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MOLECULAR CHARACTERIZATION OF *PASTEURELLA TESTUDINIS* ISOLATED FROM DESERT TORTOISES (*GOPHERUS AGASSIZII*) WITH AND WITHOUT UPPER RESPIRATORY TRACT DISEASE

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ABSTRACT: Isolates of *Pasteurella testudinis* recovered from clinically healthy desert tortoises (*Gopherus agassizii*) and tortoises with upper respiratory tract disease (URTD) were characterized in an attempt to identify strains associated with disease. Eighty-nine isolates, 52 from ill and 37 from healthy tortoises collected from Nevada (USA), June 1990 to September 1991, were genomically fingerprinted and grouped based on ribotype similarity. Twelve isolates (six from ill and six from healthy tortoises) were further characterized with regard to whole-cell protein (WCP) and outer membrane protein (OMP) composition and their ability to survive in normal tortoise plasma. The 89 isolates were initially distributed into 33 distinct ribotype groups using the restriction enzyme *Eco*RI; five ribotypes contained over 50% of the isolates. Only one *Eco*RI ribotype was comprised of multiple isolates ($n = 4$) exclusively recovered from tortoises with URTD. When the ten *Eco*RI ribotypes that contained more than one isolate per ribotype were further studied using a second restriction enzyme, *Eco*RV, one *Eco*RI/*Eco*RV ribotype contained five isolates recovered from URTD tortoises and none from healthy animals. The *Eco*RI ribotype comprised of four isolates, all from tortoises with URTD, was further separated into three distinct groups with *Eco*RV. All 12 isolates studied grew equally well in normal tortoise plasma, and when broth-grown WCP and OMP profiles were evaluated, no proteins were unique to isolates from URTD tortoises. Iron-regulated OMP's were produced in three isolates examined, but these OMP's apparently were not virulence-related.

Key words: *Pasteurella testudinis*, desert tortoise, upper respiratory tract disease, ribotyping, DNA fingerprinting, outer membrane proteins.

INTRODUCTION

Upper respiratory tract disease (URTD) appears to be a significant factor affecting the vitality of certain populations of free-ranging desert tortoises (*Gopherus agassizii*), particularly in the Mojave Desert of California (USA) (Jacobson, 1992). Clinically, URTD appears in tortoises as a rhinitis with accompanying nasal discharge. In addition to tortoises in California, free-ranging tortoises with signs of URTD also have been seen in Nevada, Utah, and Arizona (USA); this disease has contributed to the listing of the desert tortoise as threatened in the U.S. in 1990 (Jacobson et al., 1991).

The bacterium, *Pasteurella testudinis*, has been cultured from the respiratory tract of healthy desert tortoises, but more frequently has been isolated from tortoises with URTD (Snipes et al., 1980; Jacobson et al., 1991). Previous attempts to produce respiratory tract disease in tortoises by challenge with cultures of *P. testudinis* have met with equivocal results (Snipes, 1976). Recently, a previously unidentified *Mycoplasma* sp. also has been incriminated as a possible etiologic agent of URTD (Jacobson et al., 1991). Therefore, the precise role that *P. testudinis* plays in URTD in desert tortoises remains uncertain.

Our goal was to characterize strains of *P. testudinis* which may vary in their pathogenicity for tortoises. Methods used to identify and characterize strains of *P. testudinis* and techniques used to study the epizootiology and pathogenesis of other species of *Pasteurella* included ribotyping (Snipes et al., 1989, 1992), growth in normal tortoise plasma (serum or plasma resistance) (Morishita et al., 1990), and analysis of outer membrane protein (OMP) composition (Snipes et al., 1988). Ribotyp-

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ing, a technique used to fingerprint bacterial DNA, was the principal method used to differentiate strains since more traditional methods of differentiation such as serotyping have not been developed for *P. testudinis*. In addition, ribotyping is used to analyze highly conserved ribosomal RNA chromosomal genes and associated sequences, and therefore represents a desirable approach for determining genetic relatedness of bacteria (Stull et al., 1988).

Occurrence of strains that differ in pathogenicity may assist in identifying the role that *P. testudinis* plays in URTD. In addition, identification of ribotypes associated with more pathogenic strains would help direct future research into potential virulence determinants associated with *P. testudinis*. In this paper, we report the results of genotypic and phenotypic characterization of *P. testudinis*, and attempt to correlate in vitro characteristics of particular strains with the presence of URTD in desert tortoises.

