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NOTE

Bacteriology

Phylogenetic relationship of *Ornithobacterium rhinotracheale* strains

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ABSTRACT. The bacterium *Ornithobacterium rhinotracheale* is associated with respiratory disease in wild birds and poultry. In this study, the phylogenetic analysis of nine reference strains of *O. rhinotracheale* belonging to serovars A to I, and eight Mexican isolates belonging to serovar A, was performed. The analysis was extended to include sequences from another 23 strains available in the public domain. The analysis showed that the 40 sequences formed six clusters, I to VI. All eight Mexican field isolates were placed in cluster I. One of the reference strains appears to present genetic diversity not previously recognized and was placed in a new genetic cluster. In conclusion, the phylogenetic analysis of *O. rhinotracheale* strains, based on the 16S rRNA gene, is a suitable tool for epidemiologic studies.

KEY WORDS: *Ornithobacterium rhinotracheale*, phylogenetic analysis, reference strains

The Gram-negative bacterium *Ornithobacterium rhinotracheale* has been associated with respiratory disease and mortality in wild-birds and poultry and has a worldwide distribution in many avian species [3]. Isolates of *O. rhinotracheale* can be classified into 18 agar gel precipitation (AGP) serovars (A through R), and serovar A is the most prevalent among chicken and turkey isolates [3, 5, 14, 17]. Genotyping studies of *O. rhinotracheale* isolates obtained from poultry throughout the world have shown a small group of closely related clones [2, 4, 7, 10]. Six electrophoretic types (ET) obtained by multi-locus enzyme electrophoresis have been recognized in *O. rhinotracheale* isolates from eight countries around the world [2]. The six ETs were confirmed by 16S rRNA gene sequencing and rep-PCR analysis [2].

The phylogenetic relationships amongst a number of reference strains as well as Mexican isolates of *O. rhinotracheale* are unknown. Hence, the aim of the present study was to perform a phylogenetic analysis of *O. rhinotracheale* using a number of serovar reference strains and Mexican field isolates.

In the present study, a total of nine reference strains of *O. rhinotracheale* were included: B 3263/91, GGD 1261, ORV K91-201, ORV 94108 no. 2, O-95029 no. 12229, ORV 94084 K858, O-95029 no. 16279, E-94063 4.2, and BAC 96-0334 #MINN 18, representing serovars A through I [12, 17], respectively (Table 1). All reference strains were sourced from the culture collection held at the University of Queensland, Australia. A total of eight well-characterized Mexican isolates of *O. rhinotracheale* [10], were included in the study. Bacteria were cultivated on 10% sheep blood agar at 37°C in a candle jar. Brain-heart infusion broth was used for propagation and maintenance of bacterial cultures. For improved growth, this medium was supplemented with 1% (v/v) filter-sterilized and heat-inactivated horse serum [13]. The DNA was extracted directly from cell biomass cultured in brain-hearth infusion broth and purified by using DNeasy® Blood and Tissue kit (QIAGEN, Austin, TX, U.S.A.), according to the manufacturer's protocols. The 16S ribosomal RNA (rRNA) gene was amplified from the nine reference strains and the eight Mexican isolates of *O. rhinotracheale* by using primers and conditions as elsewhere reported [2]. The sequencing of the 16S rRNA gene was performed by Macrogen Inc. (Seoul, Republic of Korea) using the Sanger dideoxy terminator sequencing method. The 16S rRNA gene sequences were obtained in the region covering *Escherichia coli* positions 27-1492. A Basic Local Alignment Search Tool (BLAST) search was performed in GenBank [1]. Pairwise comparisons for similarity were performed by the program WATER included in European Molecular Biology Open Software Suite (EMBOSS) [11]. The phylogenetic analysis was performed

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Table 1. Bacterial strains used in the study

