

# Genetic analysis of a plasmid encoding haemocin production in *Haemophilus paragallinarum*

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The full sequence of plasmid p250, isolated from *Haemophilus paragallinarum* strain HP250, has been obtained. The plasmid contains seven ORFs: a putative integrase, a putative replication protein (*repB*) and five ORFs similar to those from the haemocin (bacteriocin) *hmcDCBAI* operon from *Haemophilus influenzae*. Of 19 other non-plasmid-containing *H. paragallinarum* strains screened (11 serovar reference strains and 8 field isolates), 17 strains produced haemocin and were resistant to killing by strain HP250. These strains, unlike strain HP250, have a chromosomally encoded haemocin operon. A number of other members of the family *Pasteurellaceae* were tested for haemocin sensitivity. *Pasteurella avium*, *Pasteurella volantium* and *Pasteurella* species A, all non-pathogenic bacteria found in the respiratory tract of chickens suffering from respiratory diseases, were sensitive to *H. paragallinarum* haemocin. However, amongst the pathogenic *Pasteurellaceae*, 50% of *P. multocida* isolates and all five isolates of *Pasteurella haemolytica* tested were sensitive to the haemocin. Given the prevalence of haemocin production in *H. paragallinarum* strains, it may play a role in aiding colonization by inhibiting other Gram-negative bacteria that are associated with the respiratory tract in chickens. The origin of replication from plasmid p250 has been used to generate an *Escherichia coli*–*H. paragallinarum* shuttle vector which may be useful in genetically manipulating *H. paragallinarum*.

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## INTRODUCTION

Infectious coryza is an acute upper respiratory tract disease of chickens, caused by the bacterium *Haemophilus paragallinarum* (Blackall *et al.*, 1997). Although the disease is of worldwide economic significance and affects both broiler and layer flocks, the pathogenesis of *H. paragallinarum* is poorly understood. The typical clinical signs of the disease, including nasal discharge, facial oedema, lacrimation, anorexia and diarrhoea (Blackall *et al.*, 1997), result in a drop in egg production (10–40%) in layer flocks and growth retardation due to decreased feed and water consumption in breeder and broiler flocks (Blackall *et al.*, 1997).

Studies on the pathogenesis of *H. paragallinarum* have been hampered by the lack of a genetic manipulation system for this organism. There have only been two reports of extrachromosomal plasmids in the organism. A total of 81 genetically diverse strains were surveyed and a single strain, HP250 (also known as TW1), contained a 6 kb plasmid (Blackall, 1998; Blackall *et al.*, 1991). Crude plasmid extractions from nicotinamide adenine dinucleotide

(NAD)-independent strains of *H. paragallinarum* isolated in South Africa have also been used to convert reference strains of *H. paragallinarum* into NAD-independent isolates, indicating that NAD-independence is carried on a plasmid (Bragg *et al.*, 1993). However, these plasmids have not been further characterized.

In this study, we have obtained the full sequence of the plasmid, p250, from strain HP250. The plasmid includes a haemocin-producing locus which expresses a protein capable of killing a range of other Gram-negative bacteria. A putative origin of replication for the plasmid has been identified and used to develop an *Escherichia coli*–*H. paragallinarum* shuttle vector. The production of haemocins by strains from all *H. paragallinarum* serovars and the effect of *H. paragallinarum* haemocin activity on both pathogenic and commensal chicken respiratory bacteria is described.

## METHODS

**Bacterial strains and plasmids.** *Escherichia coli* strains were cultivated in Luria–Bertani medium (LB) with appropriate antibiotics. Strain S17.1 $\lambda$ pir (*tra recA pro thi hsdR* chr::RP4-2) (Simon *et al.*, 1983) was used for conjugal transfer of derivatives of the plasmid

The GenBank accession number for the sequence reported in this paper is AY300023.

pLO1 (Lenz *et al.*, 1994). DH5 $\lambda$ pir, a lysogen of DH5 $\alpha$  containing the *pir* gene of plasmid R6K, was the host for transformations of plasmid pCVD442 (Donnenberg & Kaper, 1991), which contains the R6K replicon. The *H. paragallinarum* strains used in this study are listed in Table 3; the other avian bacteria used are listed in Table 4. All the bacteria of avian origin used in this study were obtained from the culture collection at the Animal Research Institute, Brisbane, Australia. The panel of 11 Gram-positive commensal bacteria (Table 4) was isolated from the sinus cavity of healthy chickens; all other isolates were obtained from chickens suffering respiratory disease. The bacteria of avian origin, including the *H. paragallinarum* strains, were cultured in Test medium supplemented with chicken serum and reduced NAD (TM/SN) (Reid & Blackall, 1987), either in liquid culture or on agar plates made with the addition of 15 g agar l<sup>-1</sup>.

**DNA techniques.** Restriction and DNA modification enzymes were purchased from New England Biolabs. *Taq* polymerase for PCR was purchased from Fisher Biotech. Molecular biology methods were as described in Sambrook *et al.* (1989).

