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NOTE

Aeromonas salmonicida ssp. *salmonicida* lacking pigment production, isolated from farmed salmonids in Finland

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ABSTRACT: Strains of *Aeromonas salmonicida* ssp. *salmonicida* lacking pigment production were isolated from brown trout *Salmo trutta* m. *lacustris* and sea trout *S. trutta* m. *trutta* cultivated in fresh water in south Finland. The bacteria isolated showed only minor deviations in biochemical characteristics compared to 2 strains of *A. salmonicida* ssp. *salmonicida* and the type strain of *A. salmonicida* ssp. *salmonicida* (NCMB 1102). Several characters differed when compared to the type strain of *A. salmonicida* ssp. *achromogenes* (NCMB 1110). In challenge experiments, the strain tested was highly pathogenic to rainbow trout *Oncorhynchus mykiss*.

Different forms of *Aeromonas salmonicida* are frequently isolated from diseased salmonids as well as from non-salmonids (Wichardt 1983, Böhm et al. 1986, Wiklund 1990). Traditionally *A. salmonicida* has been divided into typical strains, that is, ssp. *salmonicida* which produce a brown water-soluble pigment and 'atypical' strains which do not produce or only very slowly produce the brown pigment. The 'atypical' strains include *A. salmonicida* ssp. *achromogenes*, *A. salmonicida* ssp. *masoucida*, and several isolates not readily assigned to any of the valid subspecies. Recently a fourth subspecies, *A. salmonicida* ssp. *smithia*, which does not readily produce the brown pigment, was proposed by Austin et al. (1989).

According to 'Bergey's Manual of Systematic Bacteriology' (Popoff 1984) the production of brown pigment is one of the 9 key characteristics in separating ssp. *salmonicida* from 'atypical' strains (Table 1). The present paper, however, describes strains of *Aeromonas salmonicida* ssp. *salmonicida* lacking pigment production.

During routine examination of diseased farmed fish in our laboratory in 1991, non-pigmented variants of *Aeromonas salmonicida* were isolated from brown trout *Salmo trutta* m. *lacustris* and sea trout *S. trutta* m. *trutta* from 4 fish farms using fresh water. The fish farms, which were located in south Finland, were mainly raising smolts for stocking purposes.

Table 1. Differential characteristics of subspecies of *Aeromonas salmonicida* and corresponding results of the non-pigmented strains. +: positive reaction; –: negative reaction; NT: not tested

Characteristic	Present strains	*Aeromonas salmonicida* ssp. *salmonicida*[a]	ssp. *achromogenes*[a]	ssp. *masoucida*[a]	ssp. *smithia*[b]
Brown pigment	–	+	–	–	+/–
Indole production	–	–	+	+	–
Esculin hydrolysis	+	+	–	+	–
L-Arabinose utilization	NT	+	–	+	–
Acid from sucrose	–	–	+	+	+/–
Acid from mannitol	+	+	–	+	+/–
Voges-Proskauer	–	–	–	+	–
Gas from glucose	+	+	–	+	NT
Production of H_2S	–	–	–	+	+

[a]Popoff (1984); [b]Austin et al. (1989)

The total mortality of the affected fish stock in one of the farms was about 90%. Disease signs in the fish from this farm were more pronounced than in fish from the other farms: large necrotic lesions in the muscles, enlarged spleens, and hemorrhagic intestines. The total mortality in the other farms varied from 8 to 40%. The disease signs of the fish from these farms included minor skin ulcers, enlarged spleens, and petechial hemorrhages in the perivisceral adipose tissue.

The diseased fish in one of the farms were medicated with oxytetracycline. On 2 other farms the fish were treated with oxolinic acid. Mortality ceased during the medication period.

Material and methods. Two to eleven fish from each farm (n = 4) were examined for bacterial infection in visceral organs. Samples from kidney, liver, and spleen from the diseased fish were inoculated onto tryptic soy (TS) agar (Difco Laboratories; final NaCl concentration = 1.5%), supplemented with 5% bovine blood. The agar plates were incubated at 20°C for 7 d. Biochemical tests were carried out on the isolates using methods described by MacFaddin (1983) or Cowan (1974) and the API 50 CHE diagnostic system (Bio Merieux, France). Because the development of brown pigment was previously shown to be dependent on tyrosine and phenylalanine (Griffin et al. 1953), TS agar supplemented either with tyrosine or phenylalanine (0.1% final concentration) was used to detect the pigment production.

As reference strains we included one pigment-forming strain (isolated concurrently with one of the non-pigmented strains), one strain of *Aeromonas salmonicida* ssp. *salmonicida* isolated from diseased farmed rainbow trout *Oncorhynchus mykiss*, and type strains of *A. salmonicida* ssp. *salmonicida* (NCMB 1102) and *A. salmonicida* ssp. *achromogenes* (NCMB 1110).

Virulence tests with one of the non-pigmented strains were performed by intraperitoneally injecting rainbow trout (5 fish per concentration) with 0.1 ml sterile saline (0.9% NaCl) containing 1.3×10^7, 1.3×10^5, and 1.3×10^3 washed bacteria (CFU, colony-forming units). Control fish were injected with 0.1 ml sterile saline. The infected fish were kept in recirculating, filtered tap water at 16 to 17°C for 2 wk. Samples of spleen, kidney, and liver from dead and surviving fish were examined bacteriologically.

