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# IDENTIFICATION OF A NOVEL *MANNHEIMIA GRANULOMATIS* LINEAGE FROM LESIONS IN ROE DEER (*CAPREOLUS CAPREOLUS*)

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ABSTRACT: Eight atypical *Mannheimia* isolates were isolated from lesions in roe deer (*Capreolus capreolus*). Traditional classification based on morphologic and physiologic traits showed that they belong to a distinct biogroup (taxon) within genus *Mannheimia*. Extensive phenotypic characterization suggested that the isolates should be classified as *M. granulomatis*, although the presence of distinct traits justified their classification into a separate biogroup within this species. Phylogenetic analyses based on 16S rRNA sequences from two roe deer isolates and 41 other *Mannheimia* strains supported that the roe deer isolates form a monophyletic group within *M. granulomatis*. The *lktA* genotype was present in all roe deer isolates based on Southern blot analysis, whereas the corresponding β-hemolytic phenotype was absent in one of these isolates.

*Key words:* β-hemolytic phenotype, *Capreolus capreolus*, leukotoxin, *Mannheimia granulomatis*, roe deer.

## INTRODUCTION

The genus *Mannheimia* includes strains previously classified as trehalose-negative [*Pasteurella*] *haemolytica*, and is one of the most well-defined and robust clusters within the family of *Pasteurellaceae* Pohl 1981 (Angen et al., 1999). Members of the genus are gram-negative, nonmotile rods or cocci with a G+C content ranging between 39% and 44%. Ribotyping, multilocus enzyme electrophoresis (MLEE), DNA-DNA hybridizations, and 16S rRNA sequencing have shown that the strains can be divided into five subclades, representing at least five species: *M. haemolytica+M. glucosida*, *M. ruminalis*, *M. granulomatis*, *M. varigena*, and subclade V comprising unclassified strains (Angen et al., 1997c; Davies et al., 1997; Angen et al., 1999; Larsen et al., in press). Strains belonging to these species inhabit the mucous membranes of mammals and have been isolated from the respiratory tract of ruminants, rabbits, and hares (Devriese et al., 1991; Angen et al., 1999), the rumen of sheep and cattle, the ileum of pigs (Angen et al., 1999), and the skin of cattle (Riet-Correa et al., 2000).

Based on phenotypic traits, ruminant strains classified as trehalose-negative *M.* [*P.*] *haemolytica* could be divided into 12 biogroups or taxa (Bisgaard and Mutters, 1986; Bisgaard et al., 1986). Interestingly, the 16S rRNA phylogeny indicates that different biogroups are present among two or more species, suggesting that members of the same biogroup are not necessarily closely related (Angen et al., 1999; Larsen et al., 2007). Previous works have shown that the β-hemolytic phenotype correlates with the pathogenic potential of strains belonging to genus *Mannheimia*. The results of Angen et al. (1999) indicated that most strains isolated from lesions were β-hemolytic on bovine blood agar plates (except strains belonging to *M. granulomatis*), whereas most strains belonging to the nonpathogenic species *M. ruminalis* were nonhemolytic. Larsen et al. (2007) used Southern blot analysis, sheep blood agar plates, and a representa-



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TABLE 1. *Mannheimia* strains isolated from roe deer (*Capreolus capreolus*).

| Strains | Lesions | Geographic origin | Year | Sex | Age (yr) | *lktA* | |
|---|---|---|---|---|---|---|---|
| | | | | | | Genotype | Phenotype[a] |
| B 153/80 | Glossitis | Åseda, Sweden | 1980 | Male | <1 | + | + |
| P 4737/80[b] | Purulent necrotizing keratoconjunctivitis | Frövi, Sweden | 1980 | Female | <1 | | + |
| B 3768/80 | Purulent stomatitis and glossitis | Edsbro, Sweden | 1980 | | <1 | + | + |
| B 1829/84 | Purulent pneumonia and pleuritis | Stockholm, Sweden | 1984 | Female | <1 | + | − |
| B 926/85 | Purulent pneumonia and pleuritis | Riddarhyttan, Sweden | 1985 | Male | <1 | + | + |
| Bd 2017/92 | Neck phlegmon/abscess | Uppsala, Sweden | 1992 | Female | <1 | + | + |
| B 234/94 | Acute purulent pneumonia | Stockholm, Sweden | 1994 | Female | ~7 | + | + |
| B 1157/95 | Acute purulent pneumonia | Stockholm, Sweden | 1995 | Female | <1 | + | + |

[a] Based on β-hemolysis on sheep blood agar.

