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Molecular Characterization of a Ribonucleotide Reductase (*nrdF*) Gene Fragment of *Mycoplasma hyopneumoniae* and Assessment of the Recombinant Product as an Experimental Vaccine for Enzootic Pneumonia

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A *Mycoplasma hyopneumoniae* clone bank was screened with hyperimmune pig serum. One clone exhibited sequence homology to the prokaryotic R2 subunit of ribonucleotide reductase and was expressed as an 11-kDa protein fused to β-galactosidase. The vaccine potential of the fusion protein was assessed in pig trials. Following experimental challenge with a virulent isolate of *M. hyopneumoniae*, gross lung pathology (mean Goodwin lung score) of vaccinated animals, irrespective of adjuvant treatment, was significantly reduced compared with that of control unvaccinated pigs ($P < 0.05$).

Mycoplasma hyopneumoniae is the causative agent of porcine enzootic pneumonia, a chronic respiratory disease which affects between 30 and 80% of pigs (33) and inflicts severe economic burden on pig producers through reduced growth rates (8, 16, 24, 27). Pigs previously infected with *M. hyopneumoniae* recover with time and are highly resistant to subsequent reinfection (18, 19). Bacterins with proven efficacy have been developed for the control of porcine enzootic pneumonia (5, 23, 25); however, variation in efficacy of such vaccines, the cost of components, and the labor-intensive mode of delivery may in practice prove them to be cost prohibitive. In this report we describe the molecular and immunological analysis of an 11-kDa antigen with amino acid sequence homology to the R2 subunit of the prokaryotic class I ribonucleotide reductases. This recombinant vaccine has been shown to protect pigs from lung damage when assessed after experimental challenge with virulent *M. hyopneumoniae*.

M. hyopneumoniae J, the virulent Beaufort strain of *M. hyopneumoniae*, *Mycoplasma hyorhinis* GDL, and *Mycoplasma flocculare* Ms42 were provided by A. Pointon (Central Veterinary Laboratory, Adelaide, Australia). Porcine mycoplasmas were cultured in modified Friis medium (32). Chromosomal DNA was extracted by the method of Razin et al. (28). A recombinant gene library was constructed by ligating *Sau*3AI-digested *M. hyopneumoniae* J chromosomal DNA into the *Bam*HI site of the expression plasmids pEX1 to pEX3 (35). Ligation into this site fuses the inserted fragment to the 3′ end of the *lacZ* gene, resulting in an open reading frame which, if in phase, will be expressed as a hybrid β-galactosidase protein. After transformation into *Escherichia coli* MC1061(pCI857) (22), recombinant colonies were induced at 42°C for 2 h, lysed, and then screened for recombinant protein expression with porcine hyperimmune *M. hyopneumoniae* antiserum (32). A positive clone containing a 0.8-kb DNA insert was isolated and designated pSD9. Following heat induction at 42°C, Western blotting (immunoblotting) (1, 20, 30) of *E. coli* MC1061 (pCI857, pSD9) whole-cell extracts with porcine hyperimmune *M. hyopneumoniae* antiserum localized a strongly immunoreactive band at 128 kDa consisting of β-galactosidase (117 kDa) and a recombinant *M. hyopneumoniae* protein (11 kDa). The same antiserum did not react against *E. coli* MC1061(pCI857, pEX1) (results not shown). The 128-kDa protein was purified by urea extraction (15) and used to generate rabbit polyclonal antiserum for immunological analysis (12). Cross-reactivity against β-galactosidase was minimized by extensive absorption with β-galactosidase-impregnated nitrocellulose. Coomassie blue R250 staining of sodium dodecyl sulfate-polyacrylamide gels and Western blot analysis (using the rabbit antiserum) of *E. coli* cells containing pSD9 detected the 128-kDa fusion protein (Fig. 1, lanes 3), whereas *E. coli* MC1061(pCI857, pEX1) expressed only β-galactosidase (Fig. 1, lanes 2). Residual activity against β-galactosidase was observed after preabsorption of the rabbit antiserum in the Western blot analysis (Fig. 1B, compare lanes 2 and 3). Immunoreactive bands of less than 128 kDa identified in Fig. 1B, lanes 3 and 4, are most likely due to the presence of breakdown products of the 128-kDa fusion protein. In whole-cell *M. hyopneumoniae* protein preparations a single 42-kDa protein was identified by Western blot analysis (Fig. 1B, lane 5). The specificity of the 42-kDa protein in the Western blot was shown to be due to the *M. hyopneumoniae* component of the fusion protein, as no cross-reactivity was found with rabbit β-galactosidase antiserum against the 42-kDa *M. hyopneumoniae* protein (results not shown).

