Actinobacillus delphinicola sp. nov., a New Member of the Family *Pasteurellaceae* Pohl (1979) 1981 Isolated from Sea Mammals

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We performed phenotypic and phylogenetic studies of a gram-negative, rod-shaped bacterium isolated from cetaceans. The results of a 16S rRNA gene sequence analysis demonstrated that this bacterium represents a previously unknown line of descent in the family *Pasteurellaceae*. On the basis of the results of our phylogenetic analysis and phenotypic criteria, we propose that this organism should be classified as a new species, *Actinobacillus delphinicola* sp. nov. The type strain of *A. delphinicola* sp. nov. is strain NCTC 12870.

There have been only a few reports of infectious agents occurring in cetacean populations in the northeast Atlantic Ocean (6, 12). Considerable concern has been expressed regarding the influence of human activities on the marine environment and their possible effects on cetaceans and other marine life. An improved understanding of potentially infectious agents that occur naturally in cetacean populations is crucial to the study of environmental influences on these animals.

Postmortem and microbiological examinations have resulted in the isolation of 19 strains of gram-negative, rod-shaped bacteria whose phenotypic characteristics are consistent with identification as members of the family *Pasteurellaceae*; these bacteria were isolated from various tissues of 10 harbor porpoises (*Phocoena phocoena*), a striped dolphin (*Stenella coeruleoalba*), and a Sowerby's beaked whale (*Mesoploden bidens*). The pathological significance of these organisms for the sea mammal species from which they were isolated is not yet clear. In this paper we describe the cultural and biochemical characteristics of these bacteria and the results of a phylogenetic analysis of them.

MATERIALS AND METHODS

Samples. All of the cetaceans examined were reported under the Scottish Strandings Scheme and were located around the Scottish coastline. Postmortem examinations were carried out in accordance with a nationally agreed protocol (8). Selected tissues were removed for bacteriological investigation.

Isolation and cultivation. Primary isolations were made on Columbia agar (Difco, East Molesey, United Kingdom) supplemented with 5% citrated sheep blood (CSBA) that was incubated at 37°C in an atmosphere containing 10% CO₂ for 48 h. The sources of the strains which we investigated are shown in Table 1.

Characterization tests. Isolates were routinely grown on CSBA to provide inocula for biochemical tests; the one exception was the cultures used for the catalase test, for which tryptone soya agar (Difco) supplemented with 1% glucose was used. In the catalase test 3% H₂O₂ was pipetted onto a culture, and the culture was examined both immediately and after 5 min for the formation of gas bubbles. The oxidase test was performed by moistening a filter paper disk (diameter, 7 cm) in a petri dish with 2 or 3 drops of 1% *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (Fisons Scientific Equipment, Loughborough, United Kingdom). A small amount of a culture was smeared across the filter paper with a platinum loop, and the occurrence of a purple reaction within 30 s was considered a positive result. The effects of different atmospheres on

growth were determined by using CSBA incubated at 37°C in air without added CO₂ and prerduced CSBA incubated at 37°C in an anaerobic chamber. The ability to grow at 22, 30, and 42°C was determined on CSBA. We also tested for growth on Columbia agar supplemented with 5% serum, Columbia agar without blood or serum added, and MacConkey agar with and without salt (Difco). X- and V-factor requirements were determined on nutrient agar. Tests for oxidation or fermentation of glucose were performed with API OF medium (Biomérieux, Basingstoke, United Kingdom) and marine OF medium (10). OF medium supplemented with 2% serum and OF medium supplemented with 0.1% yeast extract were also inoculated and incubated. Acid production from carbohydrates was determined in 1% peptone water-sugar media prepared with 1% Bacto Peptone (Difco), 0.5% NaCl (Merck, Poole, United Kingdom), 1% Andrade's indicator, and carbohydrate (Merck) at a concentration of 1%, as described by Cowan and Steel (1). From a CSBA culture, a concentrated cell suspension was prepared in 0.5 ml of peptone water-sugar medium for each carbohydrate, and the preparations were incubated in a water bath at 37°C for 24 h. Arginine dihydrolase, ornithine decarboxylase, and lysine decarboxylase test media prepared by the method of Möller (11) and urea broth (Oxoid, Basingstoke, United Kingdom) were inoculated with concentrated culture suspensions, and the resulting preparations were incubated in a water bath at 37°C for 24 h. Strains were also examined with the API 20E, API 20NE, and API ZYM systems by using the manufacturer's instructions. API 20NE strips were also set up with a McFarland standard 4 inoculum. The API ZYM reactions were scored as 0, 1, 2, 3, 4, or 5 on the basis of the results of a comparison with the color chart of the manufacturer; scores of 3, 4, or 5 were considered strong positive reactions, and scores of 1 or 2 were considered weak positive reactions.