MATERIALS AND METHODS

We characterized 89 isolates of *P. testudinis* obtained from ill (URTD) and healthy desert tortoises collected from the Las Vegas Valley, Clark County, Nevada (36°14'N, 115°09'W), between June 1990 and September 1991. Tortoises were defined as ill if nasal discharge was observed. Nine of the isolates were recovered from tortoises transported to the College of Veterinary Medicine, University of Florida, Gainesville, Florida (USA), prior to culturing. Tortoises were cultured either by insertion of a cotton-tipped swab into the nares or by nasal wash with 1 ml of sterile tryptose broth introduced by a catheter attached to a 1 cc syringe. Nasal specimens were inoculated onto 5% sheep blood agar (Remel, Lenexa, Kansas, USA) and incubated at 37 C; isolates were identified as *Pasteurella testudinis* by the cultural and biochemical characteristics described by Snipes and Biberstein (1982). Cultures were transported to the laboratory for molecular characterization in CTA medium (BBL, Bectin Dickinson, Cockeysville, Maryland, USA) at ambient temperature. Following transport, cultures were checked for purity by inoculation of 5% sheep blood agar and incubation as above.

Ribotyping was performed as described by Snipes et al. (1989). Briefly, DNA from the 89 isolates was purified by the method of Wilson (1987) in which bacterial cells were lysed with sodium dodecyl sulfate; proteins and other cellular debris were removed by digestion with proteinase-K and precipitation with hexadecyltrimethyl ammonium bromide; and DNA was precipitated with isopropanol. Chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri (USA). The final concentration of DNA was determined fluorometrically (DNA fluorometer model TKO 100, Hoefer Scientific Products, San Francisco, California).

Three micrograms of purified *P. testudinis* DNA was digested for 2 hr at 37 C with 10 units of the restriction enzymes *Eco*RI or *Eco*RV (Life Technologies Inc., Gaithersburg, Maryland) in a 30 μl reaction mixture containing the appropriate buffer included by the manufacturer. Following digestion, samples were subjected to electrophoresis in a 0.7% horizontal slab agarose gel and stained with ethidium bromide (Snipes et al., 1989).

Resulting DNA restriction fragments were transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, New Hampshire, USA), using the method of Southern (1975). On completion of DNA transfer, blots were dried at 80 C in a vacuum oven and stored at 22 to 25 C until use.

Ribotyping experiments, using an *Escherichia coli* rRNA (Sigma Chemical Co.) probe and blots of *Eco*RI-digested *P. testudinis* DNA, were conducted as described by Snipes et al. (1989). Specific activity of the probe was approximately 10^7 counts per minute (cpm)/μg of RNA. Blots were then hybridized with ^{32}P-labeled RNA (10^5 to 10^6 cpm/ml of hybridization solution). The extent of hybridization of the probe with blotted DNA was analyzed by autoradiography as described by Snipes et al. (1989).

All 89 isolates were subjected to ribotyping following digestion of DNA with *Eco*RI, and isolates were placed into ribotype groups that shared identical ribotype patterns. Isolates comprising ribotype groups that contained more than one isolate were subjected to ribotyping a second time using *Eco*RV to confirm ribotype groupings. Sixty-two isolates were subjected to ribotyping in this way following digestion of DNA with *Eco*RV.

Ribotype pattern groupings were established by eye and confirmed using a digitizing tablet and a computerized program (MolMatch, UVP Inc., San Gabriel, California) for DNA and protein-band matching.

Ability to grow in normal tortoise plasma was performed as described for *Pasteurella multocida* (Morishita et al., 1990). Twelve *P. testudinis* isolates (six randomly selected from ill and six randomly selected from healthy tortoises) were incubated overnight at 22 to 25 C in 5 ml

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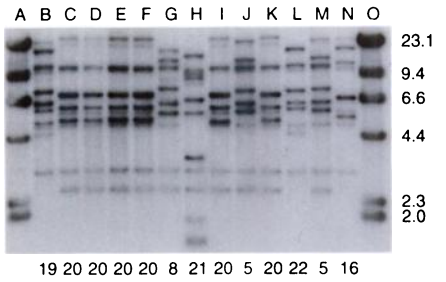


FIGURE 1. Autoradiograph of Southern blot of *Eco*RI-digested whole-cell DNA obtained from *Pasteurella testudinis* after hybridization with *Escherichia coli* rRNA probe. Lanes B through N contain DNA from selected isolates used in this study. Lanes A and O contain DNA isolated from lambda phage, digested with *Hin*dIII and hybridized with *Hae*III-digested lambda phage probe. Numbers on the right represent the number of kilobase pairs of the respective lambda phage fragments.