The 0.8-kb DNA fragment was cloned into pHSG398 (36) to form pKF1. This plasmid was used as a template to determine the complete nucleotide sequence (31) for both strands of the *M. hyopneumoniae* DNA fragment. The sequence length was

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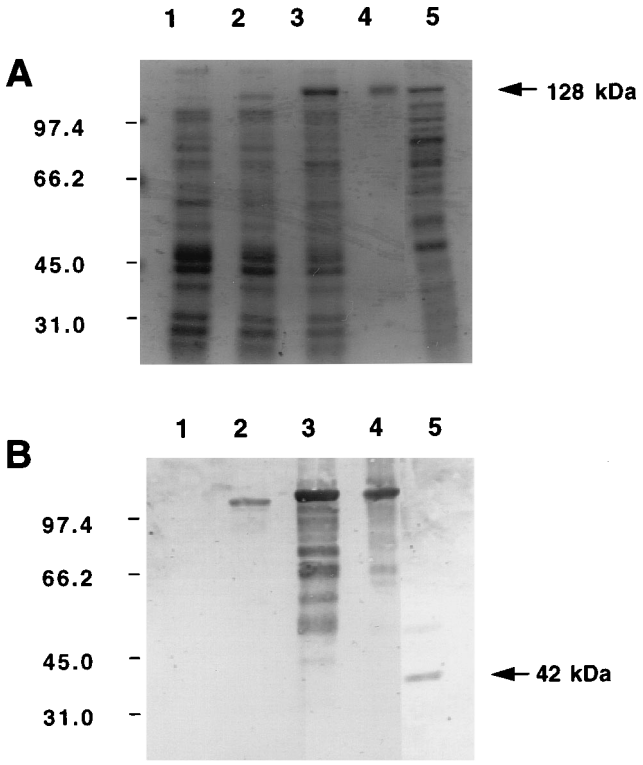


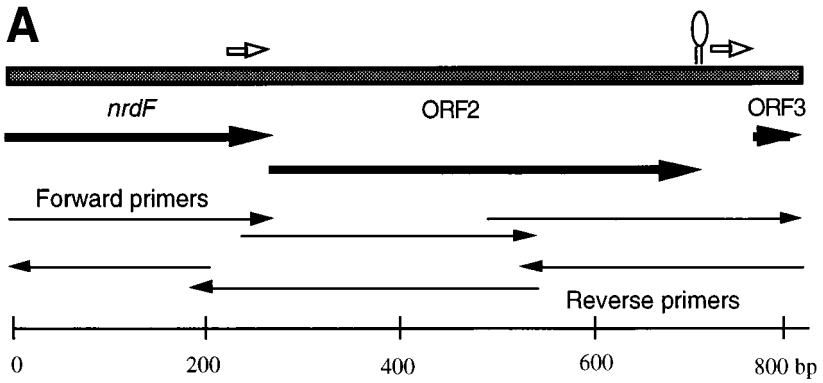
FIG. 1. Expression and identification of the *M. hyopneumoniae* fusion protein. (A) Coomassie brilliant blue-stained 11% polyacrylamide gel. Lane 1, MC1061(pCI857); lane 2, MC1061(pCI857, pEX1) (induced); lane 3, MC1061(pCI857, pSD9) (induced); lane 4, purified *M. hyopneumoniae* 128-kDa fusion protein; lane 5, whole-cell extract of *M. hyopneumoniae* J. Positions of molecular size standards (in kilodaltons) and the *M. hyopneumoniae* 128-kDa fusion protein are indicated. (B) Western blot analysis of equivalent protein samples described for panel A using rabbit *M. hyopneumoniae* 128-kDa fusion protein antiserum. The positions of molecular size markers and the 42-kDa wild-type protein present in *M. hyopneumoniae* whole-cell extracts are indicated.