#### DNA sequencing, analysis and annotation of plasmid p250.

A 1.7 kb *Hind*III fragment of p250 and a 4.2 kb *Pst*I/*Eco*RV fragment were cloned into pUC19 and sequenced using universal M13 forward and reverse primers. Oligonucleotides were designed at the ends of the cloned fragments. Initially, purified plasmid p250 was used as a template in further sequencing reactions. However, the quality of sequence data obtained was very poor so the oligonucleotide primers were used to amplify the sections of the plasmid from a midiprep (Qiagen) of plasmid p250, and the PCR product was used as the sequencing template. For each sequencing reaction, three PCRs were pooled in order to minimize the effect of *Taq* errors on sequence accuracy. After each round of sequencing, new primers were designed until a complete double-stranded sequence of the plasmid was obtained. ABI Prism Big Dye Primer Cycle Sequencing Ready Reaction with AmpliTaq DNA polymerase FS' (PE Applied Biosystems) was used for DNA sequencing. Following 2-propanol precipitation, samples were sent to the Australian Genome Research Facility (AGRF) for automated sequencing using an ABI 373A automatic sequencer (PE Applied Biosystems).

Sequence data were aligned in Sequencher (Gene Codes Corporation) and annotated using MacVector version 7.0 (Accelrys Inc.). ORFs were identified using MacVector and gene identities assigned using searches against the nucleotide and protein databases at NCBI using the tBLAST-n algorithm (Altschul *et al.*, 1997). Similarity between ORFs was calculated using BLAST analysis of two sequences with filters off.

**Southern blotting.** Bacterial genomic DNA was isolated as described by Ausubel *et al.* (1994). Restriction endonuclease (*Hind*III)-digested genomic DNA was separated on 0.7% agarose gels and transferred to GeneScreen Hybridization Transfer membrane (NEN Life Science Products) by capillary action essentially as described in Sambrook *et al.* (1989). A DNA fragment including the *hmcI* gene was amplified from p250 using primers TW25 and TW27 (Table 1). Blots were hybridized with the digoxigenin (DIG)-labelled PCR product for 16 h at 65 °C. Washes and detection were carried out (using the DIG DNA Labelling and Detection Kit, Roche) as recommended by the manufacturer.

**Conjugation of plasmids into *H. paragallinarum*.** A spontaneous nalidixic-acid-resistant mutant of *H. paragallinarum* strain 221, termed 221Nal, was isolated by plating an overnight liquid culture onto TM/SN plates containing 5 µg nalidixic acid ml<sup>-1</sup>. A 4 kb *Eco*RV/*Pst*I fragment of p250 was cloned into the *Eco*RV and *Pst*I sites of plasmid pLO1, generating plasmid pLO1/5E. *H. paragallinarum* 221Nal and *E. coli* S17.1 $\lambda$ pir/pLO1/5E were grown to mid-exponential phase in supplemented TM/SN broth containing 5 µg nalidixic acid ml<sup>-1</sup> and LB supplemented with 50 µg kanamycin ml<sup>-1</sup> respectively. One millilitre of each culture was centrifuged at 8000 g, the supernatant removed and cells washed in 1 ml 10 mM MgSO<sub>4</sub>, before being resuspended in a final volume of 300 µl of 10 mM MgSO<sub>4</sub>. 221Nal cells (100 µl) were mixed with 30 µl of S17.1 $\lambda$ pir/pLO1/5E or 30 µl of 10 mM MgSO<sub>4</sub>. Each mix was pipetted onto a TM/SN plate, and the plates were briefly air-dried. Plates were incubated overnight at 37 °C in 5% CO<sub>2</sub>. Cells were scraped off into 1 ml TM/SN and serial dilutions were plated on TM/SN plates containing 5 µg nalidixic acid ml<sup>-1</sup> and 50 µg kanamycin ml<sup>-1</sup>. Plates were incubated for 24–36 h at 37 °C in 5% CO<sub>2</sub>. Kanamycin- and nalidixic-acid-resistant colonies were sub-cultured once and then used to set up 10 ml overnight broth

**Table 1.** Oligonucleotide primer pairs used in PCR

Name	Sequence (5'–3')	Binding site*	Product size (bp)
TW25	GCTACCGCAGTCGCTTCGTT	Immediately after 3' end of <i>int</i> (C)	522
TW27	CTAATGTATGCCATAACTGC	3' region of <i>hmcA</i>	
TW13	GGAGAGAACGAACAATCC	Middle of <i>int</i> (C)	490
TW26	GATACCTGTAGTGAACGAG	Immediately after 3' end of <i>hmcI</i>	
TW12	GTATCGTGAAATTATGCAAG	3' end of <i>hmcB</i>	358
TW28	GGACGTCTAATACCGTTTAT	3' end of <i>hmcA</i> (C)	
TW08	AAGCATCGTAGCAAGAGC	3' region of <i>hmcC</i> (C)	589
TW19	TGATTCTGAATCAGACTGG	Middle of <i>hmcD</i>	
TW22	CTTGATACTTCTCACTTCCGT	3' region of <i>hmcD</i> (C)	668†
TW23	AGGTGATCGGGCATAACAATG	Immediately after 3' end of <i>rep</i>	1400‡

\*(C), binds to reverse strand.

