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Genetic diversity of North American isolates of *Renibacterium salmoninarum*

Clifford E. Starliper*

U.S. Geological Survey, Biological Resources Division, Leetown Science Center, National Fish Health Research Laboratory, 1700 Leetown Road, Kearneysville, West Virginia 25430, USA**

ABSTRACT: Genetic diversity of *Renibacterium salmoninarum* was evaluated by multilocus enzyme electrophoresis (MEE). Whole cell lysates were prepared for 40 isolates representing 5 groups based on host and geographic area. Each lysate was assessed for activity of 44 enzymes with a pH 6.5 amine-citrate and a pH 8.0 buffer. Genetic variation was scored at 26 loci. Two zones of activity (presumptive loci) were scored each for esterase (EC 3.1.1.1) and glycyl-leucine peptidase (EC 3.4.11.x). There were no monomorphic loci and there was an average of 2.65 electromorphs per locus. There were 21 electrophoretic types. Mean genetic diversity (H_T) was 0.161 and the percentage of this explained by diversity between groups was G_{st} = 8.1%; thus 91.9% of the genetic diversity was due to heterogeneity between individual isolates. The 2 groups with the highest genetic diversity were from chinook *Oncorhynchus tshawytscha* and coho *O. kisutch* salmon, both from the Manistee Weir, Michigan, USA; i.e. 0.270 and 0.298, respectively. The highest genetic diversity for a locus (h_T) was 0.587 for EST-1. At this locus, diversity between groups explained a higher percentage of the total diversity (G_{st} = 36.5%). Other loci with relatively high genetic diversity were succinate dehydrogenase (0.385; EC 1.3.99.1), cytochrome *c* oxidase (0.273; EC 1.9.3.1) and aconitase (0.311; EC 4.2.1.3). The results of this study indicate relatively low genetic diversity of *R. salmoninarum*.

KEY WORDS: Genetic diversity · *Renibacterium salmoninarum* · Electromorphs · Multilocus enzyme electrophoresis · Bacterial kidney disease

INTRODUCTION

Renibacterium salmoninarum is the Gram-positive bacillus that causes bacterial kidney disease (BKD), a significant cause of mortality among salmonid fish (Sanders & Fryer 1980). Previous serological studies have shown strains of this bacterium to be antigenically homogeneous (Bullock et al. 1974, Getchell et al. 1985). Additionally, strains of *R. salmoninarum* were similar in their lack of extracellular biological activity (Bandin et al. 1991). On the other hand, Austin & Rodgers (1980) demonstrated the existence of 2 distinct phena by evaluation of biochemical, serological and physiological studies. More recently, Bandin et al. (1992) suggested the existence of 2 antigenically distinct groups of *R. salmoninarum* based on quantitative slide agglutination and Western blot assays. However, the authors point out that it is possible that their group of 3 isolates with a major protein of 30 kDa could have been a result of the instability of the major soluble antigen (57 kDa protein) of *R. salmoninarum* as described by Griffiths & Lynch (1991).

The purpose of this study was to evaluate the presence and mobility of enzymatic activity for a representative number of *Renibacterium salmoninarum* isolates from hosts showing clinical signs of BKD. Multilocus enzyme electrophoresis (MEE) was used and the degree of genetic diversity determined.

METHODS

The isolates of *Renibacterium salmoninarum* used in this study represented 5 groups (isolate origins) based on host and geographic location (Table 1). The groups were: various hosts from the Pacific northwest

*E-mail: cliff_starliper@nbs.gov
**Formerly National Biological Service

Table 1. *Renibacterium salmoninarum*. List of 40 isolates evaluated by multilocus enzyme electrophoresis

Group[a]	Host	Isolate
Pacific Northwest (USA and Canada)	*Oncorhynchus tshawytscha*	7.29, 7.30, 7.32, ATCC 33209, RB-1-73
	O. kisutch	384, Siletz
	Salmo salar	RS18[b]
	Not known	Quinalt, Salmon Creek
Manistee Weir, Michigan, USA	*O. kisutch*	MCO1, MCO3, MCO4M, MCO5, MCO9, MCO11
Manistee Weir, Michigan, USA	*O. tshawytscha*	MCK4M, MCK26, MCK30, MCK32, MCK38, MCK49, MCK50, MCK52M, MCK56, MCK60M
Kewanee Weir, Wisconsin, USA	*O. tshawytscha*	A5, A12, A18, A22, A24M, A34, A40, A57
Strawberry Creek Wier, Wisconsin, USA	*O. tshawytscha*	B6, B13, B26, B49, B51, B58

