Atypical characteristics of the salmonid pathogen *Aeromonas salmonicida*

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Incubation of *Aeromonas salmonicida* at supra-optimal temperatures, i.e. 30–37 °C, resulted in the expression of motility by polar flagella, and changes in sugar fermentation patterns, e.g. loss of acid production from mannitol, loss of the ability to degrade complex molecules (aesculin, DNA, elastin and gelatin), and an increase in antibiotic resistance (notably co-trimoxazole). Motility was enhanced in cultures grown in brain heart infusion broth supplemented with 18% (w/v) Ficoll.

Introduction

Traditionally, the genus *Aeromonas* has been divided on the basis of motility, with non-motile species comprising *A. media* and *A. salmonicida* (Allen *et al.*, 1983; Popoff, 1984). However, the validity of this arbitrary division is now questionable, as motility has recently been reported in *A. media* (Austin & Austin, 1990). Moreover, it has been indicated that some strains of *A. salmonicida* are motile, although the flagella patterns have not been discussed (Bryant *et al.*, 1986; Lee, 1987).

In general, the characteristics of *A. salmonicida* have been recorded as a result of incubation of cultures at optimal growth temperatures, i.e. 18–24 °C (Popoff, 1984). There has been only limited interest in the effects of incubation at supra-optimal temperatures, i.e. >25 °C. Hahnel & Gould (1982) reported negligible changes in the biochemical reactions of virulent and avirulent cultures of *A. salmonicida* following incubation at 28 °C. However, it has been indicated that growth at 30 °C, which is regarded as the maximal growth temperature of *A. salmonicida* (Austin & Austin, 1987), adversely affects virulence (Ishiguro & Trust, 1981; Ishiguro *et al.*, 1981). This study has extended earlier work by examining phenotypic changes in *A. salmonicida* following incubation at ≥30 °C. In particular, emphasis has been placed on examining the expression of motility at supra-optimal temperatures.

Methods

Bacterial isolates. Five isolates of *A. salmonicida* subsp. *salmonicida* were examined: AS14 (supplied by Dr G. Dear, Anglian Water

Authority, Huntingdon), and LL, NG, S24 and S194 (recovered from the kidney of clinically diseased Atlantic salmon (*Salmo salar*). Authenticity was verified after Popoff (1984) and Austin *et al.* (1989). Briefly, the cultures comprised Gram-negative fermentative rods, which produced a diffusible brown pigment on brain heart infusion agar (BHIA; Oxoid), produced catalase and oxidase, and agglutinated with monospecific hyperimmune antiserum to *A. salmonicida* (Mono-As latex agglutination test; BioNor Aqua). Cultures were maintained at 4 °C on slopes of BHIA and subcultured every 2–3 weeks.

Growth at supra-optimal temperatures. Isolates were initially grown for 48 h at 25 °C on L-F agar: 1% (w/v) brain heart infusion (BHI, Oxoid), 0.5% (w/v) yeast extract (Oxoid), 1% (w/v) agar no. 3 (Oxoid), 10% (w/v) sucrose and 10% (v/v) inactivated (56 °C for 30 min) horse serum (Oxoid) (McIntosh & Austin, 1988). The ability to grow at supra-optimal temperatures was examined by transferring loopfuls of each culture, grown initially at 25 °C, to fresh L-F agar with incubation at 30 °C for 48 h. Resultant colonies were subcultured onto fresh plates, and incubated at 35 °C for 48 h. Inocula were then transferred to fresh L-F agar for incubation at 37 °C for 48–96 h.

Characteristics of *A. salmonicida* grown at different temperatures. Using incubation temperatures of 25, 30 and 37 °C, cultures were examined using the tests outlined by Austin *et al.* (1989). In addition, the isolates were examined for auto-aggregation in distilled water, and for susceptibility to carbenicillin (100 µg), cephaloridine (25 µg), co-trimoxazole (25 µg), sulphamethazole (200 µg) and tetracycline (100 µg) (Mast Laboratories) (after Allen *et al.*, 1977).

Wet preparations from overnight broth cultures (BHI broth, Oxoid) and dense suspensions (approx. 10⁹ cells ml⁻¹) in 0.85% saline prepared from overnight growth on BHIA and L-F agar were examined at a magnification of ×400 by phase-contrast microscopy to determine the presence of motility. The effects of increased micro-viscosity on the expression of motility were examined after growth (overnight) in BHI supplemented with 18% (w/v) Ficoll 400DL (Sigma) (after McCarter *et al.*, 1988). These cultures were diluted 1:10 with 0.85% saline prior to microscopic examination. Motile preparations were subsequently fixed for 1–2 h in 0.25% buffered (pH 7.4) glutaraldehyde (EMscope), negatively stained with 0.75% phosphotungstic acid (EMscope), and examined for the presence of flagella with an AEI EM6G transmission electron microscope.