†Product from strain HP250.

‡Product from all other haemocin-producing strains.

cultures in supplemented TMB containing  $5 \mu\text{g ml}^{-1}$  and  $50 \mu\text{g kanamycin ml}^{-1}$ . Plasmids were extracted from the overnight culture using a miniprep method (Sambrook *et al.*, 1989) and digested with *Pst*I and *Hind*III. An *H. paragallinarum*-specific PCR (Chen *et al.*, 1996) was used to confirm that the cells harbouring plasmids were *H. paragallinarum*.

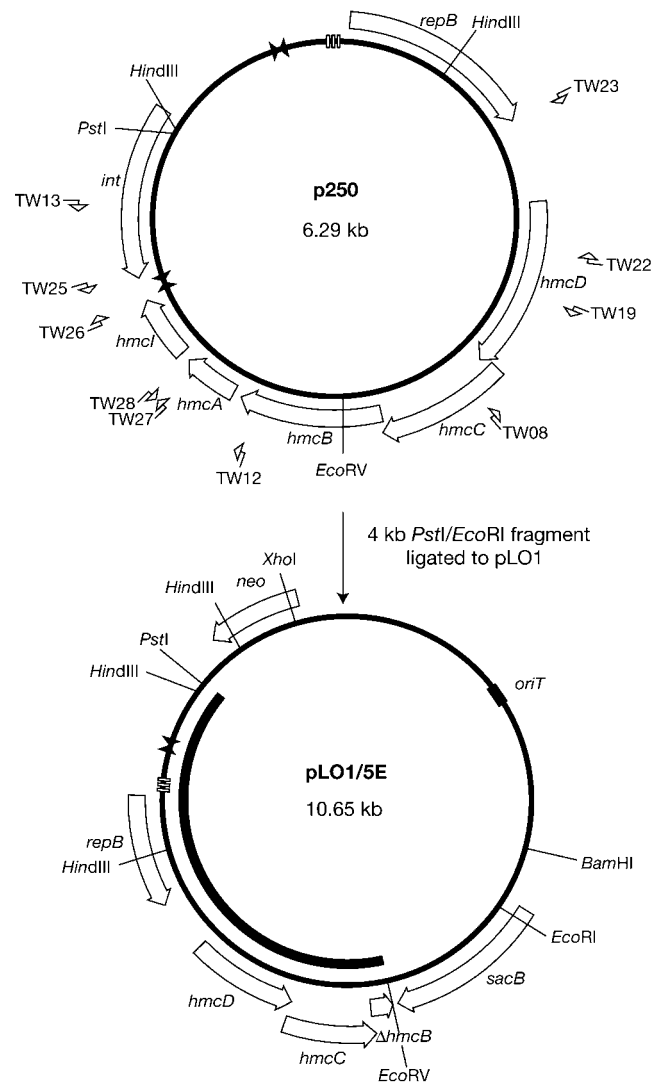
**Haemocin production and susceptibility assays.** *E. coli* strains and bacterial strains of avian origin were grown to mid-exponential phase in LB and TM/SN broth respectively. Confluent lawns of strains were made by pouring 1, 10 or 100  $\mu\text{l}$  of culture in 4 ml of TM/SN top agar (0.7%) at  $42^\circ\text{C}$  onto TM/SN plates. After cooling, 5  $\mu\text{l}$  of culture or filter-sterilized (0.22  $\mu\text{m}$  filter) culture supernatant was spotted onto the lawn and allowed to dry. After overnight incubation at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ , the agar plates were examined for clear zones around the test strains, indicative of haemocin production. Haemocin activity was scored as follows: no killing (-) (resistant); lighter zone of test strain around *H. paragallinarum* culture (+) (sensitive); inhibition of growth of test strain of <1 mm (++) (sensitive); inhibition >1 mm (+++) (highly sensitive). Sterile culture supernatants were treated with proteinase K (1 mg  $\text{ml}^{-1}$ ) at  $37^\circ\text{C}$  for 1.5 h. To test if the haemocin protein was heat stable, sterile culture supernatants were incubated at 37, 48 or  $65^\circ\text{C}$  for 1.5 h or boiled for 20 min.