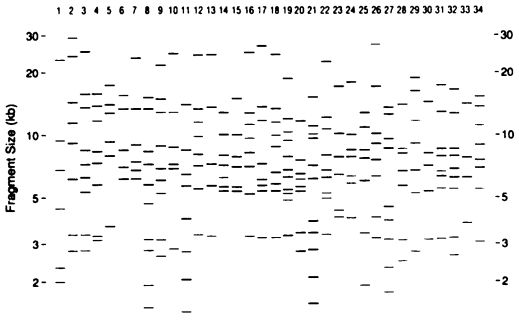


FIGURE 2. Digitized *P. testudinis* *Eco*RI ribotype patterns. Lane 1 is the digitized pattern of lambda phage digested with *Hin*dIII and hybridized with *Hae*III-digested lambda phage probe. Lanes 2 through 34 represent ribotypes 1 through 16 and 18 through 33, respectively. There is no *Eco*RI ribotype 17. The DNA fragment size is given as number of kilobase pairs (Kb).

brain-heart infusion (BHI) broth (Difco, Detroit, Michigan, USA), 1 ml of overnight cultures was transferred to a fresh 5 ml BHI broth, and the cultures incubated for 3 hr at 22 to 25 C. Following the 3 hr incubation to log phase growth (as determined by standard plate counts), bacteria were centrifuged at 8,700 × G for 5 min at 22 to 25 C, and the pellet was washed twice with sterile saline. Washed pellets were resuspended in sterile saline to an optical density of 0.3 at 540 nm (Perkin-Elmer Junior III Spectrophotometer, Coleman Instruments Division, Oak Brook, Illinois, USA), corresponding to approximately 10^8 colony-forming units/ml. Five μl of bacterial suspension were added to 0.5 ml sterile normal desert tortoise plasma, and the suspension incubated at 22 to 25 C for 1 hr. Aliquots were removed immediately, and at 1 hr after inoculation. Serial dilutions and plate counts were performed in duplicate. Survival values were calculated as the concentration after 1 hr of incubation divided by the concentration at time zero. Survival values greater than one were evidence for the survival and replication of the isolate in tortoise plasma. Statistical significance of the difference between mean plasma survival value for isolates from tortoises with URTD and for isolates from healthy tortoises was evaluated by Student's *t*-test, using an alpha value of 0.05 (Gosset, 1908).

For whole cell and outer membrane protein analysis, fresh lyophilized cultures of the same 12 *P. testudinis* isolates selected for plasma growth experiments were inoculated into 100 ml BHI broth and incubated overnight at 22 to 25 C. Following incubation, organisms were centrifuged at 28,000 × G for 15 min at 4 C and the pellets washed twice in 20 ml of sterile saline. A portion of the washed cells was suspended in distilled water and maintained at −80 C for analysis of whole-cell proteins (WCP).

Outer membrane proteins were extracted by disruption of bacterial cells to liberate bacterial membranes. A washed pellet of organisms was resuspended in 10 ml of 10 mM Tris/0.3% NaCl, pH 8.0 (Sigma Chemical Co.). The suspension was placed on ice and then disrupted for four 1 min bursts using a cell disrupter (Polytron, Brinkman Instruments, Inc., Westburg, New York, USA). After disruption, cell debris was reduced to a pellet by centrifugation at 3,000 × G for 15 min at 4 C. The supernatant containing inner and outer membranes was removed and centrifuged at 40,000 × G for 60 min at 4 C. The supernatant then was discarded and the pellet containing the membrane fraction resuspended in 5 ml 1.5% Sarkosyl (Sigma Chemical Co.) in distilled water. This suspension was left for 20 min at 22 to 25 C, and then centrifuged again at 40,000 × G for 60 min at 4 C. This process of Sarkosyl treatment to dissolve the inner membranes was repeated twice. The final pellet of outer membranes was resuspended in 150 μl of distilled water, divided into 20 to 30 μl aliquots, and frozen at −80 C until use.

