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Molecular Cloning and Sequencing of the *aroA* Gene from *Actinobacillus pleuropneumoniae* and Its Use in a PCR Assay for Rapid Identification

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The gene (*aroA*) of *Actinobacillus pleuropneumoniae*, serotype 2, encoding 5-*enol*pyruvylshikimate-3-phosphate synthase was cloned by complementation of the *aroA* mutation in *Escherichia coli* K-12 strain AB2829, and the nucleotide sequence was determined. A pair of primers from the 5′ and 3′ termini were selected to be the basis for development of a specific PCR assay. A DNA fragment of 1,025 bp was amplified from lysed *A. pleuropneumoniae* serotypes 1 to 12 of biovar 1 or from isolated DNA. No PCR products were detected when chromosomal DNAs from other genera were used as target DNAs; however, a 1,025-bp DNA fragment was amplified when *Actinobacillus equuli* chromosomal DNA was used as a target, which could be easily differentiated by its NAD independence. The PCR assay developed was very sensitive, with lower detection limits of 12 CFU with *A. pleuropneumoniae* cells and 0.8 pg with extracted DNA. Specificity and sensitivity make this PCR assay a useful method for the rapid identification and diagnosis of *A. pleuropneumoniae* infections.

Actinobacillus pleuropneumoniae causes a highly contagious respiratory disease in pigs, entailing considerable economic losses for the pig-raising industry world-wide. Detailed studies of the clinical symptoms of the disease and its characteristic lung lesions, its experimental induction in pigs with viable and sonicated *A. pleuropneumoniae*, and the endobronchial inoculation of Apx toxins exist (7, 16, 20, 25). The virulence of *A. pleuropneumoniae* may be considered multifactorial, as is the case with most pathogenic bacteria; the factors involved in pathogenesis include capsular polysaccharides (13), lipopolysaccharides (2), membrane proteins (9, 10), adhesion factors (5), exotoxins (16, 23), and urease (3). Epidemiological data suggest, however, that virulence is strongly correlated with the presence of Apx toxins, which may produce lung lesions similar to those caused by natural infection. *A. pleuropneumoniae* strains are grouped into two biovars, biovar 1 (β-NAD dependent) and biovar 2 (β-NAD independent), the former generally being the more virulent (15, 21). Biovar 1 includes 12 serotypes based on capsular polysaccharide structure, although there may be considerable variation in prevalence, presence, and virulence. Indeed, serotypes 1 and 5 are prevalent in North America, where they are responsible for major outbreaks of *A. pleuropneumoniae*-associated disease with high rates of mortality, whereas serotypes 2 and 9, which have been isolated in European countries, are less virulent and cause less mortality, although they produce lung lesions similar to those produced by serotypes 1 and 5 (8, 18). *A. pleuropneumoniae* has been detected in vivo by serological tests such as enzyme-linked immunosorbent assays and complement fixation. Conventional cultivation of *A. pleuropneumoniae* has also been improved by the development of selective media (14, 26). However, detection of this bacterium by DNA PCR amplification has proved to be more sensitive than cultivation. While in some cases specificity was incomplete, in others it allowed for unambiguous detection and identification (12, 27). In this study, we describe the molecular cloning and sequence of the *A. pleuropneumoniae aroA* gene and its use as a target DNA to be amplified by PCR assay, which results in a specific, rapid, simple, and sensitive nucleic acid-based procedure for identifying *A. pleuropneumoniae*.

Cloning and sequencing of the *aroA* gene of *A. pleuropneumoniae* serotype 2. *Sau*3A partial digestion on *A. pleuropneumoniae* serotype 2 chromosomal DNA, prepared as reported previously (22), generated fragments which were fractionated by agarose gel electrophoresis. Fragments of 3 to 9 kbp were selected to construct a genomic library in *Bam*HI-digested dephosphorylated plasmid vector pUC18 (Pharmacia), and the recombinant plasmids were used to transform the electroporated *Escherichia coli aroA* mutant AB2829 as described elsewhere (6, 24, 28). A library of 7,000 Ap^r colonies was obtained when these bacteria were plated on Luria agar (LA) medium supplemented with ampicillin and incubated at 37°C for 24 h. Two recombinant clones which complemented the *E. coli aroA* defect were isolated by replica plating of transformants onto minimal medium (24) supplemented with ampicillin and incubation at 37°C for 48 h. Recombinant plasmid DNA from both well-grown clones was isolated with a Wizard *Plus* Minipreps DNA purification system (Promega Corp.) and used to retransform *E. coli* AB2829 to confirm the ability of the plasmids to complement the *E. coli* AB2829 defect when plated on defined minimal medium. Both recombinant plasmids (designated pAP1 and pAP2) were able to complement the growth of *E. coli* AB2829. A restriction map of pAP2 is shown in Fig. 1. The nucleotide sequence of the 2.3-kb *Hin*dIII-*Eco*RI fragment of pAP2 (Fig. 2), determined by the dideoxynucleotide chain termination method with double-stranded templates by means of the *fmol* DNA sequencing system (Promega Corp.), revealed an open reading frame downstream of a *Hin*dIII site of 1,296 nucleotides, which encodes a protein of 432 amino