**Partial purification of haemocin.** Strain HP250 was inoculated into 3 ml TM/SN broth and grown for 8 h; then the culture was added to 400 ml TM/SN broth and incubated at  $37^\circ\text{C}$  with shaking overnight. Cells were pelleted by centrifugation for 10 min at 2700 g, and the haemocin was partially purified exactly as described by Venezia *et al.* (1977). Haemocin extracts were loaded onto 4–20% Nu-PAGE Novex Bistris gels (Invitrogen) in MES buffer. SeeBlue Pre-Stained protein standards (Invitrogen) were included on the gel. An overlay assay was used to locate the haemocin protein on the gel. Gels were fixed in 50% methanol, washed for 1 h in distilled water and placed on a 10 cm diameter LB agar plate containing  $50 \mu\text{g streptomycin ml}^{-1}$ . An overlay consisting of 6 ml LB top agar (0.7% agar,  $42^\circ\text{C}$ ) containing  $50 \mu\text{g streptomycin ml}^{-1}$  and 30  $\mu\text{l}$  of a 0.5 OD<sub>600</sub> culture of S17.1 $\lambda$ pir cells was poured over the gel and allowed to set. Plates were incubated overnight at  $37^\circ\text{C}$ .

## RESULTS

### Sequence analysis and genetic organization of plasmid p250

The genetic map of plasmid p250 is shown in Fig. 1. The plasmid is 6286 bp in size and detailed analysis of the sequence revealed a haemocin operon (*hmc*), a putative integrase (*int*) and a putative replication (*repB*) protein (see Table 2). The haemocin operon is homologous to the *H. influenzae* haemocin operon (Murley *et al.*, 1997, 1998) and contains the *hmcDCBA* and *I* genes, responsible for haemocin production, processing, export and immunity. Approximately 1 kb of DNA sequence obtained has no homology with any DNA or translated protein in the GenBank database. Two very small ORFs were found in this region (5986–6072, 29 aa long; 6035–6124, 30 aa) but these may not be functional. In addition, three direct repeats (22 bp each) and an inverted repeat (15 bp each) were identified in this region. The lack of large ORFs and the presence of inverted repeats suggests that the origin of replication for plasmid p250 may be located in this region.



**Fig. 1.** Schematic map of plasmid p250 and construction of plasmid pLO1/5E. White arrows indicate putative ORFs,  $\square$  indicates direct repeats,  $\blacktriangle$  indicates inverted repeats,  $\triangleright$  indicates primer binding sites. The black arc inside the map marks the section in pLO1/5E derived from p250.

### Physical characterization of the haemocin protein, HmcA

The predicted *H. paragallinarum* HmcA protein is 90 aa in length and is highly similar (85% identity/95% similarity) to the *Haemophilus influenzae* HmcA protein (AAC46225). Murley *et al.* (1998) identified a putative double-glycine leader sequence characteristic of class II bacteriocin proteins (Havarstein *et al.*, 1994; Klaenhammer, 1993) in *H. influenzae* HmcA. Whilst the *H. influenzae* and *H. paragallinarum* HmcA proteins closely match both the Gram-positive and Gram-negative double-glycine leader sequence motifs (Michiels *et al.*, 2001), the predicted leader sequence is 50 aa long, which is considerably longer than that of

**Table 2.** Summary of genes found on the p250 plasmid of *H. paragallinarum*

Gene	Nearest homology	Protein accession no.	Putative function	Protein similarity (%)	Protein size (aa/kDa)
<i>repB</i>	<i>Mannheimia varigena</i>	CAC85833	Replication protein	72	326/38.8
<i>int</i>	<i>Franciscella tularensis</i>	AAD17308	Integrase	59	293/33.7
<i>hmcA</i> *	<i>Haemophilus influenzae</i>	AAC46225	Structural protein	95	90/9.7
<i>hmcB</i> †	<i>H. influenzae</i>	AAC46224	ABC transporter	93	235/27.2
<i>hmcC</i> ‡	<i>H. influenzae</i>	AAC46223	Haemocin processing	88	198/22.7
<i>hmcD</i>	<i>H. influenzae</i>	AAC46222	Unknown	89	292/33.7
<i>hmcI</i>	<i>H. influenzae</i>	AAC46221	Immunity protein	88	104/12.5

\*Full-length *hmcA* ORF, with ATG start codon.

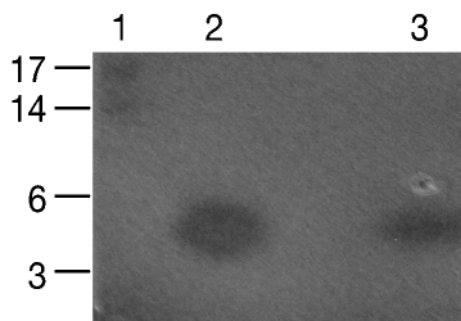
†Start codon ATG in *H. influenzae*, TTG in *H. paragallinarum*.

‡GTG start codon in both *H. influenzae* and *H. paragallinarum*.

other double-glycine leader sequences (typically 15–20 aa). Cleavage at this site results in a 40 aa mature protein. Analysis of the *H. paragallinarum* HmcA protein using SignalP (Nielsen *et al.*, 1997) identified a conventional, 19 aa long, leader, resulting in a 71 aa mature protein.