Induction of protein synthesis of three isolates under iron-depleted conditions was evaluated by preparing the OMP fractions as above except that 20 μg/ml of 2,2-dipyridyl (Sigma Chemical Co.) was included in the growth medium.

After thawing, estimation of the protein concentration in the sample was determined with the Bio-Rad Protein Assay using bovine serum

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albumin standards (Bio-Rad Laboratories, Hercules, California). Samples then were diluted 1:2 in 2X PAGE sample buffer (1% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.02% bromphenol blue, and 250 mM Tris, pH 6.8; Sigma Chemical Co.), heated to 90 to 95 C for 3 to 5 min, and then applied to the polyacrylamide gel.

Samples (1 mg/ml) of OMP or WCP were heated and 75 μl of each sample was loaded into wells made in a 5% stacking gel overlying a 19 cm separating gel (10% polyacrylamide) (Sigma Chemical Co.). Samples were subjected to electrophoresis at 30 mA for approximately 5 hr using PAGE buffer, pH 8.7. Following electrophoresis, gels were stained with 0.25% Coomassie brilliant blue (Snipes et al., 1988). Proteins of known molecular weight (ranging from 14 to 92.5 K) were included in every electrophoresis run.

RESULTS

The 89 *P. testudinis* isolates, 52 from ill and 37 from healthy tortoises, were distributed into 33 completely distinct *Eco*RI ribotype groups (Table 1, Figs. 1, 2). Nine of these ribotype groups contained more than one isolate, with *Eco*RI ribotypes 8 (n = 8) and 20 (n = 29) containing the most isolates. These two ribotypes contained 37 (71%) of 52 of the isolates recovered from tortoises with URTD, but also contained 14 isolates from clinically healthy tortoises.

Isolates from the nine *Eco*RI ribotype groups containing more than one isolate (*Eco*RI ribotypes 1, 2, 5, 8, 11, 16, 20, 24, and 25) were further evaluated with *Eco*RV. For a given isolate, ribotype patterns produced following digestion of isolate DNA with *Eco*RV differed in number of kilobase pairs (Kbp) of DNA fragments produced, compared with *Eco*RI ribotypes. However, the general size range (2 to 25 Kbp) and the number of fragments hybridizing with the probe (approximately six to eight) were similar (Fig. 3).

Restriction enzyme *Eco*RV ribotypes of isolates from *Eco*RI ribotypes 1, 2, 20, 24, and 25 were identical within the respective ribotype group. The remaining four *Eco*RI ribotypes with multiple isolates (5, 8, 11, and 16) were further subdivided when isolates were evaluated with *Eco*RV (Table 2). *Eco*RI ribotype 8 isolates, obtained from both ill and healthy tortoises, were segregated into *Eco*RV ribotypes that contained only isolates from ill or healthy animals, as were the two isolates comprising *Eco*RI ribotype 16 (Table 2).

Plasma survival values for the twelve isolates tested ranged from 1.00 to 1.92. The mean survival value for the six URTD isolates was 1.35 (SD = 0.27), compared with 1.41 (SD = 0.33) for the six isolates

TABLE 1. Ribotypes of *Pasteurella testudinis* collected from desert tortoises, June 1990 to September 1991, following digestion of DNA with *Eco*RI.

*Eco*RI ribotype	Number of isolates from tortoises with URTD*	Number of isolates from healthy tortoises
1	3	2
2	3	2
3	1	0
4	0	1
5	3	1
6	0	1
7	1	0
8	6	2
9	1	0
10	1	0
11	4	0
12	1	0
13	0	1
14	1	0
15	1	0
16	1	1
18	1	0
19	0	1
20	17	12
21	1	0
22	0	1
23	0	1
24	0	6
25	0	2
26	0	1
27	0	1
28	1	0
29	1	0
30	1	0
31	1	0
32	1	0
33	0	1
34	1	0
Totals	52	37

* Upper respiratory tract disease.