Strain	Source	Origin	Year of isolation	Serovar
B 3263/91	Broiler	South Africa	1991	A
GGD 1261	Turkey	Germany	1991	B
ORV K91-201	Broiler	U.S.A.	1991	C
ORV 94108 no. 2	Turkey	France	1994	D
O-95029 no. 12229	Broiler	France	1995	E
ORV 94084 K858	Turkey	The Netherlands	1994	F
O-95029 no. 16279	Broiler	France	1995	G
E-94063 4.2	Turkey	The Netherlands	1994	H
BAC 96-0334 MINN 18	Turkey	U.S.A.	1996	I
ESV-55	Broiler	México	2005	A
ESV-60	Turkey	México	2006	A
ESV-104	Broiler	México	2008	A
ESV-207	Peacock	México	2009	A
ESV-209	Turkey	México	2009	A
ESV-216	Layer	México	2010	A
ESV-301	Hobby chicken	México	2011	A
ESV-305	Hobby chicken	México	2012	A

by construction of a multiple alignment by ClustalX, removal of gapped columns, and analysis by the maximum likelihood method [16] and genetic distances obtained by Kimura's 2-parameter model, conducted using MEGA5 [6]. Additional sequences of well-characterized reference strains and isolates from GenBank and Green Genes data-bases were included in the analysis (Fig. 1) [2, 18, 19]. Shorter sequences (less than 700 nucleotides) were not included in the analysis [4, 8].

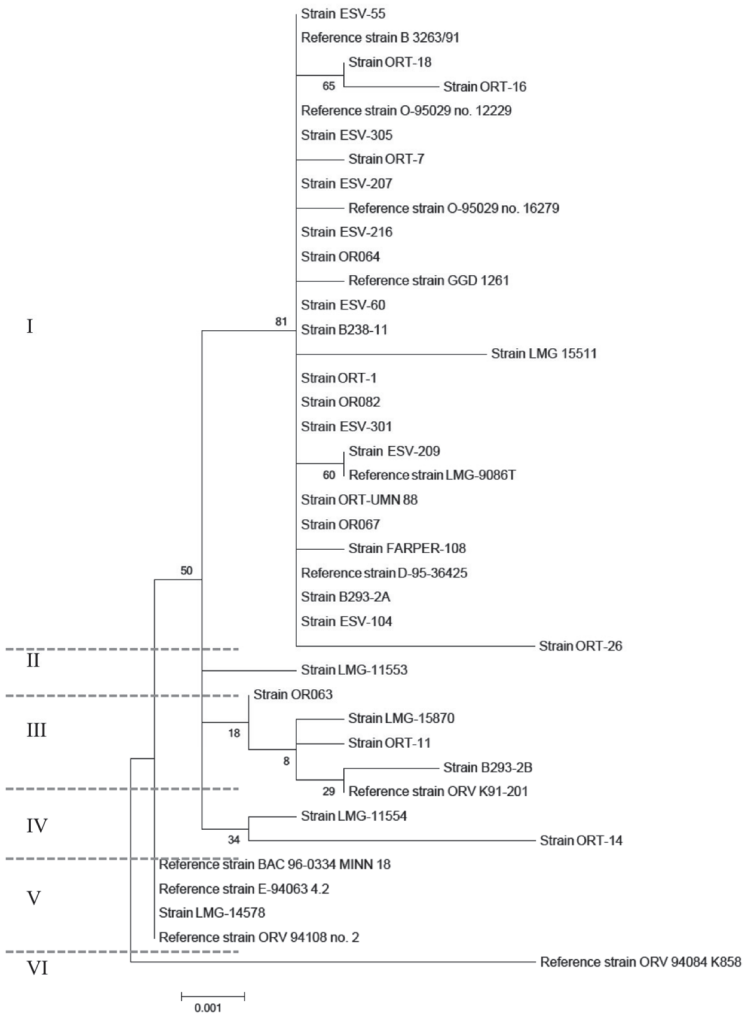
Sequences for the 16S rRNA gene of the nine reference strains and eight Mexican isolates of *O. rhinotracheale* were obtained and deposited in GenBank (accession numbers KY612254 and KY809786 to KY809801). Pairwise sequence comparisons revealed 99.1 to 100% sequence identity among the 40 sequences included in the study. The analysis of polymorphic nucleotides in the 16S rRNA gene showed differences from 1 to 10 nucleotides in comparison to a consensus 16S rRNA gene sequence of *O. rhinotracheale* (Table 2). The 40 sequences included into the phylogenetic analysis were clustered into six genetic clusters (I to VI; Fig. 1).