[b] This strain was not available for analyses of the *lktA* genotype.

tive strain collection to show that the *lktA* genotype and the corresponding β-hemolytic phenotype were present in all *Mannheimia* species, including all strains belonging to *M. haemolytica* and *M. glucosida*, *M. granulomatis*, and *M. varigena*, but only in a fraction of strains belonging to *M. ruminalis*. Murphy et al. (1995) showed that the β-hemolytic phenotype of *M. haemolytica* correlates with the presence of an active leukotoxin (*lkt*) operon on the chromosome. The structural leukotoxin (LktA) protein of *M. haemolytica*, encoded by *lktA* (Lo et al., 1987), belongs to the *E. coli* HlyA-like subfamily of cytotoxic RTX (repeats in toxin) proteins and is essential in both evasion and exploitation of the adaptive immune system during pulmonary infection (Petras et al., 1995; Tatum et al., 1998; Highlander et al., 2000). These results support the predicted correlation between pathogenic potential and the β-hemolytic phenotype.

In this study, we characterize eight atypical *Mannheimia* isolates obtained from lesions in roe deer (*Capreolus capreolus*) by using a polyphasic strategy that combines phenotypic and genotypic methods. The implications of our results for proper identification and classification of *Mannheimia* strains are discussed.

## MATERIALS AND METHODS

### Bacterial strains used

The strains used in this study and their geographic origin, time of isolation, and corresponding pathological lesions are given in Table 1. A total of eight atypical *Mannheimia* strains were isolated from lesions in roe deer submitted to the Department of Wildlife, The National Veterinary Institute, Uppsala, Sweden for post-mortem examination.

### Phenotypical characterization

All isolates were phenotypically characterized as previously described by Bisgaard et al. (1991). A total of 82 different phenotypic tests have been used. To determine presence/inactivation of the *lkt* operons, we screened the strains for the β-hemolytic phenotype on sheep blood agar plates as previously described (Murphy et al., 1995).

### Reconstruction of phylogenetic trees

The relationships of the strains in this study were inferred with 16S rRNA; these sequences have been used successfully for systematic studies in this group (Angen et al., 1999; Larsen et al., 2007). Sequences for 41 strains were obtained from GenBank (Table 2). The primers previously described by Angen et al.

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(1999) were used to amplify and directly sequence 16S rRNA from two roe deer isolates (B 234/94 and B 1829/84). The 16S rRNA sequences were aligned using both Dialign 2 (Morgenstern et al., 1999) and the NAST server (DeSantis et al., 2006), which uses a prealigned set of 16S rRNA sequences as the basis for aligning the user's sequences. The two methods yielded identical alignments. This was expected, because the average pairwise sequence similarity in the full set of 43 sequences was 97.5% and even the least similar pair of sequences was 92.5% identical, supporting that alignment errors were unlikely. Phylogenetic trees were reconstructed with the use of Bayesian techniques as implemented in the program MrBayes version 3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The best-fitting model was GTR+ invgamma based on the Akaike Information Criterion (AIC) computed by the program MrModeltest (version 2.2, Department of Systematic Zoology, Uppsala University, Uppsala, Sweden; http://www.ebc.uu.se/systzoo/staff/nylander.html). Markov chain Monte Carlo (MCMC) was run for 10,000,000 generations with four chains while sampling once every 100 generations. Convergence was confirmed by comparing the results of two independent runs. The program Tracer (version 1.3; Department of Zoology, Oxford University, Oxford, UK; http://evolve.zoo.ox.ac.uk/software.html?id=tracer) was used to determine burn-in and also for further confirmation of proper mixing and adequate run length. A burn-in of 1,000,000 generations (10,000 samples) was used in both cases. The distribution of 16S rRNA trees resulting from MCMC was summarized in the form of a consensus tree with all compatible bipartitions included by using the authors' own software in a manner that is essentially identical to what is obtained when using MrBayes' sumt command with the setting contype=allcompat, except that branch lengths were averaged over all trees; setting the branch length to zero for those trees that did not contain the corresponding bipartition, as suggested by Felsenstein (2003). The tree was rooted based on a maximum-likelihood analysis reported by Angen et al. (1999).