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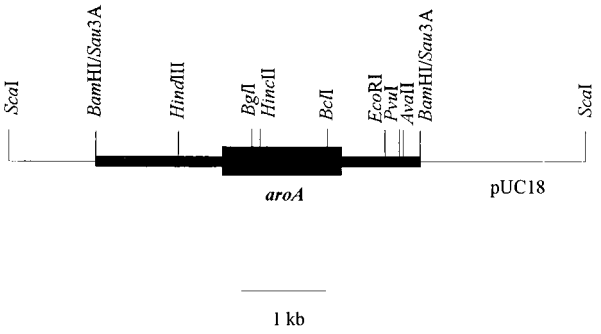

FIG. 1. Restriction endonuclease map of the *aroA* locus obtained with plasmid pAP2. Solid boxes represent *A. pleuropneumoniae*-cloned DNA. The thicker box is the *A. pleuropneumoniae aroA* gene, which was oriented from 5′ (left) to 3′ (right). The thin line represents pUC18 plasmid DNA.

acids (Fig. 2). The deduced molecular weight is 47,028, and the G+C content of the *aroA* coding region product is 43.65%. The predicted amino acid sequence of *A. pleuropneumoniae* AroA (5-*enol*pyruvylshikimate-3-phosphate [EPSP] synthase; EC 2.5.1.19) showed a high degree of homology to AroA proteins of *Haemophilus influenzae* and *Pasteurella haemolytica* (84.95% and 85.42%, respectively), the other two genera of the *Pasteurellaceae* family. Furthermore, a high degree of amino acid sequence conservation was detected when *A. pleuropneumoniae* EPSP synthase was aligned with other bacterial EPSP synthases by means of the CLUSTAL multiple-alignment program (Fig. 3).