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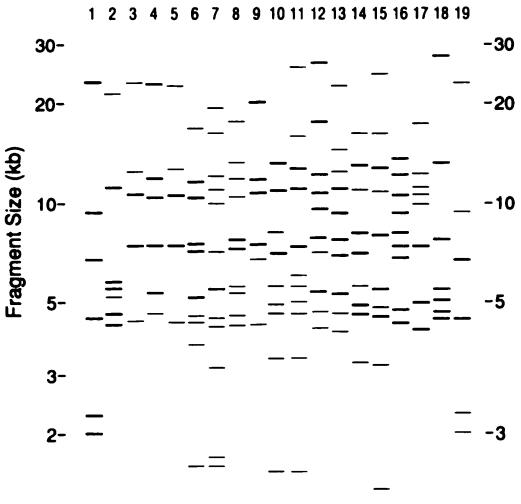


FIGURE 3. Digitized *P. testudinis* *Eco*RV ribotype patterns. The two outside lanes contain digitized pattern of lambda phage digested with *Hin*dIII and hybridized with *Hae*III-digested lambda phage probe. Lanes between the two standards contain the following *Eco*RI : *Eco*RV ribotypes: Lane 2 = 1:A; Lane 3 = 2:A; Lane 4 = 5:A; Lane 5 = 5:B; Lane 6 = 8:A; Lane 7 = 8:B; Lane 8 = 8:C; Lane 9 = 8:D; Lane 10 = 11:A; Lane 11 = 11:B; Lane 12 = 16:A; Lane 13 = 16:B; Lane 14 = 17:A; Lane 15 = 17:B; Lane 16 = 20:A; Lane 17 = 24:A; and Lane 18 = 25:A. The DNA fragment size is given as number of kilobase pairs (Kb).

obtained from healthy tortoises. This difference was not significant ($P = 0.38$).

Some protein differences between isolates were evident in WCP preparations as determined by polyacrylamide gel electrophoresis (data not shown). Whole cell proteins of molecular weight 64.1, and ranging between 28.0 and 37.4, had the most heterogeneity among the 12 isolates. When these results were compared to OMP differences, only a few of the WCP were non-OMP.

There were five OMP patterns among all 12 isolates (designated a through e, Table 3) (Fig. 4A). Non-URTD isolates generally demonstrated more variability in OMP pattern than URTD-associated isolates. Outer membrane protein 15.8 was present in all isolates. All URTD-associated isolates (and non-URTD isolates TP234, TP235, and TP236) also shared OMP 29.2 and 28.5. No OMP were unique to the

TABLE 2. Ribotypes of selected *Pasteurella testudinis* isolates from desert tortoises, following digestion of DNA with *Eco*RV. The *Eco*RV ribotypes listed are for those *Eco*RI ribotypes which had heterogeneity when digested with *Eco*RV.

*Eco*RI ribotype	*Eco*RI: *Eco*RV ribotype	Number of isolates	URTD* health status of tortoises
5	5:A	1	Ill
	5:B	3	Both ill and healthy
8	8:A	5	Ill
	8:B	1	Ill
	8:C	1	Healthy
	8:D	1	Healthy
11	11:A	2	Ill
	11:B	1	Ill
	11:C	1	Ill
16	16:A	1	Ill
	16:B	1	Healthy

* URTD, upper respiratory tract disease.

URTD-associated isolates, but two OMP's (37.4 and 28.0) were found only in non-URTD isolates TP 238 and 239. Iron-limiting conditions induced similar OMP production in the three isolates tested (Fig. 4B). These apparently iron-regulated OMP's ($n = 2$ or 3) were not produced when isolates were grown in BHI broth without dipyridyl, and had a molecular weight greater than 73.6. One iron-regulated OMP (approximate molecular weight 102.3) was evident in TP236, but not in TP234 or TP93.