As the study of Amonsin *et al*. [2] had identified a system for correlating the ET typing results with single nucleotide polymorphisms (SNP) in the 16S rRNA gene, the SNP patterns of all of the strains and field isolates in this study were examined using this correlation (Table 2). Some of the sequences used in the current study were the same as used in the Amonsin *et al*. [2] study. Hence, these strains and isolates have a known ET assignment. For a number of other strains and isolates, the correlation proposed by Amonsin *et al*. [2] was used to predict the ET (predicted ET is marked by an asterisk in Table 2). Some of the strains and isolates had the SNP pattern noted by Amonsin *et al*. [2] as well as additional SNPs not noted by Amonsin *et al*. [2]. Hence, for these strains and isolates, the predicted ET is marked as a variant (Table 2). The final group of strains and isolates had SNP profiles that were markedly different from those recorded by Amonsin *et al*. [2] and it was not possible to predict an ET (Table 2).

All of the strains previously typed as ET 1 and ET 2 by Amonsin *et al*. [2] were assigned to cluster I in the current study (Fig. 1 and Table 2). As 17 of the 19 strains and isolates allocated to cluster I aligned with the scheme suggested by Amonsin *et al*. [2] for correlating polymorphisms in the 16S rRNA gene sequence, we were able to predict that these 13 strains/isolates would be ET 1 (Table 2). A further six strains/isolates within cluster I had the same SNP pattern as assigned by Amonsin *et al*. [2] to ET 1 but also had additional SNP sites not noted by Amonsin *et al*. [2] and were thus predicted to be variants of ET 1 (termed ET 1v in Table 2). Cluster II consisted of strain LMG-11553 (GenBank accession number U87102). Cluster III consisted of strain LMG-15870 (GenBank accession number U87103) of known ET 5 and four strains that could not be assigned to a predicted ET. Cluster IV consisted of strain LMG-11554 (GenBank accession number U87104) of known ET 6 and one isolate that could not be assigned to a predicted ET. Cluster V consisted of strain LMG-14578 (GenBank accession number U87105) of known ET 4 and three reference strains that were assigned to a predicted ET. Cluster VI had only a single reference strain that could not be assigned to an ET.

In this study, we report the sequencing of 16S rRNA gene from nine reference strains of *Ornithobacterium rhinotracheale* belonging to serovars A to I. While the sequencing of 16S rRNA gene from eight reference strains of *O. rhinotracheale* belonging to serovars A to H has been previously reported [9], the sequences are not available in public data-bases.

Amonsin *et al*. [2] assigned 90.9% (50 of 55) of isolates of *O. rhinotracheale* to ET 1 and ET 2, comprising the ET 1 complex. In the present study, phylogenetic analysis of 16S rRNA gene sequences resulted in a cluster (cluster I) that consisted predominantly of strains and isolates that were known to be ET 1 or ET 2 or predicted to be ET 1 or a variant of ET 1, confirming the ET 1 complex. All of the Mexican serovar A isolates and reference strains of serovars A, B, E and G were clustered in cluster I (Fig. 1). Clearly, this complex (16S rRNA gene cluster I/ET complex 1) is a cluster of strains and isolates that are commonly distributed around the world. In the current study, more variation in the 16S rRNA gene was found than was used by the earlier study of Amonsin *et al*. [2] to correlate ET with 16S rRNA sequences. Despite this increased diversity, there was still a good