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TAAAAGTGTTGGGATAATTTAGAGAATAAAATGGAAAAAATTACATTAGCACCGATCAGC 60
                              M  E  K  I  T  L  A  P  I  S
CGTGTTGAGGGCGAGATTAATTTACCCGGCTCAAAAAGCCTTTCAAATCGTGCGTTATTA 120
 R  V  E  G  E  I  N  L  P  G  S  K  S  L  S  N  R  A  L  L
TTAGCCGCTTTAGCGAAAGGGACGACTAAGGTTACCAATTTATTAGACAGTGACGATATT 180
 L  A  A  L  A  K  G  T  T  K  V  T  N  L  L  D  S  D  D  I
CGCCATATGCTCAATGCGTTAAAAGCATTAGGCGTTAATTATTCATTATCGGAAGATAAA 240
 R  H  M  L  N  A  L  K  A  L  G  V  N  Y  S  L  S  E  D  K
ACCGTTTGTACCGTTGAAGGTGTCGGCGGTGCGTTTAATTGGAAAAACGGTTTGGCGTTA 300
 T  V  C  T  V  E  G  V  G  G  A  F  N  W  K  N  G  L  A  L
TTTTTAGGTAATGCCGGTACGGCAATGCGTCCGTTAACCGCAGCATTATGTTTAAAAGGC 360
 F  L  G  N  A  G  T  A  M  R  P  L  T  A  A  L  C  L  K  G
GCAACTGAAGCGGAAGTGGTTTTAACCGGCGAGCCTCGTATGAAAGAACGCCCGATCAAA 420
 A  T  E  A  E  V  V  L  T  G  E  P  R  M  K  E  R  P  I  K
CACTTAGTTGATGCCCTTCGTCAAGCGGGTGCAAGCGATCAATATTTGGAAAATGAAGGT 480
 H  L  V  D  A  L  R  Q  A  G  A  S  D  Q  Y  L  E  N  E  G
TATCCGCCAGTAGCAATCCGCAATAGCGGTCTAAAAGGCGGAAAAGTACAAATTGACGGC 540
 Y  P  P  V  A  I  R  N  S  G  L  K  G  G  K  V  Q  I  D  G
TCAATTTCCAGCCAATTCTTAACCGCTTTACTGATGGCGGCACCGCTTGCCGAAGGCGAT 600
 S  I  S  S  Q  F  L  T  A  L  L  M  A  A  P  L  A  E  G  D
ATGGAAATTGAAATTATCGGTGAACTGGTATCAAAACCTTATATTGATATTACCTTGGCG 660
 M  E  I  E  I  I  G  E  L  V  S  K  P  Y  I  D  I  T  L  A
ATGATGAAAGATTTTGGAGTAAAAGTTGAGAATCGAAATTACCAGACCTTTGTGGTTAAA 720
 M  M  K  D  F  G  V  K  V  E  N  R  N  Y  Q  T  F  V  V  K
GGTAATCAAAGTTATCTCTCACCGGAAAAATATTTGGTAGAAGGCGATGCGTCATCCGCT 780
 G  N  Q  S  Y  L  S  P  E  K  Y  L  V  E  G  D  A  S  S  A
TCTTATTTCTTAGCAGCCGGTGCGATTAAAGGTAAAGTAAAGGTAACCGGTATTGGTAAA 840
 S  Y  F  L  A  A  G  A  I  K  G  K  V  K  V  T  G  I  G  K
AATTCGATCCAAGGCGACCGCTTGTTTGCCAATGTGCTGGAAGCGATGGGCGCGAAAATC 900
 N  S  I  Q  G  D  R  L  F  A  N  V  L  E  A  M  G  A  K  I
ACTTGGGATGACGATTTTATCCAGGCGGAGCAAGGCGAGCTTAAAGGTGTGGATATGGAT 960
 T  W  D  D  D  F  I  Q  A  E  Q  G  E  L  K  G  V  D  M  D
ATGAACCATATTCCGGATGCAGCAATGACGATTGCGACTACCGCATTATTTGCCGAAGGG 1020
 M  N  H  I  P  D  A  A  M  T  I  A  T  T  A  L  F  A  E  G
GAAACCGTGATTCGTTATATTTATAACTGGCGTGTTAAAGAAACCGACCGTTTAGCAGCG 1080
 E  T  V  I  R  Y  I  Y  N  W  R  V  K  E  T  D  A  L  A  A
ATGGCGACTGAATTGCGCAAAGTGGGAGCAACCGTTGAGGAAGGCGAAGATTTTATTCGT 1140
 M  A  T  E  L  R  K  V  G  A  T  V  E  E  G  E  D  F  I  R
ATTCAGCCTTACCGCTCACGCAATTCCAACACGCAGAAATTGCAACCTACAATGATCAC 1200
 I  Q  P  L  P  L  T  Q  F  Q  H  A  E  I  A  T  Y  N  D  H
CGTATGGCGATGTGTTTCCCATTGATTGCATTATCTGATACACCGGTCACGATTTTAGAC 1260
 R  M  A  M  C  F  P  L  I  A  L  S  D  T  P  V  T  I  L  D
CCGAATTGTACGGCAAAAACATTCCCGACTTATTTCACCGAATTTGAAAAGTTGTCGGAA 1320
 P  N  C  T  A  K  T  F  P  T  Y  F  T  E  F  E  K  L  S  E
AGAACATAAAAAAGCGATAATAAGCGGTTAGATTTGTAAAAGAATTTGCAAATTCAACCGC 1380
 R  T  -
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FIG. 2. Nucleotide sequence of the *aroA* gene and the amino acid sequence deduced from the open reading frame of the *aroA* gene. DNA bases (top line) and amino acids (one-letter code) (below) are shown, and nucleotides are numbered to the right of the sequences. The ATG initiation code (boldface) is preceded by a potential Shine-Dalgarno sequence (double underlining). Primers which have been used for PCR analysis are underlined. The dash indicates the TGA termination codon (boldface).

