R-2433

Analysis of Two Potentially Novel *Yersinia* Species from Pennsylvania Creeks

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Abstract:

While surveying the microbial diversity in local freshwater creeks as part of a Microbiology course, the 16S rRNA sequences of two isolates suggested that they may be novel species within the Genus *Yersinia*. Subsequent polyphasic characterization and comparison to related species included morphological studies, Biolog GenIII Microplate analysis, API test panels, fatty acid methyl ester (FAME) analysis, a variety of differential and selective media, and multi-locus sequence typing.

Although the 16S rRNA sequence of *Yersinia* sp. strain AArt-01 was less similar to its closest relative (98.5-98.8% identical to *5* different species) than other strains described as distinct species, its metabolic phenotypes and housekeeping gene sequences suggest that it may be a strain of the species *Yersinia aldovae*. DNA/DNA hybridization or whole genome sequencing may be required to conclusively determine whether it is novel.

Yersinia sp. strain MAC can be clearly distinguished from other validly described species of *Yersinia* both genetically and phenotypically. It appears to belong to the same species as a *Yersinia* sp. MH-1 isolated in New Zealand as an insect pathogen. Multi Locus Sequence Typing (MLST) with the housekeeping genes recA, glnA, gyrB and hsp60 showed <90% identity to validly named species but >98% identity to *Yersinia* sp. MH-1.

Introduction/Background:

1. ***Yersinia* sp. MAC was isolated from the Loyalock Creek and was initially characterized as an "unknown" in Bio 321W – Microbiology**
2. ***Yersinia* sp AArt-01 was isolated from the Lycoming Creek as part of a microbial diversity survey**

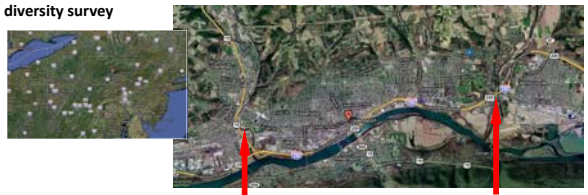

Figure 1. Collection Sites: **Lycoming Creek** **Loyalsock Creek**

Methods:

- **Environmental unknowns cultured & characterized in Microbiology course**
- **Colony PCR of 16S rDNA with primers 27f & 1492r, 1 Sanger sequencing rxn**
- **Compare sequence to validly published type strains (Eztaxon.org)**

<99.0% identical **>99.0% identical**

- **Fully sequence both strands of nearly complete 16S rDNA**
  - **Submit Sequence to GenBank**
  - **Clustal W Alignment, Neighbor Joining Tree to infer phylogenetic relationships**
- **Morphological/Metabolic characterization w/standard tests**
  - **Colony morphology, color, Gram stain, wet mount**
  - **Temperature, O_2 , pH, NaCl requirements**
  - **Carbohydrate & Nitrogen metabolism**
  - **Exoenzymes, differential and selective medium**
- **API Test Strips (50 CH, 20E/NE, ZYM)**
- **Fatty Acid Methyl Ester (FAME) Analysis (MIDI)**
- **Biolog GenIII metabolic profile**
- **Family-specific tests such as Multi Locus Sequence Analysis, pigment analysis, respiratory quinones, polar lipids, cell wall amino acids.**
- **Deposit Strains in Culture Collections (e.g. ATCC, DSMZ, CCUG, JCM, ARS/NRRL)**

Species identification assigned

Publish new species in IJSEM

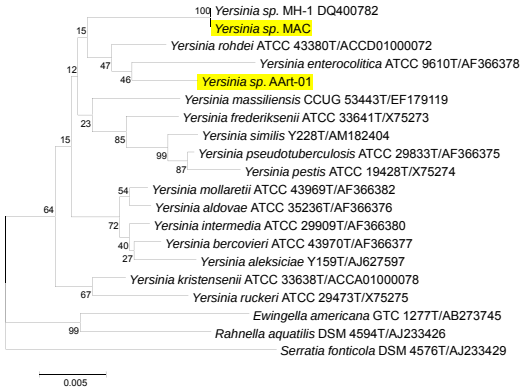

Figure 2. 16S rRNA sequence analysis.

The evolutionary history was inferred using the Neighbor-Joining method (Caitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches . The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et a., 2007).

Table 1. Pairwise comparison of *Yersinia* 16S rRNA gene sequences.