### Analyses of the *lktA* genotype

We analyzed the distribution of the *lktA* gene among roe deer isolates by using Southern blot as previously described by Larsen et al. (2007). The primers manpop_UP and manpop_DOWN previously described by Larsen et al. (2007) were used to amplify the +845/+1,302 region of *lktA*. The reaction conditions were 2.5 U *Taq* polymerase, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 0.01% Tween-20, 2.5 mM $Mg_2SO_4$, each primer at 0.5 mM, and each nucleotide at 0.1 mM. The cycling conditions were initial denaturation at 94 C followed by 25 cycles of 94 C for 30 s, 52 C for 30 sec, and 72 C for 30 sec, finishing with extension at 72 C for 10 min.

### Sequence data

Sequences have been deposited in the GenBank database (accession numbers (AY425296 and AY425297).

## RESULTS

### Bacterial strains used

The strains used in this study and their geographic origin, time of isolation, and corresponding lesions are given in Table 1. It should be noted that the strains were isolated from six different locations within a 300-km radius from Norrköping (58°36′N, 16°11′E) in southeastern Sweden. The isolates were obtained during a period from 1980 to 1995. Six of the isolates were obtained from animals $<1$ yr old, whereas a single isolate originated from a 7-yr-old female. Lesions varied from acute purulent bronchopneumonia ($n=2$), purulent pneumonia and pleuritis ($n=2$), glossitis ($n=1$), purulent stomatitis and glossitis ($n=1$), and phlegmon and abscess formation ($n=1$).

### Phenotypic characterization

All isolates were gram-negative, nonmotile, catalase and oxidase positive, and decomposed glucose fermentatively in Hugh and Leifson medium. All isolates were porphyrin, alanine aminopeptidase and phosphatase positive, and reduced nitrate. In addition, acid was produced from glycerol, D-ribose, D-xylose, meso-inositol, D-mannitol, D-fructose, D-galactose, D-glucose without gas formation, lactose, sucrose, and raffinose. Positive reactions were also obtained for all isolates in ONPG (β-galactosidase) and PGUA (β-glucuronidase). The β-hemolytic phenotype on bovine and ovine blood agar plates

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TABLE 2. Isolates of *Mannheimia*, host species, origin, GenBank accession numbers for 16S rRNA sequences used in this study.