[a]Groups were based on host and geographic area of bacterial isolation
[b]RS18 was from Nova Scotia and the only eastern North America representative; for data analysis this isolate was placed with isolates from the Pacific Northwest origin

(PNW); chinook salmon *Oncorhynchus tshawytscha* from the Manistee Weir, Michigan, USA (MCK); coho salmon *O. kisutch* from the Manistee Weir, Michigan (MCO); chinook salmon from the Kewanee Weir, Wisconsin, USA (A strains); and chinook salmon from the Strawberry Creek Weir, Wisconsin (B strains). One strain (RS18) was taken from an Atlantic salmon *Salmo salar* in Nova Scotia, Canada. Because this was the sole representative of this geographic area, for statistical analysis it was placed with the isolates from the PNW, the group having the most diverse number of hosts and sites. The PNW strains were maintained in the culture collection of the National Fish Health Research Laboratory (Kearneysville, WV, USA). The Michigan and Wisconsin origin strains were isolated in 1991 and 1992 from Lake Michigan returning broodstock displaying clinical signs of BKD (Bullock & Herman 1988, Fryer & Lannan 1993). These strains were isolated from kidney tissue by inoculation onto KDM2 (Evelyn 1977) prepared with and without 1% metabolite (Evelyn et al. 1990). Colonies were transferred to KDM2 slants and subsequent growth rinsed with fresh KDM2 broth medium and frozen at −70°C.

Preparation of whole cell lysates. A frozen aliquot of each isolate was used to inoculate 500 ml KDM2 broth supplemented with 1% metabolite. The culture was incubated on a shaker (100 rpm) for 2 to 3 wk at 15°C (New Brunswick Scientific G25R, Edison, NJ, USA). Purity of each culture was ensured by Gram stain, lack of growth on brain heart infusion agar (Difco), and the identity confirmed by the fluorescent antibody test (Bullock et al. 1980). Cells were collected by centrifugation (4100 × *g*) for 30 min at 4°C (Sorvall RC2-B, Newtown, CT, USA). The cells were suspended in 15 ml of a lysate buffer (10 mM Tris, 1 mM EDTA, pH 6.8; Selander et al. 1986), centrifuged as before and resuspended in 5 ml of lysate buffer. The cells were sonicated with 1 min bursts (5 total), while in an ice bath, using a Cole Parmer 4710 ultrasonic homogenizer (Chicago, IL, USA) set to deliver 50 W cm^{-1}. Debris was removed by centrifugation (13 000 × *g*) for 30 min at 4°C. The supernatant (whole cell lysate) was placed into 5 vials and frozen at −70°C.

Electrophoresis of whole cell lysates. Procedures employed for electrophoresis were previously described by Selander et al. (1986) using 11 to 11.5% hydrolyzed starch (Sigma). Prior to preparing the gels, the starch was passed through a 200 µm sieve to remove any particulate material. Two buffer systems were used to evaluate the isolates for each enzyme, a pH 6.5 amine-citrate (AC; Clayton & Tretiak 1972) and a pH 8.0 buffer (RW; Ridgway et al. 1970). An isolate (B58) was used on all gels as a control (100 allele) to adjust gel to gel mobility differences. Duration of electrophoresis was 3 to 4 h.

Enzyme staining. Recipes for the staining solutions have been described by Shaw & Prasad (1970), Harris & Hopkinson (1976), Allendorf et al. (1977), Schill et al. (1984), and Manchenko (1994). For each stain, the gel slice was placed into the stain mixture and incubated at 37°C for a sufficient time to allow visualization of positively stained bands. A list of the enzymes used to assay the genetic diversity of *Renibacterium salmoninarum* is presented in Table 2.

