Veterinary Microbiology 152 (2011) 353–360

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

# Alteration of virulence factors and rearrangement of pAsa5 plasmid caused by the growth of *Aeromonas salmonicida* in stressful conditions

Rana K. Daher [a,b], Geneviève Filion [a,b], Sok Gheck E. Tan [b], Stéphanie Dallaire-Dufresne [a,b], Valérie E. Paquet [a,b], Steve J. Charette [a,b,c,*]

[a] *Institut de Biologie Intégrative et des Systèmes, Pavillon Charles-Eugène-Marchand, Université Laval, 1030 Avenue de la Médecine, Quebec City, Quebec, Canada G1V 0A6*
[b] *Centre de Recherche de l'Institut Universitaire de Cardiologie et de Pneumologie de Québec (Hôpital Laval), 2725 Chemin Sainte-Foy, Quebec City, Quebec, Canada G1V 4G5*
[c] *Département de Biochimie, de Microbiologie et de Bio-informatique, Faculté des Sciences et de Génie, Université Laval, 1045 Avenue de la Médecine, Quebec City, Quebec, Canada G1V 0A6*

ARTICLE INFO

*Article history:*
Received 30 September 2010
Received in revised form 5 April 2011
Accepted 28 April 2011

*Keywords:*
Furunculosis
*Aeromonas salmonicida*
Virulence factors
Stressful conditions
Type three secretion system
Plasmid
Rearrangement
Virulence
*Dictyostelium discoideum*

ABSTRACT

*Aeromonas salmonicida*, a fish pathogen, is the causative agent of furunculosis. It was already shown that growing this bacterium in stressful conditions such as temperature above 22 °C might lead to virulence attenuation. Unfortunately, many veterinary microbiology services and reference centers still routinely cultivate *A. salmonicida* at 25 °C. Here we tested the presence of virulence factors by growth on specific medium as well as the integrity of the pAsa5 plasmid, which bears an important virulence factor, the type III secretion system (TTSS), by PCR analysis in twenty strains, most of which were grown at 25 °C in their laboratory of origin. The analysis revealed that strains, which encountered the more stressful growth conditions displayed the most frequent absence of A-layer protein and secreted proteolytic activity. Moreover, many strains had lost parts of the pAsa5 plasmid in which the TTSS region was almost always affected. To confirm the effect of stressful growth conditions on the plasmid, three strains with an intact pAsa5 were cultured at 25 °C for two weeks. A low but significant fraction of the tested colonies displayed pAsa5 rearrangements. The rearrangement always affected the TTSS region and led to a loss of virulence in the *Dictyostelium discoideum* co-culture assay. These results demonstrate that the instability of pAsa5 did not lead to its complete loss as previously proposed but to a more complex rearrangement phenomenon and emphasizes the necessity to grow *A. salmonicida* in appropriate conditions to preserve the complete virulence of the bacterium.



## 1. Introduction

*Aeromonas salmonicida* subsp. *salmonicida* (*A. salmonicida*) is a pathogenic Gram-negative bacterium found in aquatic environments and causes furunculosis, especially in salmonids (trout, salmon) (Hiney and Olivier, 1999; Wiklund and Dalsgaard, 1998). This disease has important consequences in the fish farming industry.

Many molecular elements are already known to contribute to the virulence of *A. salmonicida*. One of these is the A-layer protein, which is a 49 kDa hydrophobic protein, which is part of the bacterial envelope. This protein forms a protective shield at the surface of the bacteria, which confers resistance to bactericidal activity of host (Kay et al., 1981, 1984; Munn et al., 1982). Strains without the A-layer protein did not autoaggregate, are

* Corresponding author at: Institut de Biologie Intégrative et des Systèmes (IBIS), Pavillon Charles-Eugène-Marchand, 1030 Avenue de la Médecine, Université Laval, Quebec City, Quebec, Canada G1 V 0A6. Tel.: +1 418 656 2131x6914; fax: +1 418 656 7176.
*E-mail address:* steve.charette@bcm.ulaval.ca (S.J. Charette).

0378-1135/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2011.04.034

phage sensitive and most of all are less virulent (Ishiguro et al., 1981).

*A. salmonicida* produces also a large variety of extracellular virulence factors such as proteases and lipases (Rasch et al., 2007). These enzymes are more likely implicated in the infection process especially in the degradation of biological elements of the host tissues. The pathogenic effect of the secreted proteolytic activity is an important element in the virulence of the bacteria (Sakai, 1985). The real importance in bacterial virulence of the lipolytic activity is less clear than proteolytic activity (Lee and Ellis, 1990; Vipond et al., 1998).

*A. salmonicida* contains many plasmids (Boyd et al., 2003; L'Abee-Lund and Sorum, 2000, 2002; Reith et al., 2008; Sandaa and Enger, 1994), one of which (pAsa5 or pASvirA) is particularly important for its virulence (Reith et al., 2008; Stuber et al., 2003). pAsa5 has a locus containing the vast majority of the genes encoding the proteins required for the formation of the type III secretion system (TTSS) as well as for toxins secreted into host cells via the TTSS (Burr et al., 2002; Reith et al., 2008; Stuber et al., 2003). Studies on the mode of infection using fish and *Dictyostelium discoideum* amoeba, an alternative host model, revealed that the TTSS is required by *A. salmonicida* to infect the host (Burr et al., 2005; Dacanay et al., 2006; Froquet et al., 2007).