Determination of 16S rRNA gene sequences. DNA was isolated by a standard miniprep method described by Lawson et al. (9). A large fragment of the 16S rRNA gene was amplified by PCR by using universal primers pA (positions 8 to 28 [*Escherichia coli* numbering]) and pH* (positions 1542 to 1522). The amplified product was sequenced directly by using primers for conserved regions of the rRNA and a Sequenase version 2.0 sequencing kit (United States Biochemical Corp., Cleveland, Ohio) as described by Hutson et al. (7).

The sequences which we determined and the sequences of members of the *Haemophilus-Pasteurella* complex obtained from the EMBL Data Library were aligned by using the program PILEUP (2), and the alignment was corrected manually. A distance matrix was produced by using the program DNADIST (and the Kimura-2 parameter) of the PHYLIP package (5), and a tree was constructed by the neighbor-joining method with the program NEIGHBOR of the same package. The stability of the groups was assessed by bootstrapping by using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE (5).

Nucleotide sequence accession numbers. The nucleotide sequences of representative 16S rRNA genes which we determined have been deposited in EMBL Data Library under the following accession numbers: strain M1063/93L, X89377; strain M906/93^T (T = type strain), X89378; strain M39/94, X89380; and strain M266/94, X89381.

RESULTS AND DISCUSSION

A total of 19 strains of gram-negative, rod-shaped bacteria that were phenotypically similar to members of the *Haemophilus-Pasteurella* species complex were isolated from various tissues of 12 cetaceans. During this study bacteriological investi-

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TABLE 1. Sources of *A. delphinicola* sp. nov. strains

Strain	Host species	Tissue source
M906/93 ^T	<i>Phocoena phocoena</i>	Lung
M1063/93L	<i>Phocoena phocoena</i>	Lung
M39/94	<i>Phocoena phocoena</i>	Intestinal contents
M266/94	<i>Phocoena phocoena</i>	Lung
M1622/94	<i>Mesoploden bidens</i>	Lung
M1951/94I	<i>Phocoena phocoena</i>	Intestinal contents
M1951/94S	<i>Phocoena phocoena</i>	Stomach
M1952/94	<i>Phocoena phocoena</i>	Intestinal contents
M2060/94A	<i>Phocoena phocoena</i>	Intestinal contents
M2060/94B	<i>Phocoena phocoena</i>	Intestinal contents
M2194/94G	<i>Stenella coeruleoalba</i>	Gastric lymph node
M2194/94I	<i>Stenella coeruleoalba</i>	Intestinal contents
M2194/94L	<i>Stenella coeruleoalba</i>	Lung
M2194/94M	<i>Stenella coeruleoalba</i>	Mandibular lymph node
M446/95C	<i>Phocoena phocoena</i>	Cervix
M446/95L	<i>Phocoena phocoena</i>	Lung
M446/95U	<i>Phocoena phocoena</i>	Uterus
M529/95	<i>Phocoena phocoena</i>	Intestine
M752/95	<i>Phocoena phocoena</i>	Intestine

gations of a large number (>100) of mostly common seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) but also hooded seals (*Cystophora cristata*) and harp seals (*Phoca groenlandica*) were performed, but these investigations failed to yield similar isolates, suggesting that the group of bacteria which we studied may be adapted to cetaceans. The strains obtained from cetaceans were isolated from various tissues and are shown along with their host species in Table 1.