Partially purified haemocin was prepared from an HP250 culture supernatant and analysed by SDS-PAGE followed by an overlay assay to determine the location of the protein (Fig. 2). The active protein has an estimated molecular mass of 4.5 kDa, which closely corresponds to the predicted mass (4439.78 Da) for the 40 aa mature protein resulting from cleavage after the double glycine leader sequence.

To test whether the haemocin agent was protein-based, *H. paragallinarum* culture supernatants were treated with Proteinase K. Haemocin function was eliminated, indicating that the haemocin activity is mediated by a protein. In common with other bacteriocins, the haemocin activity of sterile culture supernatants was retained after incubation at 37, 48 and 65 °C for 1.5 h and boiling for 20 min.

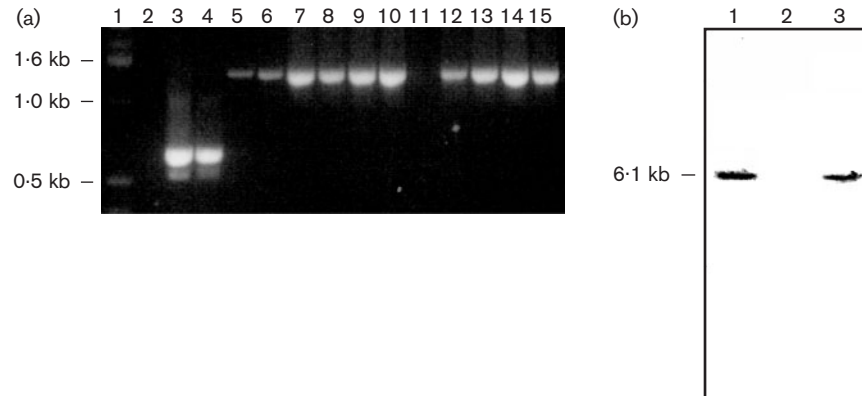


**Fig. 2.** MES-buffered SDS PAGE of the partially purified haemocin overlaid with a lawn of *E. coli* S17.1 $\Delta$ pir showing zone of inhibition. Lane 1, Pre-stained standards (kDa); lane 2, 13  $\mu$ l haemocin extract; lane 3, 2.5  $\mu$ l haemocin extract.

### Haemocin production by *H. paragallinarum* strains

A panel of strains, representing the reference strains for the Kume (Blackall *et al.*, 1990a; Kume *et al.*, 1983) and Page (Blackall *et al.*, 1990b) serotyping schemes were tested for haemocin production (Table 3). Of the 11 reference strains tested, all produced haemocin except strain 221. Strain 221 was also found to be sensitive to killing by haemocin from strain HP250, indicating that 221 has neither the genes for haemocin production nor immunity. The lack of haemocin and immunity genes in strain 221 was confirmed by PCR and Southern blotting (Fig. 3). Southern blotting also confirmed that strain 221 did not contain the *repB* gene (data not shown). A panel of eight Australian field isolates of *H. paragallinarum* (selected to represent the known serological diversity present in Australian poultry) were all found to produce haemocins with the exception of isolate HP31 (Table 3). The lack of haemocin and immunity genes in HP31 was confirmed by PCR (data not shown) and the strain was found to be sensitive to killing by haemocin from strain HP250. When combined with the two Australian serovar reference strains (Table 3), we found nine of 10 Australian *H. paragallinarum* strains produce haemocin.

In strain HP250, the haemocin genes are found on a plasmid. All the other haemocin-producing strains do not have plasmids, indicating that the genes are most probably chromosomally encoded. All of the 11 reference strains tested (Table 3) except 221 gave a 490 bp PCR product with primers TW13 and TW26 (which bind within and immediately after the end of the integrase gene respectively), a 358 bp product with primers TW12 and TW28 (which bind within *hmcB* and *hmcA* respectively), and a 589 bp product with primers TW08 and TW19 (which bind within *hmcC* and *hmcD* respectively) (data not shown). PCR using primers TW22 and TW23 (which bind within *hmcD* and immediately downstream of the putative replication protein respectively) gave a 668 bp product from purified p250 and strain HP250, but in the other 10 haemocin-producing



**Fig. 3.** (a) PCR amplification of *H. paragallinarum* strains using primers TW22 and TW23, binding *hmcD* and just outside the *repB* gene respectively. Lanes: 1, 1 kb ladder (Invitrogen); 2, negative control; 3, p250; 4, HP250; 5, 0083; 6, 0222; 7, Modesto; 8, HP14; 9, HP19; 10, 2403; 11, 221; 12, E-3C; 13, SA-3; 14, H-18; 15, 2671. (b) Southern blot of chromosomal DNA of *H. paragallinarum* strains 2403 (lane 1), 221 (lane 2) and E-3C (lane 3) digested with *Hind*III and probed with a digoxigenin-labelled PCR product containing the *hmcI* (immunity) gene.

strains a 1.4 kb fragment was obtained. This fragment was sequenced from strain 0083. The insertion consists of a 736 bp DNA sequence flanked by a 4 bp direct repeat

(TTGA). The DNA sequence is highly similar (83% identity) to the sequence of the putative integrase found in p250. However, the C-terminal region of the integrase protein is not included in the insertion, and there are two frame-shifts within the sequence. The longest ORF with an ATG initiation codon is 175 aa compared to the integrase coding sequence of 293 aa. Hence it is unlikely that this gene is functional.