Data analysis. Isolate zones of activity were scored by measuring the distance the zone had migrated from the origin. Each distance value was converted to a mobility relative to a 100 electromorph, and isolate B58 was designated the 100 electromorph. The enzyme mobility isolate profiles were evaluated and genetic diversity calculated in accordance with described methods (Nei 1977, 1978, Selander et al. 1986). Genetic diversity (h) was calculated using the equa-

Table 2. List of enzyme loci used to evaluate the genetic diversity of strains of *Renibacterium salmoninarum*. Buffer system used for scoring: AC = pH 6.5 (Clayton & Tretiak 1972); RW = pH 8.0 (Ridgway et al. 1970). NA = no activity noted

EC number	Enzyme	Buffer system
1.1.1.1	Alcohol DH[a] (ADH)	NA
1.1.1.8	Glycerol-3-phosphate DH (GPD)	NA
1.1.1.14	Sorbitol DH (SORDH)	NA
1.1.1.27	Lactate DH (LDH)	NA
1.1.1.37	Malate DH (MDH)	NA
1.1.1.40	Malic enzyme (ME)	NA
1.1.1.42	Isocitrate DH (ICD)	AC
1.1.1.44	Phosphogluconate DH (PGD)	AC
1.1.1.47	Glucose DH (GDH)	NA
1.1.1.49	Glucose-6-phosphate DH (GD)	AC
1.1.1.204	Xanthine DH (XDH)	NA
1.2.1.12	Glyceraldehyde-3-phosphate DH (GAPDH)	AC
1.3.99.1	Succinate DH (SUCDH)	RW
1.4.1.1	Alanine DH (ALADH)	AC
1.4.1.9	Leucine DH (LEUDH)	NA
1.4.1.2–4	Glutamate DH (GLUD)	RW
1.8.1.4	Diaphorase (DIA)	AC
1.6.99.2	Menadione reductase (MR)	AC
1.9.3.1	Cytochrome *c* oxidase (CTO)	AC
1.15.1.1	Superoxide dismutase (SOD)	AC
2.4.2.1	Nucleoside phosphorylase (NP)	RW
2.6.1.24	Tyrosine aminotransferase (ATRANS)	NA
2.6.1.1	Aspartate aminotransferase (AAT)	RW
2.7.1.1	Hexokinase (HK)	RW
2.7.3.2	Creatine kinase (CK)	RW
2.7.4.3	Adenylate kinase (AK)	RW
3.1.1.1	Esterase (EST-1, EST-2)	AC
3.1.3.1	Alkaline phosphatase (ALP)	RW
3.1.3.2	Acid phosphatase (ACP)	NA
3.1.3.11	Fructose bisphosphatase (FBP)	NA
3.2.1.52	Hexosaminidase (HEX)	NA
3.2.1.31	β-Glucuronidase (GUS)	NA
3.4.11.x	Peptidases	
	Glycyl-leucine (GLPEP-1, GLPEP-2)	RW
	Leucyl-glycyl-glycine (LGGPEP)	NA
3.5.4.3	Guanine deaminase (GDA)	NA
3.5.4.4	Adenosine deaminase (ADA)	NA
4.1.1.31	Phosphoenolpyruvate carboxylase (PC)	NA
4.1.2.13	Aldolase (ALD)	NA
4.2.1.2	Fumarate hydratase (FH)	AC
4.2.1.3	Aconitase (ACO)	RW
5.3.1.1	Triosephosphate isomerase (TPI)	NA
5.3.1.8	Mannose-6-phosphate isomerase (MPI)	RW
5.3.1.9	Glucose-6-phosphate isomerase (GPI)	AC
5.4.2.2	Phosphoglucomutase (PGM)	AC

[a]DH = dehydrogenase

tion: $h = (1 - \Sigma x_i^2)(n/n-1)$, where n is the number of isolates and x_i is the frequency of the electromorph. Genetic diversity was calculated for each locus (h_T) and was determined for each group at each locus. Mean genetic diversity (H_T) was calculated as the average of all h_T.

RESULTS

Forty-four enzyme staining systems were used to assay genetic variation in all of the *Renibacterium salmoninarum* isolates electrophoretically separated on the AC and RW buffer systems. No enzyme activity was detected with 20 of these, after 2 attempts with both buffer systems (Table 2). Two zones of activity (presumptive loci) were observed for the enzymes GLPEP and EST; for these, the 2 loci were designated 1 and 2, representing slow and fast mobilities, respectively. Both EST-1 and EST-2 were active when either α-naphthyl or β-naphthyl acetate was used as the substrate. In 3 enzymes activity was detected in only 1 or 2 isolates: PGM (MCK49, MCK56); GD (MCK49); and GLUD (Salmon Creek).