Colonies of the isolates grown on CSBA were circular, entire, low convex, grey, translucent or opaque, and 0.75 to 1 mm in diameter after 24 h of incubation in the presence of 10%

CO₂ at 37°C. Very slight hemolysis of sheep blood was often apparent, particularly in cultures incubated for more than 48 h. The cells were gram negative and rod shaped. The organisms were catalase negative, oxidase positive, and nonmotile. All of the isolates grew under anaerobic conditions but not in air without added CO₂. All of the isolates grew on Columbia agar without added blood or serum, but the colonies were considerably smaller than the colonies described above. Growth did not occur on MacConkey agar with or without salt. Growth occurred at 30 and 42°C but not at 22°C. Biochemically, all of the isolates were very similar. However, variable reactions were obtained for arginine dihydrolase, ornithine decarboxylase, and lysine decarboxylase activities, and four groups could be differentiated on the basis of these characteristics; four strains were positive for ornithine decarboxylase only, seven strains were positive for lysine decarboxylase and ornithine decarboxylase, seven strains were positive for arginine dihydrolase only, and one strain was positive for lysine decarboxylase only. These four groups did not correlate with the sea mammal host species from which the isolates were obtained. The urease test was negative. Nitrates were reduced to nitrites, but nitrites were not reduced. No growth occurred in methyl red Vogues-Proskauer medium, tryptone water medium, or glucose-peptone water medium. Acid was produced from glucose and mannose when the small-volume-heavy-inoculum method was used, but not from adonitol, dulcitol, galactose, inositol, inulin, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. However, oxidation-fermentation reactions were negative as determined by traditional methods and with the marine medium of Leifson, although growth was evident in both media. The results obtained for decarboxylase reactions performed with API 20E strips were consistent with the results obtained by conventional methods. However, all tests for arginine dihy-

TABLE 2. Phenotypic characteristics of *A. delphinicola* sp. nov.

Test	No. of positive strains ^a	Test	No. of positive strains
Catalase	0	Lysine decarboxylase (Møller).....	8 ^d
Oxidase	19	ONPG ^e	0
Aerobic growth.....	0	Acid produced from:	
Growth in the presence of 10% CO ₂	19	Glucose.....	19
Anaerobic growth.....	19	Mannose.....	19
Growth at 22°C.....	0	Adonitol.....	0
Growth at 30°C.....	19	Dulcitol.....	0
Growth at 42°C.....	19	Galactose.....	0
Growth enhanced by blood or serum.....	19	Inositol.....	0
X-factor requirement.....	0	Inulin.....	0
V-factor requirement.....	0	Lactose.....	0
Growth on MacConkey agar.....	0	Maltose.....	0
Nitrate reduction.....	19	Mannitol.....	0
Nitrite reduction.....	0	Melibiose.....	0
Urease	0	Raffinose.....	0
Indole	0	Rhamnose.....	0
Voges-Proskauer.....	19	Salicin.....	0
Gelatin hydrolysis.....	0	Sorbitol.....	0
Arginine dihydrolase (Møller).....	7 ^b	Trehalose.....	0
Ornithine decarboxylase (Møller).....	11 ^c	Xylose.....	0

^a A total of 19 strains were tested.

^b Strains M1951/94I, M446/95L, and M446/95U from *Phocoena phocoena* and strains M2194/94G, M2194/94I, M2194/94L, and M2194/94M from *Stenella coeruleoalba* were arginine dihydrolase positive.

^c Strains M1951/94I, M2060/94A, M446/95L, and M446/95U from *Phocoena phocoena* and strains M2194/94G, M2194/94I, M2194/94L, and M2194/94M from *Stenella coeruleoalba* were ornithine decarboxylase negative.

^d Strain M1622/94 from *Mesoploden bidens* and strains M39/94, M226/94, M1952/94, M2060/94A, M446/95C, M529/95, and M752/95 from *Phocoena phocoena* were lysine decarboxylase positive.