DISCUSSION

A considerable amount of ribotype heterogeneity was observed in this group of *P. testudinis* isolates, with 33 unique ribotype patterns detected among the 89 isolates examined with *Eco*RI. This degree of genomic heterogeneity in ribosomal RNA genes is consistent with other species of *Pasteurella* (Snipes et al., 1989, 1992). Only one *Eco*RI ribotype (ribotype 11) was comprised of several isolates exclusively recovered from URTD tortoises. When this group of four isolates was evaluated further using *Eco*RV, three distinct ribotypes were detected. Although several other

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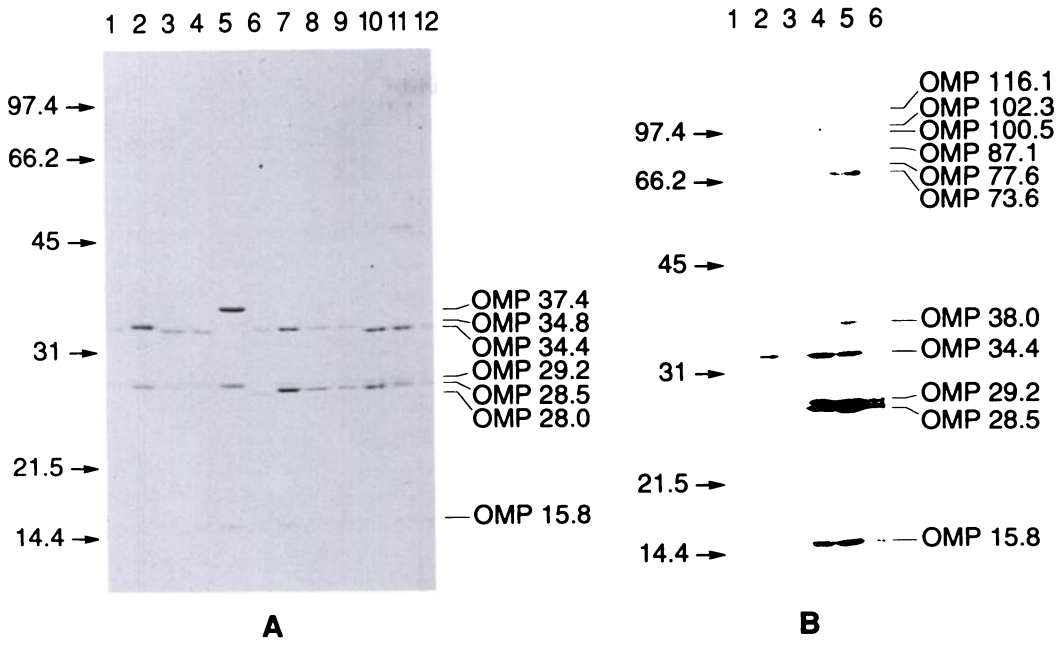


FIGURE 4A. Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins (OMP) from *P. testudinis* isolates with and without upper respiratory tract disease. Lanes 1 through 6 contain OMP from isolates TP 234, Tp 235, TP 236, TP 237, TP 238, and TP 239, respectively, all obtained from clinically healthy tortoises. Lanes 7 through 17 contain OMP from isolates TP 240, TP 242, TP 246, TP 69, TP 72, and TP 93, respectively, all obtained from tortoises with upper respiratory tract disease. Numbers in the right margin represent major OMP's. Arrows in the left margin represent position of molecular weight markers (kilodaltons).

Figure 4B. Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins (OMP) from *P. testudinis* isolates obtained from tortoises with and without upper respiratory tract disease (URTD) and grown in iron-limited (dipyridyl-containing) medium. Lanes 1 and 4, OMP from TP 234 grown without and with iron limitation, respectively; lanes 2 and 5, OMP from TP 236 grown without and with iron limitation, respectively; lanes 3 and 6, OMP from TP 93 grown without and with iron limitation, respectively. Isolates TP 234 and TP 236 were obtained from clinically healthy tortoises, isolate TP 93 was obtained from a tortoise with URTD. Numbers in the right margin represent major OMP and iron-induced OMP. Arrows in the left margin represent position of molecular weight markers (kilodaltons).

*Eco*RI ribotypes (such as ribotypes 5, 8, and 20) were found primarily among isolates from tortoises with URTD, further investigation using *Eco*RV demonstrated relatedness of strain to clinical health occurred in only one case after further evaluation with *Eco*RV. Restriction enzyme *Eco*RI ribotype 8 was subdivided into four *Eco*RV ribotype groups. One of these subgroups contained five isolates, all from URTD tortoises. Restriction enzyme *Eco*RV cuts DNA at different locations than *Eco*RI along the bacterial chromosome and therefore yields a different DNA fingerprint for a given isolate, thus enabling additional determination of relatedness of a given *Eco*RI ribotype. Further research is necessary to determine possible association of *Eco*RI/*Eco*RV ribotype profile with URTD or virulence. In contrast, the 29 isolates from *Eco*RI ribotype 20 remained a homogenous group when studied with *Eco*RV, even though these isolates were obtained from both ill and healthy tortoises.