Genetic diversity was determined for 40 *Renibacterium salmoninarum* isolates using 26 loci. Electromorph mobilities and the frequencies of each are given in Table 3. There were no monomorphic loci and there was an average of 2.65 electromorphs per locus. The number of electromorphs per locus by group was: PNW (1.23), MCO (1.77), MCK (2.12), A (1.27), and B (1.15).

Genetic diversity (h) values for each isolate origin group at each locus are presented in Table 4. The highest locus genetic diversity was recorded for EST-1 (h_T = 0.587). Of the 40 isolates 23 displayed the 100 electromorph at EST-1 (Table 3); however, 9 of the 10 PNW isolates had an electromorph which was 35% faster and only 6 other isolates had this 135 electromorph. For EST-1, the proportion of genetic diversity that is explained by variation between groups (G_{st}) was 36.5%. Other loci that had relatively high genetic diversity were SUCDH (0.385), CTO (0.273), and ACO (0.311).

Mean genetic diversity (H_T) of all isolates over the 26 loci was 0.161. The percentage of the mean genetic diversity calculated to be due to variability between isolate origin groups was G_{st} = 8.1%; therefore, 91.9% of the genetic diversity of the 40 *Renibacterium salmoninarum* isolates was due to differences between individual isolates. Mean genetic diversity of the groups was highest for the isolates from Michigan origin, MCO (0.298) and MCK (0.270) (Table 4). Genetic diversity for the PNW group was 0.058 and for the 2 groups of Wisconsin origin were: A (0.067) and B (0.051).

At 11 of the loci (GD, GAPDH, ALADH, GLUD, DIA, SOD, NP, AAT, ALP, GPI, PGM), only 1 or 2 of the 40 isolates showed variation. For SOD, the most prominent and resolved zone of activity (allele) was scored. Other SOD activity was present but resolution was not sufficient for accurate analysis; thus, variability at the SOD locus, if present, could not be determined.

The isolates comprised 21 electrophoretic types (ETs). Two ETs were represented by 9 and 10 isolates each and these differed at 1 locus, EST-1. Isolates of these 2 ETs were from all 5 groups. Seventeen of 21 ETs were represented by single isolates, many of which differed at only 1 locus and this is indicated by the low H_T relative to the ET:isolate ratio (21:40).

Table 3. *Renibacterium salmoninarum*. Electromorph frequencies of 5 groups of isolates scored at 26 loci. Isolate origins: PNW = Pacific Northwest; MCO = Michigan coho; MCK = Michigan chinook; A = Kewanee, Wisconsin, chinook; B = Strawberry Creek, Wisconsin, chinook