		Yersin sp MAC	Yersin sp MH-1	Yersin sp AArt-01	Yersin rohdi	Yersin molla.	Yersin aldov.	Yersin inter.	Yersin bercо.	Yersin krist.	Yersin mass.	Yersin freder.	Yersin aleks.	Yersin ruckeri	Yersin similis	Yersin pseudo.	Yersin entero	Yersin pestis
Yersinia sp	MAC	100.00																
Yersinia sp.	MH-1	99.90	100.00															
Yersinia sp	AArt-01	97.90	98.00	100														
Yersinia rohdei	ATCC 43380(T)	98.39	98.47	98.61	100													
Yersinia mollaretii	ATCC 43969(T)	98.46	98.56	98.32	98.77	100												
Yersinia aldovae	ATCC 35236(T)	98.32	98.43	98.53	98.97	99.59	100											
Yersinia intermedia	ATCC 29909(T)	98.11	98.22	98.6	98.84	99.38	99.52	100										
Yersinia bercovieri	ATCC 43970(T)	97.90	98.02	98.32	98.63	99.32	99.32	99.52	100									
Yersinia kristensenii	ATCC 33638(T)	98.25	98.33	98.67	99.2	98.91	99.18	99.04	98.77	100								
Yersinia massiliensis	CCUG 53443(T)	98.11	98.22	98.25	98.22	98.56	98.29	98.7	98.77	98.15	100							
Yersinia frederiksenii	ATCC 33641(T)	98.20	98.47	98.25	98.75	98.51	98.36	98.44	98.22	98.61	98.58	100						
Yersinia aleksiciae	Y159(T)	97.52	97.60	97.95	98.24	98.8	98.94	99.23	99.37	98.52	98.8	94.85	100					
Yersinia ruckeri	ATCC 29473(T)	97.76	97.91	97.76	98.18	98.29	98.15	98.08	98.29	98.79	98.15	98.18	97.96	100				
Yersinia similis	Y228(T)	97.70	97.78	97.97	98.45	98.02	98.15	98.22	98.43	98.39	98.56	98.88	98.24	98.25	100			
Yersinia pseudotuberculosis	ATCC 29833(T)	97.69	97.81	97.97	98.43	98.02	98.15	98.15	98.43	98.38	98.56	99	98.31	97.95	99.59	100		
Yersinia enterocolitica	ATCC 9610(T)	97.90	98.02	98.67	98.84	97.74	97.95	98.36	97.88	97.88	97.81	98.01	98.82	97.06	97.81	97.74	100	
Yersinia pestis	ATCC 19428(T)	97.48	97.57	97.64	98.12	97.68	97.75	97.82	97.96	98.06	98.17	96.27	97.87	97.71	99.1	99.37	97.4	100
Rahnella aquatilis	DSM 4594 (T)	97.35	97.46	96.96	97.26	97.08	97.08	97.36	96.87	97.26	97.57	94.22	97.04	96.77	97.12	96.8	97.36	96.59
Ewingella americana	GTC 1277(T)	96.86	96.97	97.06	97.11	96.99	96.85	97.12	96.78	97.04	97.33	97.33	96.62	96.88	97.16	96.85	96.64	96.57
Serratia fonticola	DSM 4576(T)	96.43	96.56	96.84	96.69	96.85	96.71	96.78	96.44	97.03	96.23	96.71	96.12	96.96	95.98	95.82	96.91	95.76

Conclusions:

1. ***Yersinia* sp. MAC is likely to belong to the same species as Y. sp. MH-1**
2. **Y. sp. MAC & Y. sp. MH-1 are sufficiently different from validly published species to merit description as new species.**
3. **Y. sp AArt-01 clusters with *Yersinia enterocolitica* but has a similar level of sequence identity with several species.**

Table 2. Fatty acid methyl ester analysis of *Yersinia* sp.

		Yersinia sp.	*Yersinia rohdei*	*Yersinia mollarettii*	*Yersinia sp.*	*Yersinia aleksicae*	*Yersinia massiliensis*
		MAC	DSM 18270	DSM18520	AArt-01	LC4	LC2
	MIDI Database ID	+ Serratia liquifaciens	+ Yersinia rohdei	+ Yersinia enterocolitica	+ Yersinia kristensenii	+ Yersinia fred/ent/int	+ Yersinia fred/ent/int
	similarity index	0.791	0.803	0.921	0.973	0.835	0.794
ECL	**fatty acid name**						
13.999	14:0	0.69	1.61	0.8	1.36	0.71	0.9
15.516	SF2 14:0 3OH/16:1 isol	1.83	1.75	1.63	1.77	1.85	1.66
15.84	SF3 16:1 w7c/16:1 w6c	38.28	11.55	13.66	31.09	31.24	31.64
16.001	16:0	37.06	37	36.46	39.57	33.24	33.1
16.918	SF3 17:0 cyclo	7.14	37.3	35.98	16.5	15.59	14.66
17.0022	17:0	0.92	-	0.15	0.27	1.09	1.11
17.845	SF8 18:1 w7c/w6c	12.63	7.17	9.02	8.04	14.26	13.81
17.999	18:0	0.54	0.41	0.33	0.35	0.66	0.84
18.929	19:0 cyclo w8c	0.17	2.51	1.4	0.04	-	-
	total	**99.3**	**99.3**	**99.3**	**99.3**	**98.64**	**97.72**
	total SF3	45.42	48.85	49.64	48.85	49.64	47.59

Conclusion: Yersinia fatty acids have insufficient variation for use in identification. This is common among the Proteobacteria.