^e ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

TABLE 3. API ZYM reactions of 19 *A. delphinicola* sp. nov. strains

Reaction ^a	No. of strains																		
	Alkaline phosphatase	Esterase	Esterase-lipase	Lipase	Leucine arylamidase	Valine arylamidase	Cystine arylamidase	Trypsin	Chymotrypsin	Acid phosphatase	Naphthol-AS-BI-phosphohydrolase	α-Galactosidase	β-Galactosidase	β-Glucuronidase	α-Glucosidase	β-Glucosidase	N-Acetyl-β-glucosaminidase	α-Mannosidase	α-Fucosidase
Strong	19	0	17	0	16	0	0	0	0	19	3	0	0	0	0	0	0	0	0
Weak	0	19	2	0	3	0	0	0	0	0	16	0	0	0	0	0	0	0	0

^a A strong reaction was a reaction in which the score was 3, 4, or 5, and a weak reaction was a reaction in which the score was 1 or 2.

drolase activity were negative with API 20NE strips when we used a no. 0.5 McFarland standard suspension, as recommended by the manufacturer, but these tests were positive when we used a no. 4 McFarland standard suspension. It is important to note that, with the exception of *Haemophilus paracuniculus*, members of the family *Pasteurellaceae* do not hydrolyze arginine. The positive arginine dihydrolase reactions of some of the isolates in this study were dependent on the methods used and therefore should be considered tentative until more detailed enzymological studies are performed. A positive Voges-Proskauer reaction was the only other positive reaction obtained with the API 20E system, and the nitrate reduction test was the only positive test obtained with API 20NE strips. When the API ZYM system was used, all of the isolates produced strong positive reactions with alkaline phosphatase and acid phosphatase. Strong or weak positive reactions were obtained with esterase lipase, leucine arylamidase, and naphthol-AS-BI-phosphohydrolase, and weak positive reactions were obtained with esterase. Detailed results of the characterization tests are shown in Tables 2 and 3.

To investigate the genetic relationships of the isolates, genes encoding 16S rRNA were amplified by PCR and subjected to a sequence analysis. An almost complete 16S rRNA gene sequence of representative strain M1063/93L was determined. Preliminary comparative sequence searches of the EMBL and GenBank libraries in which we used the FASTA program (2) revealed that the sequence which we determined was most similar (levels of sequence similarity, generally >92%) to the sequences of members of the *Haemophilus-Pasteurella* species complex. The levels of relatedness with other gram-negative taxa were significantly lower (data not shown). The sequence which we determined and the sequences of *Actinobacillus*, *Haemophilus*, and *Pasteurella* species were subjected to a pairwise analysis, and derived evolutionary distances were used to determine the phylogenetic relationships of these organisms. Figure 1 is a tree which shows the phylogenetic position of strain M1063/93L in the family *Pasteurellaceae*, and representative levels of sequence similarity are shown in Table 4. Isolate M1063/93L formed a relatively long line and was genealogically most closely associated with a group of organisms which encompassed *Actinobacillus* species, *Haemophilus parainfluenzae*, *Haemophilus parahaemolyticus*, and *Haemophilus paraphrohaemolyticus* (designated cluster 4 of the *Pasteurellaceae* by Dewhirst et al. [4]). It was evident, however, from the low levels of sequence relatedness (generally <95%) and the bootstrap data that strain M1063/93L is not particularly closely related phylogenetically to any previously described species. To determine

whether the sequence of strain M1063/93L was characteristic of our group of isolates, we determined partial sequences for eight other strains, including four strains which exhibited atypical decarboxylase reactions (e.g., arginine). The sequences of short fragments that were approximately 500 bases long (positions 40 to 530 [*E. coli* numbering system]) and included highly diagnostic regions V1 to V3 were determined, and all of the sequences were very similar (they differed by 0 to 3 bases).

FIG. 1. Unrooted tree showing the phylogenetic position of *A. delphinicola* sp. nov. in the *Haemophilus-Pasteurella* species complex. The tree is based on 1,465 16S rRNA positions and was constructed by the neighbor-joining method. Abbreviations: *P.*, *Pasteurella*; *H.*, *Haemophilus*; *A.*, *Actinobacillus*. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points.