**Table 3.** Haemocin activity in *H. paragallinarum* strains

Strain	Source country	Kume serovar	Haemocin activity*
0083†	USA	A-1	+
221‡	Japan	A-1	–
2403‡	Germany	A-2	+++
E-3C‡	Brazil	A-3	+
HP14‡	Australia	A-4	+++
0222†	USA	B-1	+
2761‡	Germany	B-1	++
H-18‡	Japan	C-1	++
Modesto†‡	USA	C-2	+++
SA-3‡	South Africa	C-3	+
HP60‡	Australia	C-4	++
HP250	Taiwan	A§	+++
HP19	Australia	A-4	+++
HP179	Australia	A-4	++
HP31	Australia	C-2	–
HP93	Australia	C-2	++
HP177	Australia	C-2	++
HP221	Australia	C-2	++
HP285	Australia	C-2	++
HP192	Australia	C-4	++

\*–, No killing; +, lighter zone of *E. coli* S17.1 cells around *H. paragallinarum* culture; ++, inhibition of <1 mm; +++, inhibition of >1 mm.

†Reference strain for the Page serotyping scheme.

‡Reference strain for the Kume serotyping scheme.

§Kume serovar unknown.

### Effect of *H. paragallinarum* haemocins on commensal and pathogenic chicken respiratory tract organisms

To assess whether haemocin production may have a role in colonization of the chicken respiratory tract, the ability of *H. paragallinarum* strains expressing haemocins to kill commensal and pathogenic chicken respiratory tract organisms was assessed (Table 4). All five *Pasteurella gallinarum* isolates were resistant to haemocin killing whereas all five avian *Pasteurella haemolytica* isolates tested were sensitive to haemocin killing. The effect of haemocins on *Pasteurella multocida* strains varied: four strains were resistant, three strains were sensitive, and one strain highly sensitive to haemocin-mediated killing. In contrast, commensal Gram-negative organisms (*Pasteurella avium*, *Pasteurella volantium* and *Pasteurella* species A) were found to be sensitive to haemocin-mediated killing. A panel of 11 different Gram-positive strains, isolated from the sinus of healthy chickens, were all resistant to haemocin-mediated killing.

### Function of the putative origin of replication of p250 in *E. coli*

To test if the putative origin of replication (*ori*) between the integrase and *repB* genes in p250 was functional in *E. coli*, a large *Pst*I/*Eco*RV fragment of the plasmid (Fig. 1) encoding part of the integrase, the putative *ori* region, *repB*, *hmcD*

**Table 4.** Haemocin sensitivity of organisms that colonize the chicken respiratory tract

Bacterial strain	Haemocin sensitivity*
<b>Gram-negative chicken pathogens</b>	
<i>Pasteurella gallinarum</i>	
BR242, BR244, BR249, BR250, BR251	—
<i>P. haemolytica</i>	
BR252, BR248, BR243, BR246, BR247	++
<i>P. multocida</i>	
PM88	+++
PM83, PM120, PM151	+
PM63, PM64, PM67, PM76	—
<b>Gram-negative commensal organisms</b>	
<i>P. avium</i>	
ATCC 29546, HP63	+
<i>P. volantium</i>	
HP37, HP63	+
<i>Pasteurella</i> species A	
HP23, HP119	+
<b>Gram-positive commensal organisms</b>	
BR263, BR264, BR265, BR266, BR267, BR268, BR269, BR270, BR271, BR272, BR273	—

\*—, No killing; +, lighter zone in test strains around *H. paragallinarum* culture; ++, inhibition of <1 mm; +++, inhibition of >1 mm.

and *hmcC* was cloned into plasmid pCVD442, which contains a *pir*-dependent *oriR6K*, and is maintained in DH5 $\alpha$ *pir* cells. The resulting plasmid, pCVD5E, was electroporated into *E. coli* DH5 $\alpha$  cells and plated on LB agar supplemented with 100  $\mu$ g ampicillin ml<sup>-1</sup>. No ampicillin-resistant colonies were found, indicating that the putative *ori* region is not functional in *E. coli*.