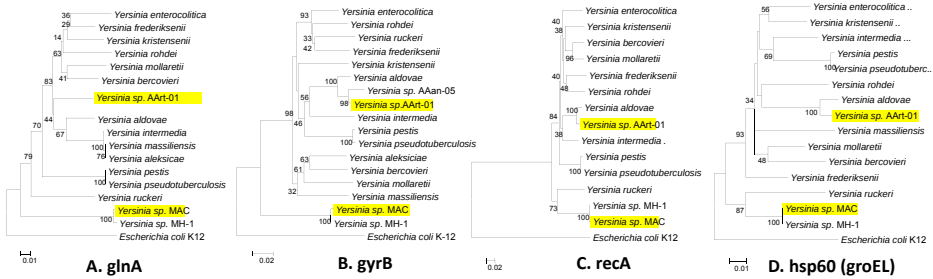

Figure 3. Multi Locus Sequence Typing of Novel Species.

The evolutionary history was inferred using the Neighbor-Joining method (Caitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches . The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et a., 2007).

Table 4. Metabolic Differences using Biolog GenIII Microplates

	species	Yersinia sp.	Yersinia rohdei	Yersinia mollaretii	Yersinia sp.
	strain	MAC	DSM 18270T	DSM 18520T	AArt-01
	Database ID	Yersinia rohdei	Yersinia rohdei	Yersinia mollaretii	Yersinia aldovae
	probability	1	1	0.984	1
	similarity	0.257	0.803	0.867	0.841
well	distance	6.922	3.678	1.755	3.033
A01	neg control	-	-	-	-
A02	dextrin	+	+	+	w
A03	D-maltose	+	+	+	+
A04	D-trehalose	+	+	+	+
A05	D-cellobiose	-	-	+	-
A06	gentiobiose	-	+	+	-
A07	sucrose	+	+	+	-
A08	D-turanose	-	-	-	-
A09	stachyose	+	+	+	-
A10	pos control	+	+	+	+
A11	pH 6	+	+	+	+
A12	pH 5	+	-	-	-
B01	D-raffinose	+	+	-	-
B02	α-D-lactose	-	-	-	-
B03	D-melibiose	w	+	-	-
B04	β-methyl-D-glucoside	+	+	+	+
B05	D-salicin	w	-	-	-
B06	N-acetyl-D-glucosamine	+	+	+	+
B07	N-acetyl-β-D-mannosamine	+	+	+	-
B08	N-acetyl-D-galactosamine	w	-	-	-
B09	N-acetyl neuraminic acid	+	+	+	-
B10	1% NaCl	+	+	-	+
B11	4% NaCl	+	-	w	-
B12	8% NaCl	-	-	-	-
C01	α-D-glucose	+	+	+	+
C02	D-mannose	+	+	+	+
C03	D-fructose	+	+	+	+
C04	D-galactose	+	-	-	-
C05	3-methyl glucose	+	-	-	w
C06	D-fucose	+	-	-	-
C07	L-fucose	+	-	-	-
C08	L-rhamnose	w	-	-	-
C09	inosine	+	+	+	-
C10	1% Na-lactate	+	+	+	w
C11	fusidic acid	-	-	-	-
C12	D-serine	w	-	+	-
D01	D-sorbitol	+	+	+	+
D02	D-mannitol	+	+	+	+
D03	D-arabitol	w	-	-	-
D04	myo-inositol	-	+	+	+
D05	glycerol	+	+	+	+
D06	D-glucose-6-PO4	+	+	+	-
D07	D-fructose-6-PO4	+	+	+	-
D08	D-aspartic acid	w	-	-	-
D09	D-serine	+	-	-	w
D10	troleandomycin	+	+	+	+
D11	rifamycin SV	+	+	+	-
D12	minocycline	-	-	-	-
E01	gelatin	-	-	-	-
E02	glycyl-L-proline	+	+	+	-
E03	L-alanine	+	+	+	+
E04	L-arginine	-	-	-	-
E05	L-aspartic acid	+	+	+	w
E06	L-glutamic acid	+	+	+	+
E07	L-histidine	+	+	+	+
E08	L-pyroglutamic acid	w	-	-	-
E09	L-serine	+	+	+	+
E10	lincomycin	+	+	+	+
E11	guanidine HCl	+	+	+	+
E12	niaproof 4	+	-	+	+
F01	pectin	+	+	+	-
F02	D-galacturonic acid	+	+	+	-
F03	L-galacturonic acid lactone	-	-	-	-
F04	D-gluconic acid	+	+	+	+
F05	D-glucuronic acid	+	+	+	+
F06	glucuronamide	+	-	+	+
F07	mucic acid	w	-	-	-
F08	quinic acid	w	-	-	-
F09	D-saccharic acid	-	-	-	-
F10	vancomycin	+	+	+	+
F11	tetrazolium violet	+	-	+	+
F12	tetrazolium blue	+	+	+	+
G01	p-hydroxy-phenylacetic acid	-	-	-	-
G02	methyl pyruvate	+	+	+	+
G03	D-lactic acid methyl ester	w	-	-	-
G04	L-lactic acid	+	-	-	-
G05	citric acid	+	+	-	+
G06	α-keto-gluteric acid	+	-	-	-
G07	D-malic acid	w	-	-	-
G08	L-malic acid	+	-	-	-
G09	bromo-succinic acid	+	-	-	-
G10	nalidixic acid	-	-	-	-
G11	LiCl	w	-	w	-
G12	K-tellurite	-	-	-	-
H01	tween-40	+	-	-	-
H02	γ-amino-butyric acid	-	-	-	-
H03	α-hydroxy-butyric acid	-	-	-	-
H04	β-hydroxy-D,L-butyric acid	-	-	-	-
H05	α-keto-butyric acid	-	-	-	-
H06	acetoacetic acid	+	-	+	+
H07	propionic acid	+	-	+	+
H08	acetic acid	+	+	+	+
H09	formic acid	+	-	-	-
H10	aztreonam	+	+	-	-
H11	Na-butyrate	+	-	w	-
H12	Na bromate	-	-	-	-