TABLE 4. Levels of 16S rRNA sequence similarity between *A. delphinicola* sp. nov. and representatives of the *Haemophilus*-*Pasteurella* species complex

Species	% 16S rRNA sequence similarity to <i>A. delphinicola</i>	Species	% 16S rRNA sequence similarity to <i>A. delphinicola</i>
<i>Actinobacillus actinomycetemcomitans</i> (M75039) ^a	92.2	<i>Haemophilus paraphrophilus</i> (M75082)	93.6
<i>Actinobacillus capsulatus</i> (M75062)	92.6	<i>Haemophilus parasuis</i> (M75065)	93.5
<i>Actinobacillus equuli</i> (M75072)	93.8	<i>Haemophilus segnis</i> (M75043)	94.3
<i>Actinobacillus hominis</i> (L06076)	94.4	<i>Haemophilus somnus</i> (M75046)	91.6
<i>Actinobacillus lignieresii</i> (M35017)	93.9	<i>Pasteurella aerogenes</i> (M75058)	92.8
<i>Actinobacillus pleuropneumoniae</i> (M75074)	93.7	<i>Pasteurella avium</i> (M75058)	94.1
<i>Actinobacillus seminis</i> (M75047)	92.6	<i>Pasteurella bettyae</i> (L06088)	92.5
<i>Actinobacillus suis</i> (M75071)	94.2	<i>Pasteurella canis</i> (M75049)	92.4
<i>Haemophilus aegyptius</i> (M75044)	94.1	<i>Pasteurella dagmatis</i> (M75051)	93.1
<i>Haemophilus aphrophilus</i> (M75041)	93.7	<i>Pasteurella gallinarum</i> (M7505)	93.3
<i>Haemophilus ducreyi</i> (M75078)	93.3	<i>Pasteurella haemolytica</i> (M75080)	93.3
<i>Haemophilus haemoglobinophilus</i> (M75064)	93.8	<i>Pasteurella langaa</i> (M75053)	92.3
<i>Haemophilus haemolyticus</i> (M75045)	94.8	<i>Pasteurella multocida</i> (M35018)	92.2
<i>Haemophilus influenzae</i> (M35019)	93.9	<i>Pasteurella mairi</i> (L06089)	92.4
<i>Haemophilus paracuniculus</i> (M75061)	92.9	<i>Pasteurella pneumotropica</i> (M75083)	92.9
<i>Haemophilus paragallinarum</i> (M75057)	94.0	<i>Pasteurella stomatis</i> (M75050)	92.4
<i>Haemophilus parahaemolyticus</i> (M75073)	93.9	<i>Pasteurella testudinis</i> (L06090)	90.2
<i>Haemophilus paraphrohaemolyticus</i> (M75076)	95.1	<i>Pasteurella trehalosi</i> (M75063)	93.6
<i>Haemophilus parainfluenzae</i> (M75081)	93.9	<i>Pasteurella volantium</i> (M75070)	93.3

^a The data in parentheses are EMBL nucleotide sequence accession numbers.

We discerned no genotypic differences between strains according to their host species (*Phocoena* and *Stenella* species).

The extensive 16S rRNA sequence analyses of Dewhirst et al. (3, 4) have shown that species belonging to the genera *Actinobacillus*, *Haemophilus*, and *Pasteurella* are phylogeneti-

cally intermixed. Although it is clear from our results that the isolates which we studied from a distinct line that is worthy of species status, the allocation of these organisms to a specific genus in the *Pasteurellaceae* is problematic given the general complexity (3, 4) of the branching pattern in this family. The

TABLE 5. Differential characteristics of *A. delphinicola* sp. nov. and other members of the genus *Actinobacillus*^a