| Taxon[a] | Strain ID | Host Species | Host Family | Country | GenBank accession no. |
|---|---|---|---|---|---|
| *M. haemolytica* | | | | | |
| Biogroup 1 | PHL213 | *Bos taurus* | Ruminantia | UK | DQ301920 |
| Biogroup 1 | CCUG 12392$^T$ | *Ovis aries* | Ruminantia | | AF060699 |
| *M. glucosida* | | | | | |
| Biogroup 3A | P731 | *O. aries* | Ruminantia | USA | AF053888 |
| Biogroup 3B | P925$^T$ | *O. aries* | Ruminantia | Scotland | AF053889 |
| Biogroup 3C | UT18 | *O. aries* | Ruminantia | Scotland | AF053890 |
| Biogroup 3D | H62 | *O. aries* | Ruminantia | Belgium | DQ301921 |
| Biogroup 3E | P741 | *O. aries* | Ruminantia | USA | DQ301922 |
| Biogroup 3F | P933 | *O. aries* | Ruminantia | Scotland | DQ301923 |
| Biogroup 3G | P737 | *O. aries* | Ruminantia | USA | AF053891 |
| Biogroup 3H | P733 | *O. aries* | Ruminantia | USA | AF053892 |
| Biogroup 9 | P730 | *O. aries* | Ruminantia | USA | AF053897 |
| *M. granulomatis* | | | | | |
| Biogroup 3J | W4672/1 | *B. taurus* | Ruminantia | Australia | DQ301924 |
| Bt 20 biovar 1 | Ph13 | *Lepus capensis* | Leporidae | France | AF053901 |
| Bt 20 biovar 2 | BJ1680.3 | *L. capensis* | Leporidae | Belgium | DQ301925 |
| [*P.*] *granulomatis* | P1135/26$^T$ | *B. taurus* | Ruminantia | Brazil | AF053902 |
| *M. varigena* | | | | | |
| Biogroup 6 | 177$^T$ | *B. taurus* | Ruminantia | Germany | AF053893 |
| Biogroup 6 | V1835 | *B. taurus* | Ruminantia | Australia | AY425282 |
| Bt 15 biovar 1 *orn*$^+$ | P655 | *Sus scrofa* | Suidae | Denmark | AF053899 |
| Bt 15 biovar 2 *orn*$^-$ | 3997/82 | *S. scrofa* | Suidae | Denmark | DQ301926 |
| Bt 36 | H39 | *B. taurus* | Ruminantia | Belgium | DQ301927 |
| *M. ruminalis* | | | | | |
| Biogroup 1 | UT26 | *O. aries* | Ruminantia | Scotland | AF053887 |
| Biogroup 8D | HPA98 | *O. aries* | Ruminantia | Scotland | AF053896 |
| Biogroup 10 | HPA95 | *O. aries* | Ruminantia | Scotland | AY425289 |
| Biogroup 10 | HPA114 | *O. aries* | Ruminantia | Scotland | AY425290 |
| Biogroup 10 | UT27 | *O. aries* | Ruminantia | Scotland | AY425291 |
| Bt 18 biovar 1 | HPA92$^T$ | *O. aries* | Ruminantia | Scotland | AF053900 |
| Bt 18 biovar 1 | HPA81 | *O. aries* | Ruminantia | UK | U57077 |
| Bt 18 biovar 2 | HPA113 | *O. aries* | Ruminantia | UK | AY425283 |
| Bt 18 biovar 2 | HPA90 | *O. aries* | Ruminantia | | AY425284 |
| Bt 18 biovar 2 | UT38 | *O. aries* | Ruminantia | | AY425285 |
| Bt 18 biovar 3 *xyl*$^+$ | HPA109 | *O. aries* | Ruminantia | UK | AY425286 |
| Bt 18 biovar 3 *xyl*$^-$ | HPA93 | *O. aries* | Ruminantia | UK | AY425287 |
| Bt 18 biovar 4 | HPA88 | *O. aries* | Ruminantia | UK | AY425288 |
| Subclade V | | | | | |
| Biogroup 7 | R19.2 | *B. taurus* | Ruminantia | Scotland | AF053894 |
| Biogroup 8A[c] | HPA102 | *O. aries* | Ruminantia | Scotland | AF053895 |
| Biogroup 8B | 274 | *B. taurus* | Ruminantia | Germany | AY425292 |
| Biogroup 8B | R108B(3) | *B. taurus* | Ruminantia | Scotland | AY425293 |
| Biogroup 8C | M14.4 | *B. taurus* | Ruminantia | Scotland | AY425294 |
| Biogroup 10 | BJ3956.1 | *O. aries* | Ruminantia | Belgium | AY425295 |
| Biogroup 10 | HPA121 | *B. taurus* | Ruminantia | Scotland | AF053898 |
| Bt 39 | BNO311 | *B. taurus* | Ruminantia | Australia | AF216870 |

[a] Bt=Bisgaard taxon.