Locus and electromorph[a]	Isolate origins PNW	MCO	MCK	A	B
ICD					
100	1.000	0.833	0.800	1.000	1.000
O		0.167	0.200		
PGD					
100	1.000	0.833	0.800	1.000	1.000
0		0.167	0.100		
108			0.100		
GD					
100			0.100		
0	1.000	1.000	0.900	1.000	1.000
GAPDH					
100	1.000	1.000	0.800	1.000	1.000
91			0.100		
100/91			0.100		
SUCDH					
100	1.000	0.333	0.600	0.875	0.833
0		0.667	0.400	0.125	0.167
ALADH					
100	0.900	0.833	1.000	1.000	1.000
0		0.167			
97	0.100				
GLUD					
100	0.100				
0	0.900	1.000	1.000	1.000	1.000
DIA					
100	1.000	1.000	0.900	1.000	1.000
0			0.100		
MR					
100	1.000	1.000	0.600	1.000	1.000
167/73			0.100		
87			0.300		
CTO					
100	0.800	0.833	0.900	0.875	0.833
0		0.167			
100/85	0.200			0.125	0.167
112			0.100		
SOD					
100	1.000	0.833	0.900	1.000	1.000
0		0.167			
97			0.100		
NP					
100	1.000	0.833	0.900	1.000	1.000
0		0.167			
90			0.100		
AAT					
100	1.000	1.000	0.900	1.000	1.000
85			0.100		
HK					
100	1.000	0.833	1.000	0.875	0.833
0		0.167		0.125	0.167
CK					
100	1.000	0.833	0.900	0.875	1.000
0		0.167		0.125	
180/100			0.100		
AK					
100	1.000	0.833	0.900	0.875	1.000
0		0.167		0.125	
180/100			0.100		
EST-1					
100	0.100	0.667	0.500	1.000	0.833
0		0.167			
135	0.900	0.167	0.200		
135/100			0.200		0.167
159			0.100		
EST-2					
100	1.000	0.667	0.900	1.000	1.000
0		0.333	0.100		
ALP					
100	1.000	1.000	0.900	1.000	1.000
0			0.100		
GLPEP-1					
100	1.000	0.667	0.900	0.875	1.000
0		0.333		0.125	
120			0.100		
GLPEP-2					
100	1.000	0.667	0.800	1.000	1.000
0		0.333	0.200		
FH					
100	1.000	0.667	0.900	1.000	1.000
0		0.333	0.100		
ACO					
100	0.800	0.667	0.800	0.875	1.000
0		0.167	0.200	0.125	
100/79	0.200	0.167			
MPI					
100	0.900	0.667	0.800	1.000	1.000
0	0.100	0.333	0.200		
GPI					
100	1.000	0.833	0.900	1.000	1.000
0		0.167			
65			0.100		
PGM					
100			0.100		
0	1.000	1.000	0.800	1.000	1.000
460			0.100		

[a] Each electromorph is the phenotypic expression of the isolate at that locus and the score is the mobility relative to the selected control or 100 allele; B58 was the control unless it was null (i.e. GD); then another isolate was selected for that locus

Table 4. *Renibacterium salmoninarum*. Genetic diversity of 40 isolates evaluated with 26 loci. For abbreviations of isolate origins, see Table 3. H_S: sample genetic diversity; H_T: mean genetic diversity

Locus	PNW	MCO	MCK	A	B	Group	Total
ICD	0.0	0.334	0.356	0.0	0.0	0.139	0.142
PGD	0.0	0.334	0.378	0.0	0.0	0.145	0.145
GD	0.0	0.0	0.200	0.0	0.0	0.050	0.050
GAPDH	0.0	0.0	0.378	0.0	0.0	0.095	0.099
SUCDH	0.0	0.533	0.533	0.250	0.334	0.313	0.385
ALADH	0.200	0.334	0.0	0.0	0.0	0.100	0.099
GLUD	0.200	0.0	0.0	0.0	0.0	0.050	0.050
DIA	0.0	0.0	0.200	0.0	0.0	0.050	0.050
MR	0.0	0.0	0.600	0.0	0.0	0.150	0.188
CTO	0.356	0.334	0.200	0.250	0.334	0.289	0.273
SOD	0.0	0.334	0.200	0.0	0.0	0.100	0.099
NP	0.0	0.334	0.200	0.0	0.0	0.100	0.099
AAT	0.0	0.0	0.200	0.0	0.0	0.050	0.050
HK	0.0	0.334	0.0	0.250	0.334	0.150	0.142
CK	0.0	0.334	0.200	0.250	0.0	0.150	0.145
AK	0.0	0.334	0.200	0.250	0.0	0.150	0.145
EST-1	0.200	0.599	0.733	0.0	0.334	0.373	0.587
EST-2	0.0	0.533	0.200	0.0	0.0	0.130	0.142
ALP	0.0	0.0	0.200	0.0	0.0	0.050	0.050
GLPEP-1	0.0	0.533	0.200	0.250	0.0	0.180	0.188
GLPEP-2	0.0	0.533	0.356	0.0	0.0	0.169	0.185
FH	0.0	0.533	0.200	0.0	0.0	0.130	0.145
ACO	0.356	0.599	0.356	0.250	0.0	0.318	0.311
MPI	0.200	0.533	0.356	0.0	0.0	0.219	0.224
GPI	0.0	0.334	0.200	0.0	0.0	0.100	0.099
PGM	0.0	0.0	0.378	0.0	0.0	0.095	0.099
Mean genetic diversity	0.058	0.298	0.270	0.067	0.051	$H_S = 0.148$	$H_T = 0.161$