PCR amplification of the *aroA* gene from *A. pleuropneumoniae*. Samples to be analyzed by PCR were either cultured bacteria or bacterium-extracted DNA. *Actinobacillus* strains were grown on brain heart infusion agar or broth (Biolife) supplemented with 0.1% NAD (wt/vol) when needed. *Aeromonas hydrophila* SO2/2 (4), used as a negative control in PCR assays, was grown on Luria broth or LA (19). *E. coli* strains were grown on Luria broth or LA. *Haemophilus parasuis* (field isolate) was grown on chocolate agar containing 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). *P. haemolytica* ATCC 33396, *Pasteurella multocida* ATCC 12048, and *Klebsiella pneumoniae* (patient isolate) were grown on blood agar plates (tryptic soy agar with 5% sheep erythrocytes). All strains were routinely cultivated at 37°C except for *A. hydrophila*, which was incubated at 28°C. PCR amplification was carried out with a DNA thermal cycler (Perkin-Elmer Cetus) and a PCR kit (Boehringer) with some modification of the manufacturer's instructions. Briefly, the reaction mixture consisted of 1 μl of DNA-containing sample, 1.25 U of *Taq* DNA polymerase, 5 μl of 10× PCR buffer (100 mM Tris-HCl, 20 mM $MgCl_2$, 500 mM KCl [pH 8.3]), a 1 μM concentration of each primer, 0.5 mM concentrations of deoxynucleoside triphosphates, and double-distilled water to a final volume of 50 μl. To minimize evaporation, 50 μl of mineral oil was added to the mixture. DNA denaturation was carried out at 94°C for 2 min, and then a total of 40 cycles were run under the following conditions: DNA denaturation at 92°C for 1 min, primer annealing at 58°C for 30 s, and DNA extension at 72°C for 40 s. After the final cycle, reactions were terminated by an extra run at 72°C for 5 min. Reactions were kept at 4°C until analyzed by endonuclease digestion and agarose gel electrophoresis (2.5% agarose gels with a Tris-borate-EDTA buffer). The pair of primers used in this study, FAP (23-nucleotide-long forward primer, 5′-GCCGCTTTAGCGAAAGGGACG AC-3′, corresponding to positions 94 to 116 of the *aroA* gene nucleotide sequence) and RAP (22-nucleotide-long reverse primer, 5′-GTAGGTTGCAATTTCTGCGTGT-3′, which corresponds to positions 1,140 to 1,161 of the *aroA* gene nucleotide sequence), successfully primed the synthesis of an expected 1,025-bp DNA fragment, which represents most of the *aroA* gene sequence (Fig. 2) of all 12 serotypes of *A. pleuropneumoniae* currently recognized and also tested for this study (Fig. 4). No PCR amplification product was obtained when *E. coli* C600-1, *A. hydrophila*, *P. haemolytica*, *P. multocida*, *K. pneumoniae*, and *H. influenzae* cells were used as sources of target DNA, with the exception of *Actinobacillus equuli* NCTC 8529, which rendered an identical band after PCR amplification (Fig. 4). Negative results were obtained when cells from other *Actinobacillus* species (*A. lignieresii* NCTC 4189, *A. ureae* NCTC 10219, *A. capsulatus* P1364, *A. suis* CCM 5586, and *A. rossii* NCTC 10801) were used as target DNAs for PCR amplification of the *aroA* gene (data not shown). A single 1,025-bp band was obtained with the 500-bp *Sau*3A fragment of the *aroA* gene cloned from *A. pleuropneumoniae* serotype 1 (data not shown). PCR assay sensitivity was evaluated by making a serial dilution of *A. pleuropneumoniae* cells in suspension, as detailed in reference 4. Amplification resulting in detectable levels of PCR product was achieved when a minimum of 12 CFU of *A. pleuropneumoniae* was lysed or 0.8 pg of extracted DNA was used.