found to be 822 bp with a GC content of 30.0%, which is typical of mycoplasmal genomes (13). Translation analysis of the 0.8-kb sequence revealed the presence of three open reading frames, designated *nrdF*, ORF2, and ORF3 (Fig. 2B). The *nrdF* gene is incomplete at the 5′ end, is read in frame with the β-galactosidase protein, is 96 amino acids in length, and ends with a TAA stop codon. A region of dyad symmetry which may act as a transcriptional terminator was not found downstream of *nrdF*, suggesting that *nrdF* and ORF2 are organized in an operon. The conserved 8-nucleotide sequence 5′-ATTGGGA G-3′ is present 7 nucleotides upstream of the ATG methionine start codon of ORF2 and ORF3. In both cases the intervening sequence is composed exclusively of A and T residues, indicative of mycoplasma ribosome binding sites (9, 37, 40). This sequence contains 4 nucleotides which can base pair with the conserved sequence 3′-CCUCCA-5′ at the 3′ end of the *M. hyopneumoniae* 16S rRNA. This degree of homology is considered sufficient to initiate translation in *E. coli* (37).

The translated product of *nrdF* shows homology to the NrdF protein of *Salmonella typhimurium* (17) (EMBL accession number X73226), an open reading frame product which is encoded by the 5′ region of the *proU* operons of *E. coli* and *S. typhimurium* (EMBL accession number X52693) (4, 11, 21). The putative *M. hyopneumoniae* NrdF protein also demonstrates homology to an open reading frame product encoded by the *esf2* random clone sequence of *Mycoplasma genitalium* (GenBank accession number U01739) (26). Homology comparisons scoring identical amino acids (Fig. 3) between the *M. hyopneumoniae* NrdF protein and the *S. typhimurium* and *E. coli* proteins gives 48.4 and 51.5%, respectively; if conserved amino acids are considered, the homology rises to 86.6 and 87.6%, respectively. The translated product of *M. hyopneumoniae nrdF* has 75.0% homology (97.9% if conserved amino acids are considered) to the protein encoded by *esf2*. In all the above reading frames the protein terminates with the same carboxy-terminal amino acid. The 5′ *proU* sequences of *S. typhimurium* and *E. coli* (4, 11, 21) correspond to the *S. typhimurium nrdF* sequence (17) and should therefore be considered to be the same gene. *S. typhimurium* NrdF functions as an R2 ribonucleotide reductase subunit; this class of enzymes is responsible for the conversion of ribonucleotides to deoxyribonucleotides for use in DNA synthesis (38). *S. typhimurium* NrdF can also detoxify hydroxyurea, thus conferring resistance against this potent DNA synthesis inhibitor (17, 29). The 42-kDa protein identified in this study may play a similar role in *M. hyopneumoniae*. There was no significant protein homology between the putative *M. hyopneumoniae* protein encoded by ORF2 and other known protein sequences deposited in GenBank. ORF3 is incomplete at the carboxyl terminus and is composed of only 8 amino acids. However, the presence of the conserved putative ribosome binding site and a putative −10 promoter sequence (5′-TATAAT-3′) 33 bp upstream of the ATG start codon of ORF3 indicates that this is the beginning of a new open reading frame (Fig. 2). As for the *rrn* promoter of *M. hyopneumoniae*, a −35 consensus sequence was not found upstream of the ORF3 −10 sequence (37).