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TABLE 3. Phenotypic characters separating roe deer isolates from related taxa of genus *Mannheimia*.[a]

| Character | Roe deer isolates | *M. granulomatis* | | | | *M. ruminalis str.* HPA92[e] |
|---|---|---|---|---|---|---|
| | | Bt 20 biovar 1[b] | Bt 20 biovar 2[c] | Biogroup 3J[d] | str. P1135/26[e] | |
| β-hemolysis[f] | +/w | + | + | + | + | − |
| Glycerol | (+) | (+) | (+) | (+) | (+) | − |
| D (+) xylose | +/(+) | + | +/(+) | + | − | − |
| Meso-inositol | (+) | (+)/− | (+)/w | − | − | − |
| D (−) sorbitol | − | + | + | + | + | − |
| Cellobiose | − | +/(+) | − | (+) | (+) | − |
| Maltose | − | +/(+) | (+) | (+) | (+) | − |
| Raffinose | (+) | +/w | (+) | − | − | − |
| Dextrin | − | (+)/− | (+)/w | (+) | w | − |
| Aesculin | − | +/(+) | − | +/(+) | + | − |
| Amygdalin | − | (+) | − | (+) | + | − |
| Arbutin | − | +/(+) | − | (+) | + | − |
| Gentiobiose | − | +/(+) | − | (+) | + | − |
| Salicin | − | +/(+) | − | (+) | + | − |
| NPG (β-glucosidase) | − | + | + | + | + | − |
| PGUA (β-glucoronidase) | + | − | − | − | + | − |

[a] +, ≥90% strains are positive; −, <10% strains are positive; w, <90 and ≥10 positive; (+), late positive (3–14 days).
[b] Based on 19 isolates from *Lepus capensis* and *Oryctolagus cuniculus*.
[c] Based on seven isolates from *Lepus capensis*.
[d] Based on three isolates from *Bos taurus*.
[e] T=Type strain.
[f] Based on bovine blood.

was present in six isolates, whereas isolate B 1828/84 was nonhemolytic. Late acid production from L-fucose was observed for two isolates, whereas a single isolate showed a positive reaction in the ONPX test (β-xylosidase).

A negative reaction was observed for all isolates in the following tests: symbiotic growth, Simmons citrate, malonate, $H_2S$/TSI, KCN growth, MR 37 C, VP 37 C, formation of gas from nitrate, urease, arginine dihydrolase, lysine and ornithine decarboxylase, phenylalanine deaminase, indole, geletinase, hydrolysis of Tween 20 and Tween 80, McConkey agar growth, pigment formation, and production of acid from meso-erythritiol, adonitol, D-arabitol, xylitol, L-arabinose, D-arabinose, L-xylose, dulcitol, D-sorbitol, D-fucose, D-mannose, L-rhamnose, L-sorbose, cellobiose, maltose, D-melibiose, trehalose, D-melezitose, dextrin, D-glycogen, inulin, aesculin, amygdalin, arbutin, gentiobiose, salicin, D-turanose, and β-N-$CH_3$-glucosamid. Negative reactions were also observed in NPG (β-glucosidase), PNPG (α-glucosidase), ONPF (α-fucosidase), α-galactosidase, and α-mannosidase. Phenotypic characters separating the isolates from biogroups belonging to *M. granulomatis* and the type strain of *M. ruminalis* (HPA92$^T$) are given in Table 3.