RFLP study. The 1,025-bp PCR-amplified products, which represent most of the *aroA* gene sequence, from all of the *A. pleuropneumoniae* serotypes and the *A. equuli* cells used in this

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APL         M-EKITLAPISRVEGEINLPGSKSLSNRALLLAALAKGTTKVTNLLDSDD  49
HINFL       M-EKITLAPISAVEGTINLPGSKSLSNRALLLAALAKGTTKVTNLLDSDD  49
PHAEM       M-EKLTLTPISRVEGEINLPGSKSLSNRALLLAALATGTTQVTNLLDSDD  49
YERPES      MLESLTLQPIALVNGTVNLPGSKSVSNRALLLAALAEGTTQLNNVLDSDD  50
SALTYPHI    M-ESLTLQPIARVDGAINLPGSKSVSNRALLLAALPCGKTALTNLLDSDD  49
ECOLI       M-ESLTLQPIARVDGTINLPGSKSVSNRALLLAALAHGKTVLTNLLDSDD  49
KLEBPNEU    M-ESLTLQPIARVDGTVNLPGSKSVSNRALLLAALARGTTVLTNLLDSDD  49
ASA         M-NSLRLEPISRVAGEVNLPGSKSVSNRALLLAALARGTTRLTNLLDSDD  49
AHY         M-NSLRLEPISRVAGEVNLPGSKSVSNRALLLAALARGTTRLTNLLDSDH  49
            * ... * **. *.*..*******.**********. *.* ..*.****.

APL         IRHMLNALKALGVNYSLSEDKTVCTVEGVGGAFNWKNGLALFLGNAGTAM  99
HINFL       IRHMLNALKALGVRYQLSDDKTICEVEGLGGTFNIQDNLSLFLGNAGTAM  99
PHAEM       IRHMLNALKALGVKYELSDDKTVCVVEGIGGAFKVQNGLSLFLGNAGTAM  99
YERPES      IRHMLNALQALGVDFRLSADRTCCEVDGLGGKLVAEQPLSLFLGNAGTAM 100
SALTYPHI    VRHMLNALSALGINYTLSADRTRCDITGNGGALRAPGALELFLGNAGTAM  99
ECOLI       VRHMLNALTALGVSYTLSADRTRCEIIGNGGPLHAEGALELFLGNAGTAM  99
KLEBPNEU    VRHMLNALSALGVHYVLSSDRTRCEVTGTGGPLQAGSALELFLGNAGTAM  99
ASA         IRHMLAALTQLGVKYKLSADKTECTVHGLGRSFAVSAPVNLFLGNAGTAM  99
AHY         IRHMLAALTQLGVKYKLSADKTECTVHGLGRSFAVSAPVNLFLGNAGTAM  99
            .****.** .**. . **.*.* * . * *  .     ..**********

APL         RPLTAALCLKGATEAEVVLTGEPRMKERPIKHLVDALRQAGASDQYLENE 149
HINFL       RPLTAALCLKGKTESEIILTGEPRMKERPILHLVDALRQAGADIRYLENE 149
PHAEM       RPLAAALCLKGAETAQIILTGEPRMKERPIKHLVDALRQVGAEVQYLENE 149
YERPES      RPLAAVLCL---GNSDIVLTGEPRMKERPIGHLVDALRQGGAQIDYLEQE 147
SALTYPHI    RPLAAALCL---GQNEIVLTGEPRMKERPIGHLVDSLRQGGANIDYLEQE 146
ECOLI       RPLAAALCL---GSNDIVLTGEPRMKERPIGHLVDALRLGGAKITYLEQE 146
KLEPNEU     RPLAAALCL---GSNDIVLTGEPRMKERPIGHLVDALRQGGAQIDYLEQE 146
ASA         RPLCAALCL---GSGEYMLGGEPRMEERPIGHLVDCLALKGAHIQYLKKD 146
AHY         RPLCAALCL---GSGEYTLGGEPRMEERPIGHLVDALRERVAHIQYLKKD 146
            *** *.***    . ..  *.*****.**** **** *    *   **...

APL         GYPPVAIRNSGLKGGKVQIDGSISSQFLTALLMAAP-LAEGDMEIEIIGE 198
HINFL       GYPPLAIRNKGIKGGKVKIDGSISSQFLTALLMSAP-LAENDTEIEIIGE 198
PHAEM       GYPPLAISNSGLQGGKVQIDGSISSQFLTALLMSAP-LAESDMEIEIIGD 198
YERPES      NYPPLRLRG-GFRGGELTVDGRVSSQFLTALLMTAP-LAEQDTTIRIMGD 195
SALTYPHI    NYPPLRLRG-GFTGGDIEVDGSVSSQFLTALLMTAP-LAPKDTIIRVKGE 194
ECOLI       NYPPLRLQG-GFTGGNVDVDGSVSSQFLTALLMTAP-LAPEDTVIRIKGD 194
KLEBPNEU    NYPPLRLRG-GFTGGDVEVDGSVSSQFLTALLMASP-LAPQDTVIAIKGE 194
ASA         GYPPLVVDAKGLWGGDVHVDGSVSSQFLTAFLMAAPAMAPVIPRIHIKGE 196
AHY         GYPPLVVDAKGLWGGDVHVDGSVSSQFLTAFLMAAPWR-PVDTRIHIKGE 195
            .***. . . *. **.. .**..******.**..*       * . *.