Results. Gram-negative, non-motile, cytochrome oxidase-positive, facultatively anaerobic, short rods were isolated from the examined fish. Pigmented (3 isolates out of 7) as well as non-pigmented (4 isolates out of 7) strains were isolated from diseased fish from one of the farms. The strains isolated from the other 3 farms were all non-pigmented, and they were isolated from 67 to 100% of the examined fish.

In the biochemical tests, the strains lacking pigment production were almost identical to the 2 pigment-forming co-isolates tested and they all showed only minor deviations (growth in 4% NaCl and at 30°C) from the type strain of *Aeromonas salmonicida* ssp. *salmonicida* (NCMB 1102). Compared to the type strain of *A. salmonicida* ssp. *achromogenes* (NCMB 1110), several different characters (19 out of 92) were encountered (Table 2). However, the strain NCMB 1110 gave a positive reaction in the VP test, although it should have been negative according to Popoff (1984).

The strains tested did not produce pigment on TS agar supplemented with phenylalanine, but after 6 to 7 d of incubation a very weak pigment production was noted on TS agar supplemented with tyrosine. The *Aeromonas salmonicida* ssp. *salmonicida* reference strains produced pigment on both agars.

In the challenge test all fish injected with 1.3×10^7 and 1.3×10^5 bacteria died within 3 d, and 4 fish out of 5 injected with 1.3×10^3 bacteria died within 5 d. One fish survived for 2 wk in the last-mentioned group. All fish in the control group were alive at the end of the experiment. The injected bacteria were re-isolated from visceral organs of all dead fish but not from the one surviving challenged fish. These isolates did not produce any pigment on TS agar.

During 1992, the same type of disease occurred in 3 of the previously affected farms, but unfortunately no attempt to isolate bacteria was made. On the fourth farm there were no disease outbreaks during 1992. However, in 1992 non-pigmented strains of *Aeromonas salmonicida* ssp. *salmonicida* were isolated from diseased fish on 2 previously unaffected farms. One of the three of these non-pigmented variants tested did not produce gas from glucose and two of them produced acid from trehalose. In all other biochemical tests they were similar to the non-pigmented strains isolated in 1991.

Discussion. To our knowledge there are very few reports of 'typical' *Aeromonas salmonicida* strains that do not produce a brown, water-soluble pigment when grown on agar containing tryptone (tyrosine or phenylalanine). Austin et al. (1989) reported one strain of *A. salmonicida* ssp. *salmonicida* out of 27 tested (4%) not producing pigment. However, several characteristics of the group described (see Austin et al. 1989) did not match the type description of *A. salmonicida* ssp. *salmonicida* (Popoff 1984). Evelyn (1971) reported an aberrant strain of *A. salmonicida* that lost its ability to produce pigment after 2 yr of subcultivation. Also, Duff & Stewart (1933) noticed that isolates lost their ability to produce the brown pigment after prolonged subcultivation. Conversely, one strain recovered from an experimentally infected goldfish was reported not to produce brown pigment until it was subcultured 5 times.

Table 2. Morphological and biochemical characteristics of non-pigmented and pigmented *Aeromonas salmonicida* ssp. *salmonicida* strains and of type strains NCMB 1102 (*A. salmonicida* ssp. *salmonicida*) and NCMB 1110 (*A. salmonicida* ssp. *achromogenes*). ASS: *A. salmonicida* ssp. *salmonicida*; ASA: *A. salmonicida* ssp. *achromogenes*. +: positive reaction; –: negative reaction; (+): weak reaction; R: resistant; S: sensitive

	Non-pigmented ASS (n = 4)[a]	Pigmented ASS (n = 2)[a]	ASS NCMB 1102	ASA NCMB 1110
Gram stain	–	–	–	–
Cell morphology	Rod	Rod	Rod	Rod
Motility	–	–	–	–
Cytochrome oxidase	+	+	+	+
Catalase	+	+	+	+
Modified O/F test	+/+	+/+	+/+	+/+
Arginine dihydrolase	+	+	+	+
Lysine decarboxylase	+	+	(+)	–
Ornithine decarboxylase	–	–	–	–
Growth in/at:				
0 % NaCl	+	+	+	+
3 % NaCl	+	+	+	+
4 % NaCl	–	–	+	+
5 % NaCl	–	–	–	–
4 °C	+	+	+	+
30 °C	+	+	–	–
37 °C	–	–	–	–
Susceptibility to:				
0/129 10 µg	R	R	R	R
150 µg	R	R	R	R
Oxytetracycline (80 µg)	S	S	S	S
Oxolinic acid (10 µg)	S	S	S	S
Trimethoprim + sulfa (5.2 + 240 µg)	S	S	S	S
Ampicillin (33 µg)	S	S	S	R
Cephalothin (66 µg)	S	S	S	R
Production of:				
Acetoin from glucose (Voges-Proskauer)	–	–	–	+
Brown pigment (Tryptic soy agar 3 d)	–	+	+	–
β-galactosidase (ONPG)	–	–	–	–
Gas from glucose	+	+	+	–
Hydrogen sulphide (SIM)	–	–	–	–
Indole (tryptone broth)	–	–	–	+
Phenylalanine deaminase	–	–	–	–
Phosphatase	–	–	–	–
Utilization of Na-citrate	–	–	–	–
Nitrate reduction	+	+	+	+
Degradation of:				
Blood (haemolysis)	+	+	+	–
Casein	+	+	+	+
DNA	+	+	+	+
Esculine	+	+	+	–
Gelatine	+	+	+	–
Starch	+	+	+	+
Tween 80	+	+	+	+
Growth on:				
Aeromonas agar	+	+	+	+
Cytophaga agar	+	+	+	+
MacConkey agar	+	+	+	+
TCBS agar	–	–	–	–