Southern hybridization analysis (34) of *M. hyopneumoniae* (strain J), *M. flocculare* (strain Ms42), and *M. hyorhinis* (strain GDL) chromosomal DNA (Fig. 4A) was carried out using the 0.8-kb *M. hyopneumoniae* DNA fragment as a probe. This DNA probe hybridized strongly to a 2.45-kb *Hin*dIII-restricted *M. hyopneumoniae* chromosomal fragment and reacted weakly against a 5.7 kb-*Hin*dIII-restricted *M. flocculare* chromosomal fragment (Fig. 4B). Following overexposure of the autoradiograph, the 0.8-kb fragment hybridized with an *M. hyorhinis* chromosomal DNA fragment of 2.6 kb (result not shown). PCR (30) primers 1469 (5′-TTTGCCGAACCTTTTTACA-3′) and 1470 (5′-AATCAGCAATAAAATTTAGC-3′) generated a 750-bp PCR product from *M. hyopneumoniae* chromosomal DNA (Fig. 4C). Under all conditions in which amplification of the 750-bp fragment was achieved with *M. hyopneumoniae* DNA, an identically sized fragment was amplified with *M. flocculare* chromosomal DNA as a template (Fig. 4C). Thus, although these primers clearly differentiate between *M. hyopneumoniae* and *M. hyorhinis* reference strains, the usefulness of such primers for routine diagnosis is limited because of cross-reaction with *M. flocculare*.

The *M. hyopneumoniae* NrdF fusion protein was purified after induction of *E. coli* MC1061(pCI857, pSD9) for 3 h at 42°C. Induced cells were collected, reduced, and fractionated with a 491 Bio-Rad PrepCell column (37-mm internal diameter) containing a 5% polyacrylamide matrix with a 4% stacking gel. Fractions (10 ml) were routinely monitored for the elution of the 128-kDa NrdF fusion protein by Western blotting using rabbit NrdF fusion protein antiserum. Samples containing the NrdF fusion protein were pooled and concentrated by ultrafiltration. NrdF fusion protein stock antigen for vaccination studies was estimated by measuring the A_{280} to contain 4.4 mg of protein per ml of PBST buffer (phosphate-buffered saline [PBS; pH 7.2], 0.2% [vol/vol] Tween 80), except for algammulin-based vaccines, which were prepared phosphate free and utilized the same stock concentration of NrdF fusion protein



