



HAL
open science

The fish pathogen: Growth in a microaerophilic atmosphere

Varpu Hirvelä-Koski

► **To cite this version:**

Varpu Hirvelä-Koski. The fish pathogen: Growth in a microaerophilic atmosphere. *Veterinary Microbiology*, 2007, 127 (1-2), pp.191. 10.1016/j.vetmic.2007.08.011 . hal-00532306

HAL Id: hal-00532306

<https://hal.science/hal-00532306>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: The fish pathogen *Renibacterium salmoninarum*:
Growth in a microaerophilic atmosphere

Author: Varpu Hirvelä-Koski

PII: S0378-1135(07)00389-6
DOI: doi:10.1016/j.vetmic.2007.08.011
Reference: VETMIC 3780

To appear in: *VETMIC*

Received date: 19-3-2007
Revised date: 2-8-2007
Accepted date: 6-8-2007



Please cite this article as: Hirvelä-Koski, V., The fish pathogen *Renibacterium salmoninarum*: Growth in a microaerophilic atmosphere, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.08.011

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

The fish pathogen *Renibacterium salmoninarum*: growth in a microaerophilic atmosphere

Varpu Hirvelä-Koski ¹⁾

Finnish Food Safety Authority (Evira)

Key words: *Renibacterium salmoninarum*, microaerophilic growth, salmonid fish

1) Varpu Hirvelä-Koski

Finnish Food Safety Authority (Evira)

Oulu Research Unit, PO Box 517, 90101 Oulu, Finland

Tel int+ 358 8 20 7724902

Fax int+358 8 20 7724915

E-mail: varpu.hirvela-koski@evira.fi

26 ABSTRACT

27 *Renibacterium salmoninarum* is the etiologic agent of bacterial kidney disease (BKD)
28 occurring worldwide in salmonid fish. This bacterium has previously been regarded as a
29 strict aerobic species. However, in this study it is shown that *R. salmoninarum* grows well
30 in microaerophilic atmosphere, the colony size being larger and the colonies being more
31 mucoid than in aerobic conditions. Microaerophilic cultivation might be one possibility to
32 increase the sensitivity of the cultivation method for the detection of this slowly growing
33 pathogen.

34

35 INTRODUCTION

36 *Renibacterium salmoninarum* is the etiologic agent of bacterial kidney disease (BKD) in
37 salmonid fish, a chronic, systemic infection characterized by granulomatous lesions in the
38 kidney and other organs (Fryer and Sanders, 1981; Fryer and Lannan, 1993). This
39 bacterium is widespread in nearly all countries where salmonid fish are cultured and it also
40 affects wild fish populations (Fryer and Sanders, 1981; Klontz, 1983). It was characterized
41 in terms of the G+C% content of the DNA, the cell wall sugar composition and the amino
42 acid composition of the peptidoglycan cell wall layer and was recognised as forming a
43 single species belonging to a new genus, *Renibacterium* (Sanders and Fryer, 1980).

44

45 *R. salmoninarum* is a Gram-positive short rod occurring frequently in pairs. It is aerobic,
46 catalase positive, cytochrome oxidase negative, non-acid fast and non-motile, does not
47 produce acid from sugars and does not form endospores. All strains require cysteine for
48 growth. Optimal growth occurs at 15 to 18°C, and growth is very slow at 5 or 22°C and
49 non-existent at 37°C. Growth on all media is slow, often requiring several weeks of
50 incubation (Sanders and Fryer, 1980; Benediktsdottir et al., 1991). *R. salmoninarum*
51 strains isolated from different regions form a very homogeneous group in terms of both

52 their morphological, biochemical and physiological (Goodfellow et al., 1985; Bruno and
53 Munro, 1986) and also their antigenic properties (Bullock et al., 1974; Getchell et al., 1985;
54 Daly and Stevenson, 1990). The bacterium is a highly conserved genospecies, and
55 studies on isolates from different parts of the world indicate relatively low genetic diversity
56 (Starliper, 1996; Grayson et al., 1999; Rhodes et al., 2000).