APL         LVSKPYIDITLAMMKDFGVKVENRNYQTFVVKGNQSYLSPE-KYLVEGDA 247
HINFL       LVSKPYIDITLAMMRDFGVQVENHHYQKFQVKGNQSYISPN-KYLVEGDA 247
PHAEM       LVSKPYIDITLSMMNDFGITVENRDYKTFLVKGKQGYVAPQGNYLVEGDA 248
YERPES      LVSKPYIDITLHLMKAFGIDVGHENYQIFHIKGGQTYRSP-GTYLVEGDA 244
SALTYPHI    LVSKPYIDITLNLMKTFGVEIANHHYQQFVVKGGQQYHSP-GRYLVEGDA 243
ECOLI       LVSKPYIDITLNLMKTFGVEIENQHYQQFVVKGGQSYQSP-GTYLVEGDA 243
KLEBPNEU    LVSRPYIDITLHLMKTFGVEVENQAYQRFIVRGNQQYQSP-GDYLVEGDA 243
ASA         LVSKPYIDITLHIMNSSGVVIEHDNYKLFYIKGNQSIVSP-GDFLVEGDA 245
AHY         LVSKPYIDITLHIMKQFGVVIEHDNYKLFYIKGNQSYVSP-GDFLVEGDA 244
            ***.******* .*.  *. ...  *. * ..* *   .*   .******

APL         SSASYFLAAGAIKG-KVKVTGIGKNSIQGDRLFANVLEAMGAKITWDDDF 296
HINFL       SSASYFLAAGAIKG-KVKVTGIGKNSIQGDRLFADVLEKMGAKITWGEDF 296
PHAEM       SSASYFLASGAIKG-KVKVTGIGKKSIQGDRLFADVLEKMGAKITWGEDF 297
YERPES      SSASYFLAAAAIKGGTVRVTGIGKKSVQGDTKFADVLEKMGAKVTWGDDY 294
SALTYPHI    SSASYFLAAGAIKGGTVKVTGIGRKSMQGDIRFADVLEKMGATITWGDDF 293
ECOLI       SSASYFLAAAAIKGGTVKVTGIGRNSMQGDIRFADVLEKMGATICWGDDY 293
KLEBPNEU    SSASYFLAAGAIKGGTVKVTGIGRNSVQGDIRFADVLEKMGATVTWGEDY 293
ASA         SSASYFLAAGAIKG-KVRVTGIGKHSI-GDIHFADVLERMGARITWGDDF 293
AHY         SSASYFLAAGAIKG-KVRVTGIGKHSIQGDIHFADVLEKMGARITWGDDF 293
            ********..**** .*.******..*. **  **.*** *** . *..*.

APL         IQAEQGELKGVDMDMNHIPDAAMTIATTALFAEGETVIRYIYNWRVKETD 346
HINFL       IQAEHAELNGIDMDMNHIPDAAMTIATTALFANSETVIRNIYNWRVKETD 346
PHAEM       IQAEQSPLKGVDMDMNHIPDAAMTIATTALFAEGETVIRNIYNWRVKETD 347
YERPES      IECSRGELQGIDMDMNHIPDAAMTIATTALFATGPTTIRNIYNWRVKETD 344
SALTYPHI    IACTRGELHAIDMDMNHIPDAAMTIATTALFAKGTTTLRNIYNWRVKETD 343
ECOLI       ISCTRGELNAIDMDMNHIPDAAMTIATAALFAKGTTTLRNIYNWRVKETD 343
KLEBPNEU    IACTRGELNAIDMDMNHIPDAAMTIATAALFARGTTTLRNIYNWRVKETD 343
ASA         IEAEQGPLHGVDMDMNHIPDVGHDHSGQSHCLPRVPPHSQHLQLAVRDDR 343
AHY         IEAEQARLHGIDMDMNHIPDAAMTIAVAAFVCRGAHIHSQHLQLAGEGDG 343
            *  ... *...*********.. . .  .       .  .    ..

