IPTG. One ml of the culture was harvested and mixed with 200 μl of SDS-PAGE sample buffer. Ten μl samples were electrophoresed (0.1% SDS- 15% PA gel) and transferred to nitrocellulose membrane. ORF99 proteins were probed by antiserum as described in the legend to Fig. 1. Lane M; size markers, lanes 1 and 2; JM83[pCL1920], lanes 3 and 4; JM83[pCLMG1], lanes 5 and 6; JM83[pCLMG2]. 0.5 mM IPTG was added (+) or not added (-). ORF99 proteins are indicated by an arrow.

TABLE I

Thermal Induction of Mucts62

Strain	Phage titer (pfu/ml)	
	−IPTG	+IPTG
YK1151[pCL1920]	2.8×10^9 (1.3×10^6)	3.4×10^9 (1.5×10^6)
YK1344[pCL1920]	2.2×10^3 (1.0)	1.7×10^3 (0.77)
YK1344[pCLMG1]	1.6×10^5 (7.3×10^1)	4.8×10^8 (2.2×10^5)
YK1344[pCLMG2]	3.8×10^8 (1.7×10^5)	3.0×10^8 (1.4×10^5)

Note. Relative titer is listed in parentheses.

E. coli requires HU protein. HU catalyzes replicative transposition step of this phage (15, 24). We, therefore, attempted to use Mu phage transposition system to evaluate the HU activity of ORF99 protein. For this purpose, we cloned ORF99 DNA and its flanking regions into plasmid pCL1920 (Fig. 3A).

As can be seen in Fig. 3B, 11 kDa ORF99 protein was detected in JM83[pCLMG1] in the presence of IPTG (lane 4), but this protein could not be detected in the absence of IPTG (lane 3). ORF99 protein was also detected in the cell extracts of JM83[pCLMG2] both in the presence and in the absence of IPTG (lanes 5 and 6). These results showed that ORF99 was expressed from the recombinant plasmids using *E. coli* promoters of pCL1920 vector; transcription of ORF99 DNA in pCLMG1 started from the *lacZ* promoter in the presence of IPTG. On the other hand, constitutively read-through mRNA directed from Sp^R/Sm^R gene was used for ORF99 expression in pCLMG2 (see Fig. 3A). This mRNA must have been translated from the native start codon of ORF99 sequence since the detected protein was 11 kDa (Fig. 3B, lanes 5 and 6).

We then introduced pCL1920, pCLMG1 and pCLMG2 into the heat-inducible Mucts62 lysogen of *E. coli* and thermal induction of Mucts62 was analyzed. As shown in Table I, heat induction of Mucts62 occurred at 42°C in a wt strain YK1151[pCL1920] (1.3×10^6-fold increase in the absence of IPTG and 1.5×10^6-fold increase in the presence of IPTG, compared to the HU mutant strain YK1344[pCL1920]). Heat-induction was promoted strongly in the YK1344[pCLMG1] strain in the presence of 0.4 mM IPTG (2.2×10^5-fold), but very weakly in the absence of IPTG (7.3×10^1-fold). Heat-induction was also promoted strongly in the YK1344 [pCLMG2] strain both in the presence of 0.4 mM IPTG (1.7×10^5-fold) and in the absence of IPTG (1.4×10^5-fold). The effect of IPTG on Mu induction paralleled the amount of ORF99 protein expressed from pCLMG1