Table 3. MLST Sequence Pairwise Similarities

Species	*Yersinia* sp. MAC			
	glnA	gyrB	recA	Y-HSP60
Yersinia rohdei	83.4%	83.8%	84.7%	90.0%
Yersinia mollaretii	83.2%	85.7%	83.2%	90.2%
Yersinia sp. MH-1	99.6%	98.5%	99.4%	99.6%

Table 5. Additional Distinguishing Phenotypic Traits

		Yersinia sp.	*Yersinia rohdei*	*Yersinia mollarettii*	*Yersinia sp*
		MAC	DSM 18270T	DSM 18520T	AArt-01
API 20E	β-galactosidase	w	+	-	-
API 20E	ornithine decarboxylase	+	-	+	w
API 20E	urease	-	+	+	+
API 20E	acetoin production	-	+	-	+
API 20E	acid from D-sorbitol	-	+	+	-
API 20E	acid from D-melibiose	+	-	-	+
API 20E	acid from amygdalin	-	-	+	-
API 20E	acid from L-arabinose	-	-	+	+
	DNase	+	-	-	-
	brilliant green agar	+	+	+	+ red
	Eosin Methylene Blue agar	+	+ metallic	+ metallic	+
	Hektoen Enteric Agar	+	+ orange	+ orange	+ green

Conclusions:

1. ***Yersinia sp*. MAC and can be distinguished genetically and phenotypically from related validly published species.**
2. **Yersinia sp. AArt-01 appears to be most closely related to *Yersinia aldovae* based on MLST and Biolog Phenotypes.|**
3. **To complete the preparation of these strains for publication in the *International Journal of Systematic and Evolutionary Microbiology*, several additional reference strains must be obtained for comparison, including *Yersinia sp*. MH-1, *Yersinia aldovae*, and *Yersinia enterocolitica.***
4. **Given the available genome sequences for most members of the *Yersinia* Genus (Chen *et al*., 2010), whole genome sequencing of the strains described here can be justified.**

Acknowledgements

The Biolog and MIDI/FAME instruments were purchased with funding from NSF award DBI-0960114 to JDN, **"MRI-R2: Acquisition of Instrumentation for Novel Microbe Characterization by Undergraduate Researchers."**

References

Chen, P.E., Cook, C., Stewart, A.C., Nagarajan, N., Sommer, D.D., Pop, M., Thomason, B., Kiley, M.P., Lentz, S., Nolan, N., Sozhamannan, S., Sulakvelidze, A., Mateczun, A., Du, L., Zwick, M.E., and Read, T.D. (2010). Genomic characterization of the Yersinia genus. *Genome Biology **11***, R1.

Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* 57, 2259-2261

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.

McNeill, M.R., and Hurst, M.R. (2008). Yersinia sp. (MH96) - a potential biopesticide of migratory locust Locusta migratoria L *N.Zea.Plant Protec.* ***61***:236-242.

Saitou N & Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4:406-425.

Tamura K, Nei M & Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *PNAS* 101:11030-11035.

Tamura K, Dudley J, Nei M & Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol.Evol.* 24:1596-1599.