B

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                                                                    50
GAT CTA TTA TAT AAA CTA ATT GAA TTA GAA AAA GAT TAT CTC TAT GAT TTA TAT
Asp Leu Leu Tyr Lys Leu Ile Glu Leu Glu Lys Asp Tyr Leu Tyr Asp Leu Tyr
                                                                   100
TCT GAA GTT GGA CTT GCT GAA TCA GCA ATA AAA TTT AGC ATT TAT AAT GCC GGG
Ser Glu Val Gly Leu Ala Glu Ser Ala Ile Lys Phe Ser Ile Tyr Asn Ala Gly
                                                                   150
AAA TTC TTG CAA AAT CTA GGA TAT GAT TCA CCT TTT TCA AAA GAG GAA ACC GAA
Lys Phe Leu Gln Asn Leu Gly Tyr Asp Ser Pro Phe Ser Lys Glu Glu Thr Glu
                                                                   200
ATT GAA CCT GAA ATT TTT AGT CAA TTA TCG GCT CGA GCT GAT GAA AAT CAT GAC
Ile Glu Pro Glu Ile Phe Ser Gln Leu Ser Ala Arg Ala Asp Glu Asn His Asp
                                                                   250
TTT TTT TCA GGA AAT GGC TCT TCT TAT GTA ATG GCG CTT GCT GAA GAA ACC GAA
Phe Phe Ser Gly Asn Gly Ser Ser Tyr Val Met Ala Leu Ala Glu Glu Thr Glu
                                                                   300
GAT GAA GAT TGG GAG TTT TAA A ATG ACA AAT GAT GAA AAA TAT GAT ATA ATA
Asp Glu Asp Trp Glu Phe Stop   Met Thr Asn Asp Glu Lys Tyr Asp Ile Ile
                                                                   350
AAC AAA TCT AAA TTA AGA GGA GAA ATT TTT GTT GTT TAT TTT TCC TCA ATT TCG
Asn Lys Ser Lys Leu Arg Gly Glu Ile Phe Val Val Tyr Phe Ser Ser Ile Ser
                                                                   400
AAT AAT ACC CAT CGG TTT GTC GAA AAA TTA AAC TTT GAA AAA GCA AGA ATT CCT
Asn Asn Thr His Arg Phe Val Glu Lys Leu Asn Phe Glu Lys Ala Arg Ile Pro
                                                                   450
GTT GAG ATT GAC CAA GAA TTA GTA GTT GAT AAA GAT TAT GTA CTT TTT TGT CCA
Val Glu Ile Asp Gln Glu Leu Val Val Asp Lys Asp Tyr Val Leu Phe Cys Pro
                                                                   500
ACT TAT AGT GGC GGG AAA GGT CTA AGA AGT GGG GCT GTC CCA AAA CAG GTA
Thr Tyr Ser Gly Gly Lys Gly Leu Arg Ser Gly Ala Val Pro Lys Gln Val
                                                                   550
ATA AAA TTT TTA AAC AAT GAA CAA AAT CGT CGA TTT TGT AAA GCT GTA ATC GCT
Ile Lys Phe Leu Asn Asn Glu Gln Asn Arg Arg Phe Cys Lys Ala Val Ile Ala
                                                                   600
TCT GGA AAC ACT AAT TTT GGC GAT ACT TTT GCG CTT GCA GGC ACA ATT ATT TCC
Ser Gly Asn Thr Asn Phe Gly Asp Thr Phe Ala Leu Ala Gly Thr Ile Ile Ser
                                                                   650
CAA AAA TTA AAA GTT CCT TTT TTA TAT AGT TTT GAA TTA TTA GGA ACA AAT GAT
Gln Lys Leu Lys Val Pro Phe Leu Tyr Ser Phe Glu Leu Leu Gly Thr Asn Asp
                                                                   700
GAT GTA AAA AAG GTT CGG CAA ATT TTA GAA GAT TTT TGA GGA AAA TAA
Asp Val Lys Lys Val Arg Gln Ile Leu Glu Asp Phe Trp Gly Lys Stop
                                                                   750
                                  ---------->      <----------
GCTAA AAAAT AATGA ATATA ATAGT TAGTT CGCTA ACTAA ATTGG GAGAA
                                                                   800
AATAA ATG AAA AAA GTG GGT TTG GAT C
      Met Lys Lys Gly Cys Asp Val
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FIG. 2. DNA sequence analysis of the cloned 0.8-kb *M. hyopneumoniae* chromosomal DNA fragment. (A) Diagrammatic representation of the strategy used to obtain the nucleotide sequence of the 0.8-kb *M. hyopneumoniae* DNA fragment (shaded). Filled arrows indicate the positions of open reading frames, the first of which has sequence homology to the *nrdF* gene of *S. typhimurium*. Thin arrows indicate the extent of the sequence reaction from synthesized oligonucleotide primers. The open arrows indicate the positions of putative *M. hyopneumoniae* ribosome binding sites, while the keyhole symbol indicates the presence of inverted repeats. Scale is given underneath in base pairs. (B) Nucleotide sequence of the *M. hyopneumoniae* DNA fragment. Three open reading frame products are given three-letter amino acid codes. Underlining denotes the conserved 8-nucleotide sequence which is 7 bases upstream of the ATG start codons. Dashed arrows above the sequence indicate an inverted repeat structure.

solubilized in 0.85% NaCl containing 0.2% (vol/vol) Tween 80 (pH 7.4). Pigs (n = 18) used for vaccination studies were from an *M. hyopneumoniae*-free piggery and were randomly allocated into pens of 4 to 5 pigs. All pigs were housed in an experimental piggery which had no other resident pigs and were fed an antibiotic-free commercial pelleted grower ration. Pigs (n = 12) were inoculated intramuscularly at 42 and 75 days of age with NrdF fusion protein (1 mg per pig) complexed with one of four different adjuvants (3 pigs per adjuvant): alhydrogel (Cyanamid Websters), algammulin (3), DEAE-dextran-mineral oil (14), and DEAE-dextran-Auspharm vegetable oil (Auspharm International). All pigs, including six control animals, were challenged at 85 days of age with 20 ml of lung homogenate (equivalent to 9.2 g [wet weight] in 20 ml of PBS per pig) containing the virulent Beaufort strain (7) of *M. hyopneumoniae*. Prior to challenge, all pigs were sedated with