Host	Origin	Year	Serovar	GenBank
Broiler	**Mexico**	**2005**	**A**	**KY809795**
Broiler	**South Africa**	**1991**	**A**	**KY809786**
Chicken	China	2012	-	KC454303
Chicken	China	2012	-	KC454301
Broiler	**France**	**1995**	**E**	**KY809790**
Chicken	**Mexico**	**2012**	**A**	**KY612254**
Chicken	China	2012	-	KC454292
Peacock	**Mexico**	**2009**	**A**	**KY809798**
Broiler	**France**	**1995**	**G**	**KY809792**
Chicken	**Mexico**	**2012**	**A**	**KY809800**
Chicken	Hungary	2013	A	KX998691
Turkey	**Germany**	**1991**	**B**	**KY809787**
Turkey	**Mexico**	**2006**	**A**	**KY809796**
Pheasant	England	2009	-	HF548213
Turkey	Israel	1993	-	U87106
Chicken	China	2012	-	KC454287
Turkey	Hungary	2015	B	KX998701
Chicken	**Mexico**	**2011**	**A**	**KY809801**
Turkey	**Mexico**	**2009**	**A**	**KY809799**
Turkey	United Kingdom	1988	-	U87101
Turkey	USA	1995	A	CP006828
Turkey	Hungary	2015	A	KX998694
Broiler	Peru	2013	-	KP128016
Turkey	USA	1995	-	U87100
Pheasant	England	2010	-	HF548214
Broiler	**Mexico**	**2008**	**A**	**KY809797**
Chicken	China	2012	-	KC454311
Rook	Germany	1983	-	U87102
Chicken	Hungary	2013	D (C)	KX998690
Guinea Fowl	Belgium	1992	-	U87103
Chicken	China	2012	-	KC454296
Pheasant	England	2010	-	HF548215
Broiler	**USA**	**1991**	**C**	**KY809788**
Rook	Germany	1983	-	U87104
Turkey	China	2012	-	KC454299
Turkey	**USA**	**1996**	**I**	**KY809794**
Turkey	**The Netherlands**	**1994**	**H**	**KY809793**
Turkey	The Netherlands	1994	-	U87105
Turkey	**France**	**1994**	**D**	**KY809789**
Turkey	**The Netherlands**	**1994**	**F**	**KY809791**

Fig. 1. Phylogenetic relationship of *O. rhinotracheale* reference strains and isolates included in this study based on the neighbor-joining analysis of 16S rRNA gene sequences. The numbers at nodes indicate bootstrap values obtained from 1,000 resamplings. The scale bar represents sequence variation. In bold, strains sequenced in the present study.

correlation between ET results (predicted or known) and the phylogeny.

Reference strain ORV 94084 K858 of serovar F of *O. rhinotracheale* was allocated to cluster VI as the sole member of that cluster. Ten nucleotide changes were recorded in this strain (Table 2). It is clear that this strain represents significant new diversity that has not been previously recognized and association with antigenic and pathogenic traits need to be studied. This reference strain was included in a multilocus sequence typing (MLST) analysis that included seven housekeeping genes, and showed the most nucleotide polymorphisms [15, 16]. Two distinct phylogenetic clusters were identified in the phylogenetic tree generated from MLST sequences and reference strain ORV 94084 K858 of serovar F of *O. rhinotracheale* was allocated, along with other two strains, to cluster A and differed considerably from the vast majority of *O. rhinotracheale* strains included in that study [16]. Further studies comparing phylogenetic analysis based on 16S rRNA gene and MLST are needed.

Identical genotypes (16S rRNA gene phylogeny and ERIC-PCR pattern) of Mexican field isolates included in the study, recovered over a long time period in two main Mexican geographic areas (Puebla and Jalisco), support that *O. rhinotracheale* population is predominantly clonal as has been previously suggested [2, 19].

In conclusion, new information was added to the knowledge of population structure of *O. rhinotracheale* by the phylogenetic analysis of the 16S rRNA gene, allowing a worldwide comparison of sequence data and evidencing the existence of greater genetic variability among strains than previously contemplated.