### Construction of an *E. coli*–*H. paragallinarum* shuttle vector

The *Pst*I/*Eco*RV fragment containing the putative p250 origin of replication (Fig. 1) was cloned into plasmid pLO1, resulting in a kanamycin-resistant plasmid with a ColE1-based origin of replication, pLO1/5E (Fig. 1). Plasmid pLO1/5E was transformed into the conjugative *E. coli* donor strain S17.1 $\lambda$ *pir* and the resulting strain was used in a conjugation with nalidixic-acid-resistant 221Nal (haemocin-negative) *H. paragallinarum* cells. After overnight conjugation followed by a 36 h incubation on TM/SN plates containing both nalidixic acid and kanamycin, numerous colonies were obtained on plates from conjugations where 221Nal was mixed with the *E. coli* donor strain harbouring pLO1/5E, but no colonies were obtained on plates from the 221Nal- or S17.1 $\lambda$ *pir*/pLO1/5E-only controls. Nalidixic-acid- and kanamycin-resistant colonies were subcultured once and were confirmed as *H. paragallinarum* cells by PCR and the absence of growth on LB agar plates. Plasmids with

the identical restriction pattern to pLO1/5E were extracted from all five colonies tested, indicating that pLO1/5E can be transferred into *H. paragallinarum* strain 221Nal by conjugation and stably maintained under antibiotic selection.

## DISCUSSION

Plasmid p250 contains seven ORFs, five of which are highly similar to the haemocin production locus of *H. influenzae*. The presence of both the haemocin structural gene and the haemocin immunity gene, which protects cells from destruction by haemocin, ensures that the plasmid is maintained. Any cells that have lost the plasmid in a population of HP250 would be destroyed by haemocin secreted by plasmid-containing cells. In all other strains of *H. paragallinarum* tested, except 221 and HP31, which lacked haemocin, the haemocin locus appears to be located on the bacterial chromosome. The integrase region of the plasmid is also included in the chromosomal insertion of the haemocin locus, suggesting that at some stage the p250 plasmid has either integrated into the *H. paragallinarum* chromosome in most strains, or been excised from the chromosome in strain HP250.

In *H. influenzae*, the haemocin locus is also found on the bacterial chromosome of type b stains. In a survey of type b strains, 93 %, representing 25 of 29 unique electrophoretic types (ETs) found to be clonally distinct by multilocus enzyme electrophoresis (MLEE) produced haemocin, and only type b strains from highly divergent ETs did not produce haemocins (LiPuma *et al.*, 1990). Although these four non-haemocin-producing ETs represent genetically highly divergent strains, ETs that are closely related to these divergent ETs do produce haemocin, so in *H. influenzae* there is no clear correlation between extent of genetic divergence and haemocin production. There is no evidence that either strain 221 or isolate HP31 represent an unusual genotype of *H. paragallinarum*. Australian isolates of *H. paragallinarum* have a limited genetic diversity, with the 10 isolates, including HP31, examined by restriction endonuclease analysis showing very limited pattern variation as compared with a selection of 15 overseas isolates (Blackall *et al.*, 1991). In an MLEE study involving 118 strains and isolates, all 49 Australian isolates (HP31 was not examined) and strain 221 were allocated to the dominant MLEE Cluster A, which consisted of 85 isolates from six continents that formed seven ETs (Bowles *et al.*, 1993).

The mechanism of action of the haemocin protein is unknown. Early work by Streker *et al.* (1978, 1981) indicated that the haemocin of *H. influenzae* caused elongation of sensitive *H. influenzae* cells and inhibited DNA synthesis but not RNA or protein synthesis. The proposed 4.4 kDa (40 aa) mature haemocin protein has a predicted pI of 11.29 and has six Lys or Arg residues. The haemocin is heat stable, but activity is destroyed by proteinase K. There are four Cys residues in the mature protein that may contribute to the retention of activity after heating. Mature HmcA

has a molecular mass of approximately 4.5 kDa, consistent with cleavage after the double-glycine motif. Initiation of translation at the ATG start codon assigned by Murley *et al.* (1998) would result in an unusually long (50 aa) leader sequence. In *H. paragallinarum*, there is an additional potential start codon for the *hmcA* gene 30 bp upstream of this codon, which is not found in *H. influenzae*. Murley *et al.* (1998) suggested that the *H. influenzae* *hmcA* gene could be initiated from a GTG codon downstream of the ATG, resulting in a 27 aa leader peptide. In *H. paragallinarum*, the equivalent codon is CTA and it is not known if this codon can function as a start codon in *H. paragallinarum*.

Little is known about how the Hmcl protein product confers immunity to haemocin-mediated killing. The *H. paragallinarum* Hmcl is a small (104 aa) protein, highly similar to the Hmcl protein from *H. influenzae* (80 % identity, 88 % similarity) and, like the Hmcl protein, is highly positively charged (20 % Lys or Arg), with a predicted pI of 9.74. The *H. influenzae* Hmcl ORF is 105 aa in length. However, there is an alternative Met initiation codon located upstream of both the *H. influenzae* and *H. paragallinarum* ORFs, giving an Hmcl protein of 119 or 118 aa in length, respectively. However this ORF may not be functional, as the start codon is 4 bp upstream of the RNA transcript start determined by Murley *et al.* (1997). Both the *H. influenzae* and *H. paragallinarum* Hmcl proteins have identical KHKRKAKK Lys-rich motifs. The Hmcl proteins also share homology with an uncharacterized protein from *Mesorhizobium loti* (31 % identity, 53 % similarity, NP 108470).