#### 16S rRNA phylogeny

BLAST searches of the NCBI nonredundant (nr) database with the 16S rRNA sequences of isolates B 234/94 and B 1829/84 as queries suggested that they are most closely related to *M. granulomatis*. Because the closest BLAST hits are not necessarily each other's closest relative (Koski and Golding, 2001), we reconstructed the phylogeny with the use of these sequences, along with sequences of

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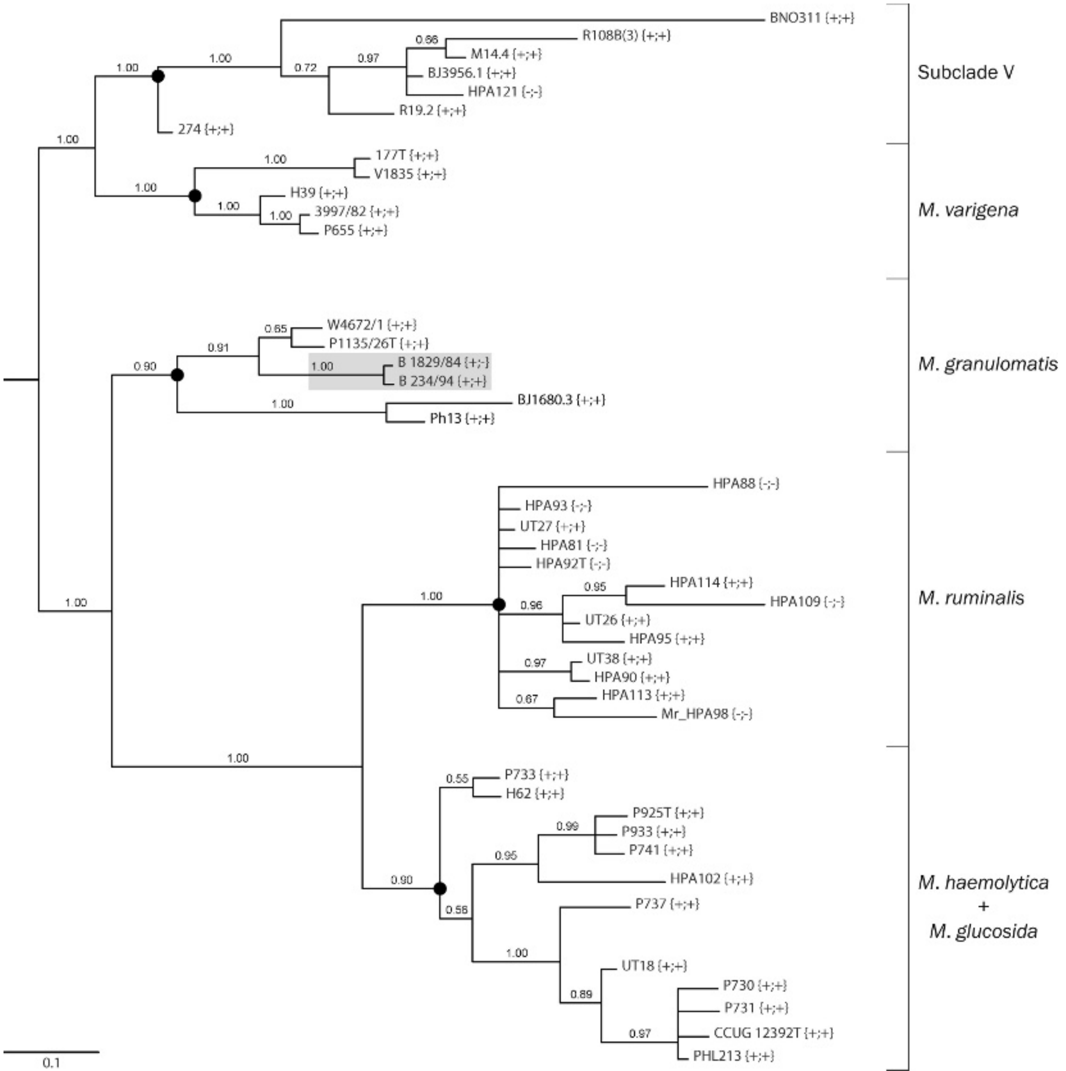


FIGURE 1. Distribution of 16S rRNA trees from Markov chain Monte Carlo (MCMC) summarized in the form of a consensus tree with all compatible bipartitions included and with branch lengths averaged over all trees, setting the branch length to zero for those trees that did not contain the corresponding bipartition. The tree was rooted based on a maximum-likelihood analysis reported by Angen et al. (1999). Posterior probability values are shown for all compatible bipartitions. Presence/absence of the *lktA* genotype and the corresponding β-hemolytic phenotype is indicated by {±;±}. Monophyly of the five subclades reported by Angen et al. (1999) is indicated by black circles. Nomenclature: Sequence names contain the corresponding strain ID as listed in Table 2.