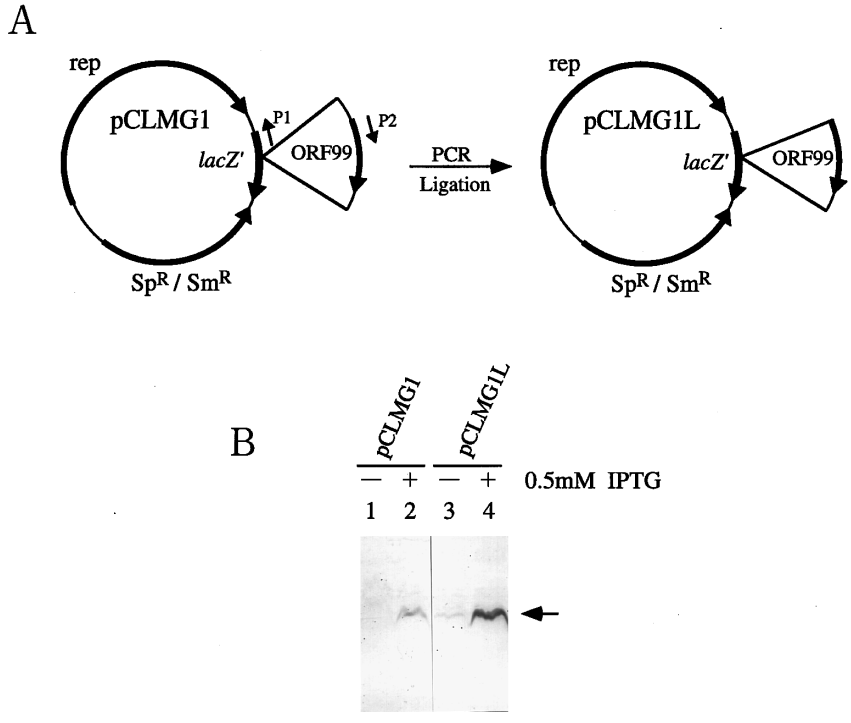

FIG. 4. High-level expression of ORF99 in *E. coli* cells. (A) Construction of a high-level expression plasmid of ORF99. High expression plasmid pCLMG1L was constructed by PCR method from pCLMG1 plasmid. A DNA fragment was amplified from pCLMG1 plasmid by PCR using the pair of oligodeoxyribonucleotide primers, P1 (5′GCTGTTTCCTGTGTGAAA3′) and P2 (5′TATGGCAAAAATCAAATCA3′) to eliminate the upstream region of ORF99 DNA. The PCR-generated fragment was blunt-ended with T4 polymerase and ligated. (B) Western blot analysis of ORF99 protein. JM83 harboring pCLMG1 or pCLMG1L was analyzed as described in the legend to Fig. 3. Lanes 1 and 3; JM83[pCLMG1], lanes 2 and 4; JM83[pCLMG1L]. ORF99 proteins are indicated by an arrow.

TABLE II

Effect of ORF99 on Plasmid Maintenance in *himA* Mutant

Strain (recipient)[a]	Marker transduced	Sp^R + Tc^R/Tc^R (%)[a]
YK1100[pCL1920]	*him*A::TN10 (Tc^R)	0/50 (0)
YK1100[pCLMG1]	*him*A::TN10 (Tc^R)	0/50 (0)
YK1100[pCLMG2]	*him*A::TN10 (Tc^R)	0/50 (0)
YK1100[pCLMG1L]	*him*A::TN10 (Tc^R)	50/50 (100)
YK1100[pCL1920]	*zih*35::TN10 (Tc^R)	50/50 (100)
YK1100[pCLMG1]	*zih*35::TN10 (Tc^R)	50/50 (100)
YK1100[pCLMG2]	*zih*35::TN10 (Tc^R)	50/50 (100)
YK1100[pCLMG1L]	*zih*35::TN10 (Tc^R)	50/50 (100)

[a] Fifty colonies of each Tc^R transductant were transferred to LB agar plates containing 50 μg of Sp + 12 μg of Tc/ml to test the Sp^R marker of the plasmid. The numbers of Sp^R colonies/tested colonies are shown. The rates of plasmid maintenance are shown in parentheses.

and pCLMG2 (Fig. 4B). It was, therefore, suggested that ORF99 protein has an activity like that of *E. coli* HU and stimulates transposition of Mu phage.

Compensation for the absence of IHF by ORF99. *E. coli* HU is a histone-like protein closely related to protein IHF (Integration host factor), which consists of IHFα and IHFβ subunits encoded by *himA* and *hip* /*himD* genes, respectively (2). It was reported that HU can replace IHF in several *in vivo* and *in vitro* reactions (12), but pSC101 replication, which requires IHF, was not supported by *E. coli* HU in cells deficient in IHF (25). When *himA*::Tn*10* mutation was introduced in *E. coli* wt strain YK1100 harboring pCL1920, pCLMG1 and pCLMG2 by P1-mediated transduction, these plasmids were not maintained in the transductants, while these plasmids were maintained in the *zih*35::Tn*10* transductant whose genotype is *himA*$^+$ (Table II). On the other hand, the plasmid pCLMG1L, which expresses ORF99 at a high level (see Fig. 4) was maintained stably after the introduction of *himA*::Tn*10* mutation (Table II). The result suggested that the high-level expression of ORF99 compensated for the absence of IHF in the replication of pSC101 replicon.

CONCLUSIONS

(1) Antiserum against GST-ORF99 fusion protein was prepared. Using this antiserum, we found that ORF99 protein is present in *M. gallisepticum* cells, indicating that ORF99 is a functional gene.

(2) ORF99 protein bound to DNA-cellulose, although the binding affinity of the protein was weaker than that of *E. coli* HU.

(3) ORF99-expressing plasmids exhibited strong promotion of the growth of bacteriophage Mu in *E. coli* cells depleted of HU.

(4) pSC101-derived plasmid was maintained stably in *E. coli* deficient in IHF when a high level of ORF99 protein was present, *i.e.*, ORF99 protein compensates for the lack of IHF.

These results suggested that ORF99 protein of *Mycoplasma gallisepticum* is a counterpart of *E. coli* histone-like protein HU.

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