Characteristic	<i>Actinobacillus delphinicola</i>	<i>Actinobacillus actinomycetemcomitans</i>	<i>Actinobacillus capsulatus</i>	<i>Actinobacillus equuli</i>	<i>Actinobacillus hominis</i>	<i>Actinobacillus lignieresii</i>	<i>Actinobacillus muris</i>	<i>Actinobacillus pleuropneumoniae</i>	<i>Actinobacillus rossii</i>	<i>Actinobacillus seminis</i>	<i>Actinobacillus suis</i>
Catalase	- ^b	+	+	d	-	d	+	d	+	+	+
Oxidase	+	+	+	(+)	+	(+)	+	d	+	d	(+)
β-Galactosidase	-	-	+	d	+	d	-	+	(+)	-	(+)
Ornithine decarboxylase	d	-	-	-	-	-	-	-	-	d	-
Urease	-	-	+	+	+	+	+	+	+	-	+
Growth on MacConkey agar	-	d	+	+	-	+	-	-	(+)	-	+
Beta-hemolysis (sheep blood)	-	-	-	-	-	-	-	+	d	-	+
Acid production from:											
Galactose	-	+	+	d	+	+	d, w	+, w	+	d, L	(+)
Inositol	-	-	-	-	-	-	d, w	-	+	d	-
Lactose	-	-	+	+	+	d, L	-	d	d	-	+
Maltose	-	+	+	+	+	+	+	+	(-)	d, L	+
Mannitol	-	(+)	+	+	+	+	+	+	+	d, L	-
Mannose	+	+	+	+	-	+	+	+	d	-	+
Melibiose	-	-	+	+	+, L	-	+, L	-	-	-	+
Raffinose	-	-	+	+	+	d	+	d	(-)	-	+
Salicin	-	-	+	-	d	-	+	+	-	-	+
Sorbitol	-	(-)	+	d	-	(-)	-	-	+	-	-
Sucrose	-	-	+	+	+	+	+	+	-	-	+
Trehalose	-	-	+	+	+	-	+	-	-	-	+
Xylose	-	d	+	+	+	+	-	+	+	-	+

^a Data from this study and reference 13.

^b +, 90 to 100% of the strains are positive; (+), 80 to 89% of the strains are positive; d, 21 to 79% of the strains are positive; (-), 11 to 20% of the strains are positive; -, 0 to 10% of the strains are positive; w, weak reaction; L, late reaction.

new species differs from members of the genus *Pasteurella* by its negative catalase reaction and can be readily distinguished from the genus *Haemophilus* by its lack of a requirement for X and V factors. Phenotypically, the new species is most similar to members of the genus *Actinobacillus*. The cetacean bacterium, however, differs from most *Actinobacillus* spp. by its negative *o*-nitrophenyl- β -D-galactopyranoside reaction. However, some *Actinobacillus* spp. (e.g., *Actinobacillus muris* and *Actinobacillus seminis*) also are *o*-nitrophenyl- β -D-galactopyranoside negative or exhibit variable reactions (13), and therefore this criterion does not preclude classification of the cetacean bacterium in the genus *Actinobacillus*. We recognize that the current taxonomy of the family *Pasteurellaceae* is very unsatisfactory (3, 4). Assignment of the cetacean bacterium to the genus *Actinobacillus* is therefore considered a placement of convenience, and the position of this organism may have to be changed depending on the outcome of a future comprehensive taxonomic restructuring of the family *Pasteurellaceae*.

Description of *Actinobacillus delphinicola* sp. nov. *Actinobacillus delphinicola* (del.phi.ni'co.la. L. n. *delphinus*, dolphin; L. n. *cola*, dweller: M.L.n. *delphinicola*, dolphin dweller) cells are pleomorphic gram-negative rods. They are nonmotile and facultatively anaerobic, but added CO₂ is required for growth. Colonies on CSBA incubated in an atmosphere containing 10% added CO₂ at 37°C are circular, entire, low, convex, smooth, grey, and 0.75 to 1 mm in diameter after 24 h. Non-hemolytic or weakly hemolytic. Blood or serum enhances growth. No growth occurs on MacConkey agar. Growth occurs at 42°C but not at 22°C. Catalase negative and oxidase positive. Nitrates are reduced to nitrites. Nitrite is not reduced. Acid is produced from glucose and mannose but not from adonitol, dulcitol, galactose, inositol, inulin, lactose, maltose, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. Voges-Proskauer positive. Urease, H₂S, and indole negative. Alkaline phosphatase, esterase-lipase, leucine arylamidase, and acid phosphatase are produced in large amounts, naphthol-AS-BI-phosphohydrolase is produced, usually in large amounts, and esterase is produced in lesser amounts when the API ZYM system is used. Arginine may be hydrolyzed by some strains. Some strains decarboxylate ornithine and/or lysine. Isolated from cetaceans. The type strain is strain NCTC 12870 (= M906/93). The description of the type strain corresponds to that of the species except that the type strain is ornithine decarboxylase positive and arginine dihydrolase and lysine decarboxylase negative.

Characteristics useful for differentiating *A. delphinicola* from some other *Actinobacillus* spp. are shown in Table 5.

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