41 *Mannheimia* strains. The 16S rRNA data set included 1,257 bp after removal of ambiguous bases. Both runs converged and the consensus tree with all compatible bipartitions included and with branch lengths averaged over all trees, setting the branch length to zero for those trees that did not contain the corresponding bipartition, is shown in Figure 1, along with posterior probabilities (PP). This phylogram is largely topologically concordant with the Bayesian analysis reported by Larsen et al. (2007) and suggest that the two roe deer isolates form a cluster within *M. granulomatis* (PP=0.90%). There was strong PP support for mono-

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phyly of the roe deer isolates (PP=100%), supporting that they belong to a distinct lineage of *M. granulomatis* (PP=100%).

#### Analyses of the *lktA* genotype

The *lktA* genotype and the corresponding β-hemolytic phenotype are given in Table 1. It should be noted that isolate P 4737/80 died during storage and could not be included in these analyses. Southern blot analysis indicated that the *lktA* gene was present in all seven isolates. The +845/+1,302 region of the *lktA* gene was amplified by PCR in the six β-hemolytic isolates. We could not amplify *lktA* from the nonhemolytic isolate B 1829/84, presumably because substitutions have resulted in imperfect matches of the primers.

## DISCUSSION

Traditional classification based on morphologic and physiologic traits showed that the eight roe deer isolates belonged to a distinct biogroup (taxon) within genus *Mannheimia* (data not shown). Extensive phenotypic characterization suggested that the isolates should be classified as *M. granulomatis*, although the presence of distinct traits justified their classification into a separate biogroup within this species (Table 3). The 16S rRNA tree supported that the roe deer isolates form a monophyletic group within *M. granulomatis*. The 16S rRNA sequences from the roe deer isolates were almost identical despite having been isolated in 1984 and 1994, respectively (data not shown), supporting that they constitute a clonal complex within *M. granulomatis*.

The bovine *M. granulomatis* strains W4672/1 (biogroup 3J) and P1135/26$^{T}$ (*M.* [*P.*] *granulomatis*) were their closest neighbors on the 16S rRNA tree, whereas the leporine strains Ph13 (Bisgaard taxon 20 biovar 1) and BJ1680.3 (Bisgaard taxon 20 biovar 2) constituted a more distantly related group (Fig. 1). The results presented here suggest the divergence of at least three distinct *M. granulomatis* lineages that may have adapted to cervine, bovine, and leporine hosts, respectively.

Prior to this work, nonhemolytic *Mannheimia* strains had never been isolated outside the bovine and ovine rumen and they had never been associated with disease conditions, supporting the predicted correlation between pathogenic potential and the β-hemolytic phenotype. However, our analyses showed that isolate B 1829/84 was nonhemolytic, although the *lktA* gene was present on the chromosome. The presence of an inactivated *lkt* operon has been observed in a single *M. ruminalis* strain (HPA88), whereas chromosomal deletions of ~7,400 bp, corresponding to the entire *lkt* operon, have occurred in the remaining nonhemolytic *M. ruminalis* strains (Larsen et al., in press). If one assumes that the evolving *M. granulomatis* populations adapted to different ecologic niches (host species and anatomic niches) offering different conditions for growth, then this diversity could imply that different pools of unused genes have been lost in those populations. This hypothesis predicts the existence of variable morphologic and physiologic traits between strains belonging to the same species and constitutes the most parsimonious explanation for the observed phenotypic variation within *M. granulomatis* and other *Mannheimia* species (Angen et al., 1997a, b). These results support the use of a polyphasic strategy, combining phenotypic and genotypic methods, for proper identification and classification of *Mannheimia* strains.

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