(Table continued on next page)

Table 2 (continued)

	Non-pigmented ASS (n = 4)[a]	Pigmented ASS (n = 2)[a]	ASS NCMB 1102	ASA NCMB 1110
Acid production (API 50 CHE):				
Glycerol	+	+	+	+
Erythritol	–	–	–	–
D-Arabinose	–	–	–	–
L-Arabinose	+	+	+	–
Ribose	+	+	+	+
D-Xylose	–	–	–	–
L-Xylose	–	–	–	–
Adonitol	–	–	–	–
β Methyl-xyloside	–	–	–	–
Galactose	+	+	+	(+)
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
D-Mannose	±	–	–	+
L-Sorbose	–	–	–	–
Rhamnose	–	–	–	–
Dulcitol	–	–	–	–
Inositol	–	–	–	–
Mannitol	+	+	+	+
Sorbitol	–	–	–	–
α Methyl-D-mannoside	–	–	–	–
α Methyl-D-glucoside	+	+	+	–
N Acetyl glucosamine	+	+	+	–
Amygdaline	–	–	–	–
Arbutine	+	+	+	–
Salicine	+	+	+	–
Cellobiose	–	–	–	–
Maltose	+	+	+	+
Lactose	–	–	–	–
Melibiose	–	–	–	–
Saccharose	–	–	–	+
Trehalose	–	–	–	+
Inuline	–	–	–	–
Melezitose	–	–	–	–
D-Raffinose	–	–	–	–
Amidon	+	+	+	+
Glycogen	+	+	+	+
Xylitol	–	–	–	–
β Gentiobiose	–	–	–	–
D-Turanose	–	–	–	–
D-Lyxose	–	–	–	–
D-Tagatose	–	–	–	–
D-Fucose	–	–	–	–
L-Fucose	–	–	–	–
D-Arabitol	–	–	–	–
L-Arabitol	–	–	–	–
Gluconate	+	+	+	–
2 ceto-gluconate	–	–	–	–
5 ceto-gluconate	–	–	–	–

[a]Current isolates

On the other hand, there are reports that motile *Aeromonas* species and *Vibrio anguillarum* can produce the same type of pigment as *A. salmonicida* (Paterson 1974, Allen et al. 1983, Evelyn & Ketcheson 1990). Recently, a strain of *Pseudomonas fluorescens* producing brown pigment was also reported (Frerichs & Holliman 1991).

So far most strains of *Aeromonas salmonicida* not producing pigment have been considered to be 'atypical' ones and they have been assigned to the ssp. *achromogenes*, ssp. *masoucida* (Kimura 1969), ssp. *smithia* (Austin et al. 1989), or simply identified as 'atypical' strains. Our results show that the present isolates should not be included in ssp. *achromogenes*, ssp. *masoucida* or ssp. *smithia*. A comparison between the results of the present non-pigmented isolates and the key characteristics of different subspecies of *A. salmonicida* proposed by Popoff (1984) shows that the present isolates differ from ssp. *achromogenes* in 5 tests (indole production, esculin hydrolysis, acid production from sucrose and mannitol, and gas production from glucose), from ssp. *masoucida* in 4 tests (indole production, acid production from sucrose, Voges-Proskauer test, production of H_2S), and from ssp. *smithia* (Austin et al. 1989) in 2 tests (esculin hydrolysis, production of H_2S) (Table 1).

Based on our results, we conclude that the present isolates should be considered as *Aeromonas salmonicida* ssp. *salmonicida*. Thus the use of pigment production as one of the key diagnostic features for separating ssp. *salmonicida* from the other subspecies has to be re-evaluated. The reliability of using pigment production in diagnosing typical *A. salmonicida* has also previously been questioned (Austin & Austin 1987).

The challenge test showed that the injected strain was highly pathogenic for rainbow trout.

The origin of the present strains is unclear, but the development of non-pigmented variants of typical *Aeromonas salmonicida* may be due to a mutation in pigment producing strains. This hypothesis is supported by the co-isolation of typical pigment-producing and pigment-lacking strains from one of the farms. Fingerlings from one of the affected farms were transferred in the beginning of the summer to the 3 other affected farms and obviously the described bacterium was transferred with the fish.

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