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                        10                  20
M.h  D L L Y K  L I E L E  K D Y L Y  D L Y S E  V G L A E
M.g  e L m Y r  L I E L E  K D Y L e  e L Y e g  f G i v d
E.c  D L L l e  L y d n E  l g Y t d  e L Y a E  t p w A d
S.t  D L L m e  L y d n E  i r Y t e  a L Y a E  t G w v n

              30                  40                  50
M.h  S A M K F  I - Y N A  G K F L Q  S N L G Y  D S P F S
M.g  d A i K F  s V Y N A  G K F L Q  - N L G Y  D S P F t
E.c  d v k a F  n - a N K  a L M L c  Y N L G Y  e p l F p
S.t  d v k a F  n - a N K  a L M L c  Y N L G Y  e a l F p

                        60                  70
M.h  K E E T E  I E P E I  F S Q L S  A R A D E  N H D F F
M.g  a a E T r  I E P E I  F a Q L S  A R A D E  N H D F F
E.c  a E m a E  v n P a I  l a a L S  p n A D E  N H D F F
S.t  p E m a d  v n P a I  l a a v S  p n A D E  N H D F F

              80                  90
M.h  S G N G S  S Y V M A  L A E E T  E D E D W  E F
M.g  S G N G S  S Y V M g  v s E E T  n D E D W  n F
E.c  S G s G S  S Y V M g  k A v E T  E D E D W  E F
S.t  S G s G S  S Y V M g  k t v E T  E D E D W  E F
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FIG. 3. Sequence homology for the *M. hyopneumoniae* (*M.h*) NrdF 11-kDa protein aligned with the product of the gene immediately upstream of the *E. coli proU* (*E.c*) operon, *S. typhimurium* NrdF protein (*S.t*), and *M. genitalium esf2* gene product (*M.g*). Homology to identical amino acids is 51.5, 48.4, and 75.0%, respectively; however, when conserved amino acids are considered, homology rises to 87.6, 86.6, and 97.9%, respectively. Amino acids are given in one-letter codes. Identical amino acids are in boldface capital letters, conserved amino acids are in underlined boldface lowercase letters, and variant amino acids are denoted by lowercase letters. DNA sequences were compared by using the Australian National Genomic Information Service to search the GenBank sequence database. Homology was calculated with the MacVector sequence analysis program, version 3.5 (IBI-Kodak).

azaperone and anesthetized by induction with halothane and nitrous oxide via face mask and then more deeply anesthetized (sufficient for intubation) with thiopentone (3.5 mg/kg of body weight) given through a marginal ear vein. Animals were weighed weekly and slaughtered at 126 days of age, when lungs were collected for Goodwin lung scoring (10). Samples of infected lung containing lesions were cultured for the bacteria *Pasteurella multocida* (2) and *Actinobacillus pleuropneumoniae* (39), which are commonly associated with porcine pneumonia; neither organism was detected. The effects on lung score of vaccination and of adjuvants within the vaccinated groups were assessed in an analysis of variance of logistic-transformed scores in which tests of significance were performed using an estimate of experimental error pooled from this and four similar trials (6). Pigs vaccinated with NrdF fusion protein (n = 12) had a significantly lower ($P < 0.05$) mean logistic-transformed lung score (mean lung score = 8.0; -2.02 ± 0.27 standard error) than the unvaccinated controls (n = 6) (mean lung score = 15.3; -0.97 ± 0.38 standard error), but there were no significant differences detected among the four adjuvant groups (alhydrogel, algammulin, DEAE-dextran-mineral oil, and DEAE-dextran-Auspharm vegetable oil). The mean average daily live weight gain of vaccinates (0.562 ± 0.024 kg/day) was not significantly greater ($P > 0.05$) than that of the controls (0.506 ± 0.034 kg/day), and no significant differences in gain were detected among the four adjuvant treatment groups. We are currently assessing the protective efficacy of the 11-kDa NrdF antigen in combination with other antigens derived from *M. hyopneumoniae*.