57

58 *R. salmoninarum* has been described as an aerobic bacterial species (Sanders and Fryer,
59 1980). In BKD there are often large granulomatous lesions in the internal organs of the
60 diseased fish, pointing to an ability to survive and proliferate in circumstances involving a
61 decrease in oxygen concentration. In this study we tested the growth of *R. salmoninarum*
62 under aerobic, microaerophilic and anaerobic conditions.

63

64 MATERIALS AND METHODS

65

66 **Trial 1:**

67 Three strains of *R. salmoninarum* were used: type strain of *R. salmoninarum* (ATCC
68 33209) and two strains, which had been isolated from rainbow trout in northern Finland in
69 the years 1997 and 2002 through the fish health control programme, using the icelandic
70 cultivation method (Benediktsdottir et al., 1991). All the strains had been kept frozen at –
71 80°C prior to the experiment. The bacterial population obtained on KDM2 agar (Evelyn,
72 1977) after 14 d was suspended in 0.1% peptone-saline to OD 0.08 (615 nm). Ten-fold
73 serial dilutions were prepared using peptone-saline, and 0.1 ml of each test dilution was
74 streaked onto two parallel KDM2 agar plates. One of the parallel plates was incubated
75 aerobically and the other under microaerophilic conditions, both at 16±1°C. The
76 microaerophilic atmosphere containing 5-8 % oxygen and 12-15 % carbon dioxide was
77 generated in sealed jars using the CampyGen™ equipment (Oxoid, Hants, U.K.), and was

78 tested using *Campylobacter fetus* subsp. *fetus* (ATCC 27374) as a control strain. The
79 KDM2 plates were observed for growth after 5 and 12 wks.

80

81 One microaerophilic subculture per strain used in Trial 1 was identified as *R.*
82 *salmoninarum* (Table 1), based on the observation of small Gram-positive rods occurring
83 in pairs in Gram staining, a negative oxidase test (Spot test, Difco/BBL), a positive
84 catalase test, a positive result in fluorescent antibody (FA) staining, typical results with API
85 ZYM test strips (bioMérieux, France) and little or no growth on TSA agar. A polymerase
86 chain reaction (PCR) was also used as a confirmatory test. In the FA staining a fixed
87 smear of bacterial culture was covered with polyclonal rabbit anti-*Renibacterium*
88 *salmoninarum* serum, incubated in a moist chamber at $37 \pm 1^\circ\text{C}$ for 30 min, rinsed with
89 PBS and covered with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG
90 (Nordic Immunological Laboratories, the Netherlands). It was then incubated as above,
91 rinsed and covered with phosphate-buffered glycerol and examined under an
92 epifluorescence microscope. The API ZYM test strips were incubated at $16 \pm 1^\circ\text{C}$ for 18-24
93 h and the results were compared with those described in the World Organisation for
94 Animal Health's Diagnostic Manual for Aquatic Animal Diseases (OIE, 2006), combined
95 with those obtained with *R. salmoninarum* ATCC 33209 in our laboratory. PCR was
96 performed according to Miriam et al. (1997) with some minor modifications (Hirvelä-Koski
97 et al., 2006). Amplification was performed in a 50 μl volume containing 0.3 μM primers FL7
98 and RL5, 0.2 mM each of nucleotide triphosphate and 2.5 mM MgCl_2 .

99

100 **Trial 2:**

101 A total of 19 strains of *R. salmoninarum* were used, ten of which had been isolated from
102 different fish species in northern Finland during the years 1997-2002 through the fish
103 health control programme, using the icelandic cultivation method (Benediktsdottir et al.,

104 1991). Six strains were obtained from Sweden and three from Denmark. The bacterial
105 strains were cultivated on three parallel KDM2 agar plates. One of them was incubated
106 aerobically, one anaerobically and one in a microaerophilic atmosphere. The anaerobic
107 atmosphere (carbon dioxide content 8-14 %, oxygen content below 1 %) was generated
108 using AnaeroGenTM (Oxoid) and anaerobiosis in the incubation jars was checked using
109 Anaerotest strips (Merck,Darmstadt, Germany). The incubation temperature was 16±1°C.
110 The plates were observed for growth after 15 d.