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Table 2. Polymorphic sites in *O. rhinotracheale* in 16S rRNA gene sequences

Strain[a)]	ET or predicted ET[b)]	ERIC-PCR genotype[c)] /AGP serovar	Cluster[d)]	Base at the following nucleotide position[e)]																										
				128	164	171	181	186	187	276	309	320	374	432	443	455	558	570	598	819	831	984	985	994	996	1002	1136	1197	1229
Consensus				A	A	G	G	A	A	G	G	G	C	G	T	A	C	G	C	T	G	G	A	A	T	C	A	C	C
D95-36425	1		I	.[h)]	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
B 3263/91	1*	I / A	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
O-95029 no. 12229	1*	IV / E	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
ORT-UMN 88	1*	? / A	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
ESV-55	1*	VIII / A	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
ESV-60	1*	VIII / A	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
ESV-104	1*	VIII / A	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
ESV-207	1*	VIII / A	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
ESV-216	1*	VIII / A	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
ESV-301	1*	VIII / A	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
ESV-305	1*	VIII / A	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
B238-11	1*		I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
B293-2A	1*		I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
OR064	1*	A	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
OR067	1*	A	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
OR082	1*	B	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
ORT-1	1*		I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
GGD 1261	1v*	I / B	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	T	.	.	.
O-95029 no. 16279	1v*	VI / G	I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	T	.
ORT-7	1v*		I	.	.	.	.	.	.	.	.	.	T	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
ORT-18	1v*		I	.	.	.	.	.	.	.	.	.	.	.	C	.	T	.	.	.	A	.	.	.	.	.	.	.	.
ORT-16	1v*		I	.	.	.	.	.	.	.	A	A	.	.	C	.	T	.	.	.	A	.	.	.	.	.	.	.	.
ORT-26[f)]	1v*		I	.	.	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
LMG-9086T	1		I	.	.	.	.	.	.	T	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
ESV-209	1*	VIII / A	I	.	.	.	.	.	.	T	.	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
LMG-15511	2		I	.	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.
FARPER-108	?		I	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.
LMG-11553	3		II	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T
LMG-15870	5		III	G	G	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
OR063	?	D (C)	III	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B-293-2B	?		III	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T
ORT-11	?		III	.	G	.	.	G	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
ORV K91-201	?	II / C	III	.	G	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
LMG-11554	6		IV	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	G	.	.	.	.	.
ORT-14[g)]	?		IV	.	.	A	.	.	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	.	.	.	.	.	.
LMG-14578	4		V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
ORV 94108 no. 2	4*	III / D	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
E-94063 4.2	4*	III / H	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
BAC 96-0334 MINN 18	4*	VII / I	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
ORV 94084 K858	?	V / F	VI	.	.	.	A	G	G	.	.	.	.	.	.	.	.	A	T	C	.	A	G	.	C	.	G	.	.

a) Strains with underlining were examined in the study of Amonsin *et al.* [2]. The polymorphisms reported for these strains are based on the Genbank sequence. b) ET=Electrophoretic type. The results that are underlined are those reported by Amonsin *et al.* [2]. The ET results with an asterisk (*) are predicted results based on the current sequencing data and an exact alignment with the polymorphism pattern reported by Amonsin *et al.* [2]. Where additional polymorphisms were found outside the base positions used by Amonsin *et al.* [2], the ET is a predicted variant ET (1v). Where the polymorphisms in the strains do not match at all with the polymorphisms used by Amonsin *et al.* [2], the result is recorded as ? c) ERIC-PCR genotype=the enterobacterial repetitive intergenic consensus-type as previously defined [10]. d) Cluster assignment is based on Fig. 1. e) Base positions that are underlined are the base positions used by Amonsin *et al.* [2] to define ET by 16S rDNA polymorphisms. The other base positions are those found in the current study. f) ORT-26, further base nucleotide positions at 65 (G), 74 (G), 76 (G), 165 (T) and 172 (G). g) ORT-14, further base nucleotide positions at 403 (T), 418 (C), 420 (A) and 428 (A). h) Dots indicate no change from the consensus sequence.

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