DISCUSSION

MEE has been shown to offer estimates of bacterial population genetic diversity similar to results using DNA hybridization studies (Gilmour et al. 1987). Such analysis also provides a relatively inexpensive method to determine genotypic profiles for a large number of isolates (Manchenko 1994). Homogeneity of individuals is determined by statistically evaluating the isolate profiles, which has been done for a number of bacterial species including *Campylobacter* spp. (Aeschbacher & Piffaretti 1989), *Legionella pneumophila* (Selander et al. 1985), Mycobacteria (Wasem et al. 1991) and some *Salmonella* spp. (Reeves et al. 1989). The genetic diversity of certain fish pathogenic bacteria has also been evaluated using MEE: *Aeromonas salmonicida* (Boyd et al. 1994), *Edwardsiella ictaluri* (Starliper et al. 1988) and *Yersinia ruckeri* (Schill et al. 1984).

In this study of *Renibacterium salmoninarum* isolates from various origins, the mean genetic diversity (H_T = 0.161) was relatively low and comparable to other genetically homogeneous bacteria such as *Vibrio anguillarum* and *V. ordalii* (H_T = 0.130; Starliper et al. 1989); and *Bordetella* spp. (H_T = 0.284; Musser et al. 1986). However, H_T of *R. salmoninarum* was greater than that determined for 2 other fish pathogenic bacteria, namely *Aeromonas salmonicida* (H_T = 0.037; Boyd et al. 1994) and *Yersinia ruckeri* (H_T = 0.014; Schill et al. 1984).

Absence of enzyme activity, denoted as null, may result not only result from a bacterium lacking an allele at a particular locus, but could reflect insufficient enzyme concentration in the whole cell lysate or use of an inappropriate buffer system to detect activity of a particular enzyme (Selander et al. 1986). Two of the most frequently used buffer systems were used in the present study. It is possible that another buffer system might detect activity in some of the enzymes which had no positive isolates in this study.

Noteworthy was the activity of CTO in the *Renibacterium salmoninarum* isolates using the MEE technique. To determine CTO, N,N-dimethyl-*p*-phenylenediamine was used as the substrate and the activity developed for visualization by addition of α-naphthol to the staining solution. The visible product of the reaction was indophenol blue. It has been previously reported that *R. salmoninarum* is cytochrome oxidase

negative when the standard biochemical method is used for determination (Sanders & Fryer 1980). This contrast in results could be explained by the difference in the preparation of the whole cell lysates for the MEE method of CTO determination. With MEE, the cells were disrupted by sonication, perhaps releasing the enzyme into the lysate solution. Using the standard biochemical method which employs colonial growth, the cells remain intact.

Mean genetic diversity for each of the isolate origins was greatest for the MCO and MCK groups (Table 4). To determine if the isolates of Michigan origins were different from the rest and, perhaps, explain a portion of the mean genetic diversity, the 40 isolate profiles were regrouped—all strains of Michigan origin in one group and all other strains in another. Frequencies and genetic diversity values were recalculated (data not presented). Using this grouping, there was no activity of any Michigan strains at the GLUD locus, and there was variation at all other loci. For the group with strains from the other 3 origins, there were 13 monomorphic loci and 2 (GD, PGM) were void of detectable enzyme activity. The number of monomorphic loci detected among the isolates when strains were placed in 2 groups contrasts to that in the 5 groups, in which no monomorphic loci were scored. With 2 groups, the mean genetic diversity (H_T) remains the same as with 5 groups, and if a larger percentage of the total genetic diversity explained by group differences (G_{st}) became evident, the sample genetic diversity (H_S) would be reduced. However, with the isolates in 2 groups, the H_S actually increased to 0.154, from 0.148, for the isolates placed into 5 groups. Accordingly, G_{st} for the 2 group arrangement was reduced to 4.3%, from 8.1%, for the 5 groups. The results of this study are indicative of relatively low genetic diversity of *Renibacterium salmoninarum*. Furthermore, the genetic diversity present within the species is explained by variability among isolates regardless of their origin and not by differences between groups based on their host and geographic origin.

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