111

112 RESULTS AND DISCUSSION

113

114 A dense growth was observed with all the *Renibacterium salmoninarum* strains after
115 incubation under microaerophilic conditions in both trials. All isolates were in pure culture
116 and confirmed as *R. salmoninarum* by PCR. The number of colonies was slightly smaller
117 than on the aerobically incubated plates in Trial 1 (Table 1), but the colony size was larger
118 than in the aerobically incubated cultures (Fig. 1). After 12 wks the diameter of the single
119 colonies was 1-2 mm in aerobic cultivation and 3-4 mm under microaerophilic conditions,
120 and their appearance was more mucoid and more yellowish in the latter case. The fact that
121 the bacterial suspensions were prepared using aerobically cultivated plates may
122 nevertheless have influenced the number of colonies detected, for if the bacterial strains
123 tested here had been incubated in a microaerophilic atmosphere several times before the
124 trial, the result might have been different. No growth could be observed in any of the 19 *R.*
125 *salmoninarum* strains after anaerobic incubation.

126

127 Even though the incubation temperature used here was not optimal for *Campylobacter*
128 *fetus* subsp. *fetus*, growth of this bacterium was observed, indicating the presence of a
129 microaerophilic atmosphere in the incubation jars.

130

131 The results show that *R. salmoninarum* grows well in a microaerophilic atmosphere. This
132 is the first report on this phenomenon for *R. salmoninarum*, which has been regarded as a
133 strict aerobic species (Sanders and Fryer, 1980). The phylogenetic position of *R.*
134 *salmoninarum* was determined as being the actinomycetes subdivision (Stackebrandt et
135 al., 1988), and the bacterium was first grouped within the *Arthrobacter/Micrococcus*
136 subline, as supported by the strict aerobic metabolism of these genera (Stackebrandt et
137 al., 1988). Later, based on an almost complete 16S rRNA sequence and the unique
138 phenotypic characteristics of this bacterium, it was stated that *R. salmoninarum* should be
139 considered a separate genus of the high G+C% Gram-positive eubacterial subdivision
140 (Gutenberger et al., 1991; Banner et al., 1991). The microaerophilic growth of *R.*
141 *salmoninarum* observed here also supports the suggestions of Gutenberger et al. (1991).

142

143 It is well known that *R. salmoninarum* is very difficult to detect in subclinically infected fish
144 populations. Several authors have reported that, even though its presence in kidney tissue
145 is demonstrable by FA, ELISA or PCR, for example, efforts to isolate it by a culture method
146 can be unsuccessful (Mitchum et al., 1979; Paterson et al., 1979; Evelyn et al., 1981;
147 Pascho & Mulcahy, 1987; Griffiths et al., 1991; Sakai & Kobayashi, 1992; Gudmundsdottir
148 et al., 1993; Jansson et al., 1996; Miriam et al., 1997; Cvitanich, 2004). Microaerophilic
149 cultivation could be one possibility for increasing the sensitivity of the cultivation method
150 for detecting *R. salmoninarum* in fish tissues.

151

152

153 ACKNOWLEDGEMENTS

154

155 I warmly thank Dr. Eva Jansson (National Veterinary Institute, Sweden, SVA) and Dr. Nils-
156 Jörgen Olesen (Danish Institute for Food and Veterinary Research, DFVF) for providing
157 me with *R. salmoninarum* strains.

158

159 REFERENCES

160

161 Banner, C.R., Rohovec, J.S., Fryer, J.L., 1991. A new value for mol percent guanine +
162 cytosine of DNA for the salmonid fish pathogen *Renibacterium salmoninarum*. FEMS
163 Microbiol. Lett. 79, 57-60.

164 Benediktsdottir, E., Helgason, S., Gudmundsdottir, S., 1991. Incubation time for the
165 cultivation of *Renibacterium salmoninarum* from Atlantic salmon, *Salmo salar* L., broodfish.
166 J. Fish Dis. 14, 97-102.

167 Bruno, D.W., Munro, A.L.S., 1986. Uniformity in the biochemical properties of
168 *Renibacterium salmoninarum* isolates obtained from several sources. FEMS Microbiol.
169 Lett. 33, 247-250.

170 Bullock, G.L., Stuckey, H.M., Chen, P.K., 1974. Corynebacterial kidney disease of
171 salmonids: growth and serological studies on the causative bacterium. Appl. Microbiol. 28,
172 811-814.