Resistance to bactericidal effects of normal plasma is virulence-related in *Pasteurella multocida*, and this trait has been adapted to provide an in vitro virulence model for turkeys (Morishita et al., 1990).

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TABLE 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoesis patterns of major outer membrane proteins (OMP) from *Pasteurella testudinis* collected from desert tortoises, June 1990 to September 1991, Nevada.

Strain	Major OMP[a]							OMP pattern[b]	*Eco*RI : *Eco*RV ribotype
	37.4	34.8	34.4	29.2	28.5	28.0	15.8		
TP234	–[c]	–	+	+	+	–	+	a	26:–
TP235	–	+	–	+	+	–	+	b	23:–
TP236	–	–	+	+	+	–	+	a	2:A
TP237	–	–	+	+	–	–	+	c	8:C
TP238	+	–	–	+	–	–	+	d	24:A
TP239	–	+	–	–	–	+	+	e	27:–
TP240[d]	–	+	–	+	+	–	+	b	20:A
TP242[d]	–	+	–	+	+	–	+	b	20:A
TP246[d]	–	+	–	+	+	–	+	b	20:A
TP69[d]	–	–	+	+	+	–	+	a	8:A
TP72[d]	–	–	+	+	+	–	+	a	8:A
TP93[d]	–	–	+	+	+	–	+	a	8:A

[a] Molecular weight (kilodaltons) for major OMP.
[b] Arbitrary letter designations representing similar OMP patterns.
[c] +, OMP produced; –, no OMP produced.
[d] Isolates from tortoises with upper respiratory tract disease.

By evaluating the ability of *P. testudinis* to survive in fresh normal tortoise plasma, we determined whether resistance to tortoise plasma bactericidal factors could be exploited in studying *P. testudinis* virulence in vitro. Apparently this does not apply to *P. testudinis*; isolates from healthy and ill tortoises of different ribotypes survived and grew equally well in plasma. This finding may reflect differences in pathogenesis, as URTD, unlike *P. multocida* infection in turkeys, is confined primarily to the tortoise upper respiratory tract and usually is not a septicemic disease.

The major differences in protein composition among isolates of *P. testudinis* examined appeared to be with OMP's rather than WCP's. Outer membrane protein differences were evident among the 12 isolates examined, but none were unique to isolates from URTD tortoises or a particular ribotype. One OMP was common to all isolates, and it might be possible to exploit this protein for serologic and immunologic purposes in the future. Outer membrane protein patterns of isolates from healthy tortoises generally had more heterogeneity than those from tortoises affected by URTD. Therefore, it still might be possible to identify OMP unique to disease-associated strains as, for example, those comprising *Eco*RI : *Eco*RV ribotype 8A, OMP pattern a (Table 3). A number of OMP's are associated with virulence traits in other bacteria, and may be induced under iron limited conditions (Litwin and Calderwood, 1993). We observed production of iron-regulated OMP in *P. testudinis*, but their role in pathogenicity remains unclear.

The population of desert tortoises sampled in this study was from a single geographic location, the Las Vegas Valley in Nevada. Therefore, the results presented in this paper may or may not be representative of tortoise populations elsewhere. However, the tortoises studied from this area provided a convenient and large sample of isolates to examine heterogeneity of *P. testudinis*. In addition, tortoises suffering from URTD do not always die, and therefore some of the animals defined as "healthy" may actually be recovered carriers of certain strains of *P. testudinis*, complicating interpretation of results.

Based on the results of this study, particular strains of *P. testudinis*, as characterized by ribotype or OMP analysis, are not readily associated with URTD in desert tortoises. One *Eco*RI/*Eco*RV ribotype (8:A) contained five isolates exclusively from ill tortoises, and this group may warrant further study. However, this is the only group of isolates of any significant number to have all originated from tortoises with URTD. Failure to identify a particular "pathogenic" *P. testudinis* strain is somewhat in agreement with previous pathogenesis studies involving this organism and captive desert tortoises (Snipes, 1976), and I believe that further study of the epizootiology of URTD in free-ranging tortoises is warranted.

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