Murley *et al.* (1997) identified a  $\sigma^{70} - 10$  region but could not identify a  $-35$  region upstream of the *H. influenzae* *hmcI* transcription start site and showed that the Hmcl protein could be expressed from this promoter in *E. coli* and provide protection from haemocin-mediated killing. However, when the *H. paragallinarum* *hmcI* gene was cloned into the same vector (pGEM-T Easy, Promega) the resulting plasmid was unable to protect against haemocin-mediated killing (data not shown), suggesting that the *H. paragallinarum* *hmcI* gene does not have a promoter immediately upstream. Differences in the  $\sigma^{70} - 10$  region identified by Murley *et al.* (1997) (TAAAAAT) and the same region of the *H. paragallinarum* sequence (TAAATTT) or the as-yet-unidentified  $-35$  region of the gene may account for the lack of expression from the *H. paragallinarum* construct in *E. coli*.

We tested a number of close relatives of *H. paragallinarum*, all members of the family *Pasteurellaceae*, for sensitivity to the *H. paragallinarum* haemocin. While one of the phylogenetically most closely related species to *H. paragallinarum* was uniformly resistant to the haemocin (*P. gallinarum*) other close relatives were sensitive (e.g. *P. avium*, *P. volantium* and *Pasteurella* species A). Hence, from the small number of strains tested here there appears to be no underlying phylogenetic association in the family *Pasteurellaceae* in terms of sensitivity to the *H. paragallinarum* haemocin.

The haemocin produced by *H. paragallinarum* may play a role in enhancing colonization of the sinus of the chicken. We found that isolates of *P. avium*, *P. volantium* and *Pasteurella* species A, all non-pathogenic Gram-negative bacteria that are commonly found in the upper respiratory tract of chickens suffering from respiratory disease due to other agents (Blackall *et al.*, 1997), were sensitive to the *H. paragallinarum* haemocin. Similarly, around 50 % of the isolates of the primary pathogen *P. multocida* and all five isolates of the secondary pathogen *P. haemolytica* we tested were sensitive to the haemocin. The 11 Gram-positive commensal bacteria isolated from healthy chickens that we examined were selected to represent a cross-section of the different normal flora present in the healthy chicken sinus and included isolates that appeared to be members of the genera *Micrococcus*, *Staphylococcus* and *Streptococcus*. All of these Gram-positive bacteria were resistant to the haemocin. When the sinus of a healthy chicken is cultured, the resultant organisms tend to be dominated by Gram-positive bacteria, while cultures of sinuses of chickens suffering respiratory disease tend to be dominated by Gram-negative organisms. Our results for the activity of the *H. paragallinarum* haemocin would tend to suggest that the haemocin may aid *H. paragallinarum* in colonizing the sinus of the chicken by helping to inhibit the growth of some of the other Gram-negative bacteria that are associated with respiratory disease in chickens.

The putative integrase gene found in plasmid p250 is also found in all haemocin-producing strains tested, including those that encode the haemocin operon on the chromosome. Downstream of the integrase are two 17 bp inverted repeats (AATCCCCGTGATTATTA) separated by 11 bp of unknown function. The integrase gene may be involved in the integration of the haemocin operon into the *H. paragallinarum* chromosome. An additional 'remnant' integrase is located between the *repB* and *hmcD* genes in strains where the haemocin operon is chromosomally located.

Plasmid p250 replication appears to be mediated by a replication initiation protein (RepB). The plasmid includes an ORF for a putative 326 aa protein which is closely related to plasmid RepB-like replication proteins from *Mannheimia varigena* (54 % identity, 72 % similarity) and the plasmid pFA3 replication protein from *Neisseria gonorrhoeae* (54 % identity, 72 % similarity). The RepB family of replication proteins possess nicking-closing (topoisomerase I)-like activity and are involved in initiation of plasmid replication via the theta mechanism (del Solar *et al.*, 1998). The non-coding region in plasmid p250 between the *repB* and *int* genes is 1074 bp in length. This region includes three adjacent 22 nucleotide repeats (GGTATAGAAAATGCG-GTCAAT), similar to those of repeat-containing (iteron) origins of DNA replication, and may function as binding sites for the RepB protein (del Solar *et al.*, 1998). The presence of a RepB homologue and iteron-type repeats suggests that p250 replicates by a theta mechanism. There is also a 15 bp inverted repeat (TAAAATCCGTCATTC

separated by 21 bp) of unknown function in the non-coding region of p250.

The p250 replication origin does not function in *E. coli*. However, an *E. coli*-*H. paragallinarum* shuttle vector has been developed and can be readily transferred by conjugation into *H. paragallinarum* strain 221NaI. This is believed to be the first report of a shuttle vector system for *H. paragallinarum* and will facilitate the genetic manipulation of *H. paragallinarum* to investigate pathogenesis of the organism.

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