Previous studies have shown that when *A. salmonicida* strains were cultured at 25 °C and above, there was a loss of the expression of the A-layer protein or a loss of pAsa5 plasmid. In both cases, this was accompanied by loss of virulence (Ishiguro et al., 1981; Stuber et al., 2003). On the other hand, reference publications such as Bergey's Manual of Systematic Bacteriology (Brenner et al., 2005) indicate that *A. salmonicida* can be routinely grown at 25 °C which, in fact, represents a stressful condition that may affect the virulence of the bacteria (Ishiguro et al., 1981; Stuber et al., 2003). Since many veterinary microbiology services and reference centers follow this recommendation to grow *A. salmonicida* strains at 25 °C and since this is done repeatedly in some cases, we thus investigated the presence of various virulence factors in environmental isolates of *A. salmonicida* grown for many of them in stressful conditions over many years. It appears that growing *A. salmonicida* in stressful conditions correlated with the absence of many virulence factors including a previously undescribed rearrangement of pAsa5 rather than a complete loss of this plasmid which leads to TTSS loss and concomitant virulence loss.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The *A. salmonicida* strains used are listed in Table 1. The strains were grown at 18 °C on furunculosis agar (Bacto-Tryptone 10 g, yeast extract 5 g, L-tyrosine 1 g, NaCl 2.5 g, agar 15 g; per liter of distilled water) (Hanninen and Hirvela-Koski, 1997).

Before they were obtained by our group, the HER strains have been grown repeatedly at 25 °C by the Félix d'Hérelle Reference Center. All the strains from the Faculté de médecine vétérinaire, Université de Montréal (FMVUM, Quebec, Canada) were grown at 25 °C at least one time by its veterinary microbiology service. In the case of A449 strain, information was unavailable as to whether the bacteria had been routinely (or sporadically) grown at 25 °C prior to our analysis. Finally 01-B516, 01-B522 and 01-B526 strains were always grown at temperature below 20 °C.

**Table 1**
*A. salmonicida* strains.

| Strain | Origin[a] | Source and/or reference[b] |
|---|---|---|
| A449 | Brown trout (France) | Dacanay et al. (2006) |
| HER1084 (95-68) | INA (France) | FHRC (Popoff, 1971) |
| HER1085 (170-68) | INA | FHRC |
| HER1098 (866) | INA (USA) | FHRC (Udey, 1978) |
| HER1104 (132-66) | INA | FHRC |
| HER1107 (01-J3000) | INA | FHRC |
| HER1108 (10-69) | INA (Denmark) | FHRC (Popoff, 1971) |
| HER1110 (35-69) | INA (Japan) | FHRC (Popoff, 1971) |
| 07-5957 | Atlantic salmon (Canada) | FMVUM |
| 07-7287 | Perch (Canada) | FMVUM |
| 07-7346 | Atlantic salmon (Canada) | FMVUM |
| 07-7817 | INA (Canada) | FMVUM |
| 07-9324 | Brook trout (Canada) | FMVUM |
| 08-2647 | Brook trout (Canada) | FMVUM |
| 08-2783 | Brook trout (Canada) | FMVUM |
| 08-4188 | Brook trout (Canada) | FMVUM |
| 09-0167 | Atlantic salmon (Canada) | FMVUM |
| 01-B516 | Brook trout (Canada) | FMVUM |
| 01-B522 | Brook trout (Canada) | FMVUM |
| 01-B526 | Brook trout (Canada) | FMVUM (Dautremepuits et al., 2006) |

[a] INA: Information not available or not traceable.
[b] FMVUM: Faculté de médecine vétérinaire, Université de Montréal (Quebec, Canada). FHRC: Félix d'Hérelle Reference Center, Département de biochimie, de microbiologie et de bio-informatique, Université Laval (Quebec, Canada).

To mimic the effect of harsh growth conditions on the stability of pAsa5 and especially to imitate the cumulative effect of repeated growth periods at 25 °C as those imposed to HER strains, three wild-type strains (A449, 01-B516 and 01-B526) were inoculated from frozen stock on furunculosis agar plates, which were incubated for one week at 25 °C. Five isolated colonies from each agar plate were picked and streaked on five separate furunculosis agar plates and incubated for another week at 25 °C. The isolated colonies were split in two: half were directly used for PCR genotyping and the other half were used to prepare frozen stock. For the stock, isolated colonies were cultured in furunculosis broth at 18 °C and than 30% of glycerol were added. Aliquots of the bacterial suspensions were stored at −80 °C until their used.

### 2.2. Presence of A-layer and secreted lipases and proteases

The presence of the A-layer protein was detected by growing the bacteria on Coomassie brilliant blue (CBB)