Abbreviation: BHI, brain heart infusion.

Table 1. Differences in characteristics of five isolates of *Aeromonas salmonicida* recorded after incubation at 25, 30 and 37 °C

Characteristic	Incubation temperature (°C)		
	25	30	37
Colony and micro-morphology			
Production of brown diffusible pigment	+	—	—
Motility in BHI	—	—(1)*	+
Auto-aggregation of cells	+(4)*	+(3)*	—
Biochemical test			
Catalase production	+	—	—
Degradation of:			
Aesculin	+	—	—
DNA	+	+	—
Elastin	+	+	—
Gelatin	+	+	—
Production of acid from:			
Galactose	+	+	+(3)*
Mannitol	+	+	—
Susceptibility to:			
Co-trimoxazole (25 µg)	+	—	—

* Numbers in parentheses refer to the number of positive responses.

Results and Discussion

All five isolates of *A. salmonicida* grew at 37 °C when cultured on L-F agar or in BHI. However, there were changes in the characteristics of the organisms at temperatures of ≥ 30 °C (Table 1). For example, pigment production, which is typical of *bona fide* isolates of *A. salmonicida* subsp. *salmonicida* (Austin & Austin, 1987), did not occur at ≥ 30 °C. In addition, the ability to produce catalase and to degrade aesculin, DNA, elastin and gelatin was lost at 37 °C. Loss of these enzymic activities, which appeared to be transient insofar as the activities were subsequently detected upon re-incubation of subcultures at 25 °C, may contribute to changes in virulence. Indeed, virulence appears to be diminished when cultures are incubated at 30 °C rather than at the optimum growth temperature, i.e. < 25 °C (Ishiguro *et al.*, 1981). In addition, it was observed that all isolates became resistant to co-trimoxazole (25 µg) at 30 and 37 °C. Parallel changes in antibiotic sensitivity patterns have been observed by Hahnel & Gould (1982), who noted that some isolates of *A. salmonicida* became increasingly resistant to novobiocin and sulphisoxazole when incubated at 26 °C rather than 18 °C.

Following incubation at 37 °C, BHI cultures of all isolates were observed to contain low numbers (approx. 1%) of motile cells, which demonstrated a twisting/tum-

Fig. 1. Negatively stained cells of *A. salmonicida* subsp. *salmonicida*. (a) Strain S24 grown in Ficoll broth at 30 °C, showing the presence of a tuft of polar flagella. (b) Strain NG grown on L-F medium at 37 °C, showing the presence of a single polar flagellum. Bars, 1 µm.

bling movement. It should be emphasized that isolate NG was also motile at 30 °C. Examination of suspensions prepared from colonies on L-F agar, when incubated at 30 and 37 °C, also revealed the presence of low numbers of motile cells. However, motility was not observed in any culture in BHI or on L-F agar when incubated at 25 °C. Nevertheless, motility was recorded in cultures which were grown at 25 °C in BHI supplemented with Ficoll. Using transmission electron microscopy, flagella were observed in suspensions prepared from cultures on L-F agar and in BHI supplemented with Ficoll. Essentially, the nature of the growth medium appeared to influence the thickness of the flagella on the bacterial cells. Thus, thick flagella were produced in the presence of Ficoll. Conversely, only thin flagella were observed around cells grown on L-F agar. For example, in BHI supplemented with Ficoll, each cell of *A.*

salmonicida S24 demonstrated two or three, apparently sheathed polar-located flagella (Fig. 1a), which resembled those observed on motile cells of *A. media* (Austin & Austin, 1990). Colonies of *A. salmonicida* NG from L-F agar possessed cells with a single apparently unsheathed, polar-located flagellum (Fig. 1b). Flagella were not observed on cells from cultures in BHI. Furthermore, flagella were only recorded from freshly prepared negatively-stained specimens. Flagella were not seen on preparations stored overnight at room temperature prior to examination.

The benefit of using Ficoll to enhance the production of flagella agrees with previous work on *Vibrio parahaemolyticus*, which was observed to produce lateral and polar flagella (McCarter *et al.*, 1988). Thus, it was reasoned that *V. parahaemolyticus*, which normally possesses only polar flagella when grown in liquid medium, produced lateral flagella with physical support, as provided by Ficoll.

Certainly, this study has demonstrated motility in *A. salmonicida* as a result of incubation at supra-optimal temperatures and/or increased micro-viscosity of liquid cultures, i.e. by the addition of Ficoll. Possibly, the possession of flagella represents a different stage of the life cycle, as suggested for *A. media* (Austin & Austin, 1990). The mechanisms which might induce motility of *A. salmonicida* in the natural environment are obscure. Perhaps, the presence of mucus on fish surfaces could be mimicking the effects of Ficoll, i.e. by increasing the micro-viscosity.

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