APL         RLAAMATELR-KVGATVEEGEDFIRIQPLPLTQFQHAEIATYNDHRMAMC 395
HINFL       RLTAMATELR-KVGAEVEGGEDFIRIQPLPLNQFKHANIETYNDHRMAMC 395
PHAEM       RLTAMATELR-KVGAEVEEGEDFIRIQPLALENFQHAEIETYNDHRMAMC 396
YERPES      RLTAMATELR-KVGAEVEEGEDYIRVVPPL--QLTAADIGTYDDHRMAMC 391
SALTYPHI    RLFAMATELR-KVGAEVEEGHDYIRITPPA--KLQHADIGTYNDHRMAMC 390
ECOLI       RLFAMATELR-KVGAEVEEGHDYIRITPPE--KLNFAEIATYNDHRMAMC 390
KLEBPNEU    RLFAMATELR-KVGAEVEEGEDYIRITPPL--TLQFAEIGTYNDHRMAMC 390
ASA         CTPCTHGHRRAQAGVS-EEGTTFITRDAA--DPAQARRDRHLQRSRIAMC 390
AHY         SSA-RHGHRAAQVGVEVEEGHDFITVTPP--AQLNHAAIDTYNDHRIAMC 390
                  ..   ..*.. *.* ..*   .              .  *.***

APL         FPLIALSDTPVTILDPNCTAKTFPTYFTEFEKLSERT    432
HINFL       FSLIALSNTPVTILDPKCTAKTFPTFTNEFEKICLKN    432
PHAEM       FSLIALSNTEVTILDPNCTAKTFPTYFRELEKLSVR-    432
YERPES      FSLVALSDTPVTILDPKCTAKTFPDYFEQFARK----    424
SALTYPHI    FSLVALSDTPVTILDPKCTAKTFPDYFEQLARMSTPA    427
ECOLI       FSLVALSDTPVTILDPKCTAKTFPDYFEQLARISQAA    427
KLEBPNEU    FSLVALSDTPVTILDPKCTAKTFPDYFGQLARISTLA    427
ASA         FSLVALSDIAVTINDPGCTSKTFPDYFDKLASVSQAV    427
AHY         FSLVALSDTAVTINDPGCTSKTFPDYFDKLACVSQAV    427
            *.*.***.. *** ** **.****..* ***
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FIG. 3. CLUSTAL computer alignment of the deduced amino acid sequences encoded by the *aroA* gene from *A. pleuropneumoniae* (APL), *H. influenzae* (HINFL), *P. haemolytica* (PHAEM), *Yersinia pestis* (YERPES), *Salmonella typhimurium* (SALTYPHI), *E. coli* (ECOLI), *K. pneumoniae* (KLEBPNEU), *Aeromonas salmonicida* (ASA), and *A. hydrophila* (AHY). Identical amino acids in all species are indicated by an asterisk; conservative substitutions are indicated by a dot.

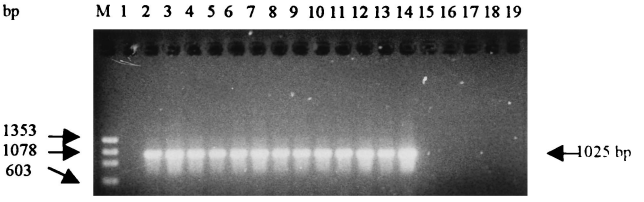

FIG. 4. Agarose gel electrophoresis of PCR amplification products from total DNA from *E. coli* (lane 1), *A. pleuropneumoniae* serotypes 1 to 12 (ATCC 27088, ATCC 27089, ATCC 27090, NCTC 11384, NCTC 11383, ATCC 33590, WF 83, 405, CVJ 13261, D 13039, 16153, and 8329, respectively) (lanes 2 to 13), *A. equuli* NCTC 8529 (lane 14), *H. influenzae* ATCC 35056 (lane 15), *P. haemolytica* ATCC 33396 (lane 16), *P. multocida* ATCC 12048 (lane 17), *K. pneumoniae* (patient isolate) (lane 18), and *A. hydrophila* SO2/2 (lane 19). M, standard DNA size markers (*Hae*III-digested ϕX174).