173 Cvitanich, J.D., 2004. *Renibacterium salmoninarum* bar forms: characterization,
174 occurrence, and evidence of a host response to a *R. salmoninarum* infection. J. Fish Dis.
175 27, 193-212.

176 Daly, J.G., Stevenson, M.W., 1990. Characterization of the *Renibacterium salmoninarum*
177 haemagglutinin. J. Gen. Microbiol. 136, 949-953.

178 Evelyn, T.P.T., 1977. An improved growth medium for the kidney disease bacterium and
179 some notes on using the medium. Bull. Off. int. Epiz. 87, 511-513.

180 Evelyn, T.P.T., Ketcheson, J.E., Prospero-Porta, L., 1981. The clinical significance of
181 immunofluorescence-based diagnoses of the bacterial kidney disease carrier. Fish Pathol.
182 15, 293-300.

- 183 Fryer, J.L., Sanders, J.E., 1981. Bacterial kidney disease of salmonid fish. *Ann. Rev.*
184 *Microbiol.* 35, 273-298.
- 185 Fryer, J.L., Lannan, C.N., 1993. The history and current status of *Renibacterium*
186 *salmoninarum*, the causative agent of bacterial kidney disease in Pacific salmon. *Fish.*
187 *Res.* 17, 15-33.
- 188 Getchell, R.G., Rohovec, J.S., Fryer, J.L., 1985. Comparison of *Renibacterium*
189 *salmoninarum* isolates by antigenic analysis. *Fish Pathol.* 20, 149-159.
- 190 Grayson, T.H., Cooper, L.F., Atienzar, F.A., Knowles, M.R., Gilpin, M.L., 1999. Molecular
191 differentiation of *Renibacterium salmoninarum* isolates from worldwide locations. *Appl.*
192 *Environ. Microbiol.* 65, 961-968.
- 193 Goodfellow, M., Embley, T.M., Austin, B., 1985. Numerical taxonomy and emended
194 description of *Renibacterium salmoninarum*. *J. Gen. Microbiol.* 131, 2739-2752.
- 195 Griffiths, S.G., Olivier, G., Fildes, J., Lynch W.H., 1991. Comparison of western blot, direct
196 fluorescent antibody and drop plate culture methods for the detection of *Renibacterium*
197 *salmoninarum* in Atlantic salmon (*Salmo salar* L.). *Aquaculture* 97, 117-129.
- 198 Gudmundsdottir, S., Benediktsdottir, E., Helgason, S., 1993. Detection of *Renibacterium*
199 *salmoninarum* in salmonid kidney samples: a comparison of results using double-sandwich
200 ELISA and isolation on selective medium. *J. Fish Dis.* 16, 185-195.
- 201 Gutenberger, S.K., Giovannoni, S.J., Field, K.G., Fryer, J.L., Rohovec, J.S., 1991. A
202 phylogenetic comparison of the 16S rRNA sequence of the fish pathogen, *Renibacterium*
203 *salmoninarum*, to Gram-positive bacteria. *FEMS Microbiol. Lett.* 77, 151-156.
- 204 Hirvelä-Koski, V., Koski, P., Pohjanvirta, T., Sukura, A., 2006. Atypical growth of
205 *Renibacterium salmoninarum* in subclinical infection. *J. Fish Dis.* 29, 21-29.
- 206 Jansson, E., Hongslo, T., Höglund, J., Ljungberg, O., 1996. Comparative evaluation of
207 bacterial culture and two ELISA techniques for the detection of *Renibacterium*
208 *salmoninarum* antigens in salmonid kidney tissues. *Dis. Aquat. Org.* 27, 197-206.