While a number of bacterins are currently available or under evaluation for the control of porcine enzootic pneumonia (5, 23, 25), the particle size of such vaccines precludes entrapment within certain novel adjuvant delivery systems which can target specific arms of the effector immune system. Identification and cloning of protective antigens of *M. hyopneumoniae* provide the basis for the development of new generation vaccines against enzootic pneumonia.

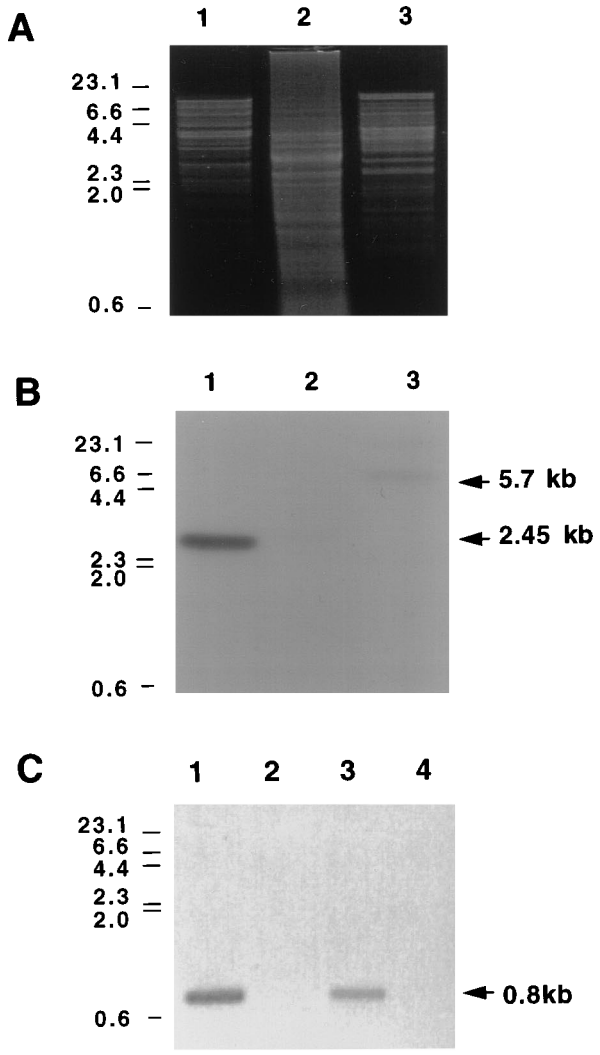

FIG. 4. Southern hybridization and PCR analysis of *M. hyopneumoniae* DNA, *M. hyorhinis* DNA, and *M. flocculare* DNA. (A and B) Lanes 1, *M. hyopneumoniae* chromosomal DNA digested with *Hin*dIII; lanes 2, *M. hyorhinis* chromosomal DNA digested with *Hin*dIII; lanes 3, *M. flocculare* chromosomal DNA digested with *Hin*dIII. Molecular size markers (in kilobases) are indicated. (A) Ethidium bromide-stained 1% agarose gel; (B) Southern hybridization analysis using the 0.8-kb DNA fragment as a probe. Arrows indicate the 2.45-kb *Hin*dIII *M. hyopneumoniae* DNA fragment and 5.7-kb *M. flocculare Hin*dIII fragment. (C) PCR amplification of porcine mycoplasma DNA using primers 1469 and 1470, which target the *M. hyopneumoniae* 0.8-kb DNA fragment. Genomic DNAs from *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* (lanes 1 to 3, respectively) were used as templates for the PCR. Lane 4 is a control without a template. The hybridization temperature used was 55°C. Molecular size markers (in kilobases) and the 0.8-kb PCR products are indicated.

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