study, were analyzed with restriction endonucleases. From the restriction enzyme digestion of these PCR products, we found three different restriction fragment length polymorphism (RFLP) patterns with *Sau*3A (Fig. 5). Serotypes 1, 4, 5, 9, 11, and 12 presented RFLP pattern 1, which rendered fragments of 500, 220, 190, and 115 bp when PCR products were digested with *Sau*3A. Serotypes 2, 3, 6, 7, and 8, as well as *A. equuli*, presented RFLP pattern 2, with fragment sizes of 450, 240, 220, and 115 bp after *Sau*3A digestion. RFLP pattern 3 was presented by serotype 10, which rendered fragments of 450, 220, 190, 115, and 50 bp. Identical grouping was obtained when *aroA*-PCR-amplified products from all *A. pleuropneumoniae* serotypes were restricted with *Hpa*II endonuclease, with the exception of serotype 7, which presented a clearly different RFLP pattern. In this case, serotype 10 was grouped within RFLP 2 serotypes (data not shown).

Discussion. In this study, we developed a method for the rapid and easy identification of *A. pleuropneumoniae*. The *aroA* gene, encoding 5-*enol*pyruvylshikimate-3-phosphate synthase, contributes to aromatic amino acids and the folic acid universal pathway in bacteria and has been used successfully for taxonomic purposes before. The *Aeromonas* genus was analyzed by PCR-RFLP of the *aroA* gene (4) with good results. PCR-RFLP of the *aroA* gene has also been used for identifying and typing *Staphylococcus aureus* (29). The results obtained in each case were related to genetic diversity of strains and species. Several PCR assays have been proposed for rapid detection and identification of *A. pleuropneumoniae* (11, 12, 17, 27); the one proposed here is able to rapidly identify all 12 serotypes of *A. pleuropneumoniae* so far described by PCR amplification of an expected 1,025-bp fragment representing most of the *aroA* gene sequence. No other species or genera yielded positive reactions in our PCR assays, with the exception of *A. equuli*. PCR amplification of the *A. pleuropneumoniae aroA* gene is further enhanced when combined with RFLP after *Sau*3A PCR amplification product digestion, which allows reasonable discrimination among serotypes. This PCR-RFLP combination allowed *A. pleuropneumoniae* to be divided into three groups: group 1, with serotypes 1, 4, 5, 9, 11, and 12; group 2, with serotypes 2, 3, 6, 7, and 8 and *A. equuli*; and group 3, with

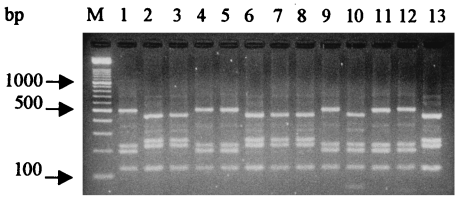

FIG. 5. Agarose gel electrophoresis of fragments produced by *Sau*3A digestion of *A. pleuropneumoniae aroA* genes amplified by PCR. Lanes 1 to 12, serotypes 1 to 12; lane 13, *A. equuli*. M, standard DNA size markers (*Hae*III-digested ϕX174).

serotype 10. The fact that *A. equuli* gave a positive PCR amplification did not present a problem, since *A. equuli* can be easily differentiated by its NAD-independent growth. These results accord well with those of Gram and Ahrens (11), in whose study the 12 serotypes of *A. pleuropneumoniae* were divided into three groups based on *omlA* gene nucleotide sequences of reference serotypes. Some discrepancies with Gram and Ahrens' results could be observed; serotypes 5 and 10, for example, are closely related on the basis of *omlA* gene sequences (11), although our PCR-RFLP procedure assigns them to different groups. Moreover, *omlA* gene nucleotide sequences would place serotype 4 in our group 2 and serotype 2 in our group 1. These discrepancies have also been observed when groupings based on *omlA* nucleotide sequences and Apx toxin patterns are compared (1, 11). In conclusion, our PCR-RFLP assay may be considered a useful taxonomic tool for the identification of *A. pleuropneumoniae* because of its simplicity, specificity, and sensitivity and because it has a reasonable discriminatory power among serotypes. Furthermore, based on its high sensitivity, our PCR-RFLP test may be used to detect *A. pleuropneumoniae* in clinical samples.

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