- 209 Klontz, G.W., 1983. Bacterial kidney disease in salmonids: an overview. In: Antigenes of
210 fish pathogens (Anderson, D.P., Dorson, M., Dubourget, P.H., Eds.), pp 177-199.
211 Collection Fondation Marcel Merieux, Paris, France.
- 212 Miriam, A., Griffiths, S.G., Lovely, J.E., Lynch, W.H., 1997. PCR and probe-PCR assays to
213 monitor broodstock Atlantic salmon (*Salmo salar* L.) ovarian fluid and kidney tissue for
214 presence of DNA of the fish pathogen *Renibacterium salmoninarum*. J. Clin. Microbiol. 35,
215 1322-1326.
- 216 Mitchum, D.L., Sherman, L.E., Baxter, G.T., 1979. Bacterial kidney disease in feral
217 populations of brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), and rainbow
218 trout (*Salmo gairdneri*). J. Fish. Res. Board Can. 36, 1370-1376.
- 219 OIE, 2006. Bacterial kidney disease (*Renibacterium salmoninarum*). Manual of diagnostic
220 tests for aquatic animals 2006, Chapter 2.1.11. World Organisation for Animal Health
221 (Office International des Épizooties), Paris.
- 222 Pascho, R.J., Mulcahy, D., 1987. Enzyme-linked immunosorbent assay for a soluble
223 antigen of *Renibacterium salmoninarum*, the causative agent of salmonid bacterial kidney
224 disease. Can.J. Fish. Aquat. Sci. 44, 183-191.
- 225 Paterson, W.D., Gallant, C., Desautels, D., 1979. Detection of bacterial kidney disease in
226 wild salmonids in the Margaree River system and adjacent waters using an indirect
227 fluorescent antibody technique. J. Fish. Res. Board Can. 36, 1464-1468.
- 228 Rhodes, L.D., Grayson, T.H., Alexander, S.M., Strom, M.S., 2000. Description and
229 characterization of IS994, a putative IS3 family insertion sequence from the salmon
230 pathogen, *Renibacterium salmoninarum*. Gene 244, 97-107.
- 231 Sakai, M. and Kobayashi, M., 1992. Detection of *Renibacterium salmoninarum*, the
232 causative agent of bacterial kidney disease in salmonid fish, from pen-cultured coho
233 salmon. Appl. Environ. Microbiol. 58, 1061-1063.
- 234 Sanders, J.E., Fryer, J.L., 1980. *Renibacterium salmoninarum* gen. nov., sp. nov., the
235 causative agent of bacterial kidney disease in salmonid fishes. Int. J. Syst. Bacteriol. 30,
236 496-502.

237 Stackebrandt, E., Wehmeyer, U., Nader, H., Fiedler, F., 1988. Phylogenetic relationship of
238 the fish pathogenic *Renibacterium salmoninarum* to *Arthrobacter*, *Micrococcus* and related
239 taxa. FEMS Microbiol. Lett. 50, 117-120.

240 Starliper, C.E., 1996. Genetic diversity of North American isolates of *Renibacterium*
241 *salmoninarum*. Dis. Aquat. Org. 27, 207-213.

242

Accepted Manuscript

243 Table 1

244 The number of bacterial colonies on KDM2 agar plate of three strains of *Renibacterium salmoninarum* after incubation in an aerobic
 245 or microaerophilic atmosphere for 12 weeks.

246

Dilution	<i>R. salmoninarum</i> : type		Isolate 1		Isolate 2	
	strain		Aer	CO ₂	Aer	CO ₂
10 ⁻⁴	Aer ^a	CO ₂ ^b	Aer	CO ₂	Aer	CO ₂
	>200 ^c	>200	>200	>200	>200	>200
10 ⁻⁵	>200	>200	>200	>200	>200	>200
	>200	>200	>200	66	>200	>200
10 ⁻⁶	45	27	84	8	>200	44
	45	27	84	15	>200	174
10 ⁻⁷	5	8 ^d	70	2 ^d	114	30 ^d
	5	8	74	20	114	40
10 ⁻⁸	0	0	35	0	42	0
	0	0	35	12	47	5

247 ^a Incubation in an aerobic atmosphere

248 ^b Incubation in a microaerophilic atmosphere

249 ^c The number of colonies could not be counted exactly because of dense growth

250 ^d Microaerophilic growth identified as *R. salmoninarum*

Accepted Manuscript

251 Figure 1. Colonies of *Renibacterium salmoninarum* on KDM2 agar plate. Left: the plates incubated
252 aerobically; right: the plates incubated in microaerophilic atmosphere.

Accepted Manuscript

Manuscript

