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

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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.

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; Kay et al., 1984; Munn et al., 1982). Strains without the A-layer protein did not autoaggregate, are 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 (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.

MATERIAL AND METHODS

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.

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.

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) agar (Cipriano and Bertolini, 1988). The CBB agar medium was prepared by adding

0.01 % of Coomassie brilliant blue R-250 to tryptic soy agar (BD, Mississauga, ON, Canada). The bacteria were first grown on furunculosis agar for 3 days at 18°C, streaked on CBB agar, and then incubated at 18°C for 3 days. Blue colonies indicate that the A-layer protein is present while white colonies indicated that it is absent (Cipriano and Bertolini, 1988).

The secretion of lipases was detected by growing the bacteria on tributyrin agar. The tributyrin agar medium consists of 0.5% of bactopeptone, 0.3% of yeast extract, 1% of tributyrin and 1.5% of agar. The bacteria were first grown on furunculosis agar for 3 days at 18°C, streaked on tributyrin agar, and then incubated at 18°C for 3 days. The lipolytic activity was observed by the formation of a clear zone around individual colonies (Rasch et al., 2007).

The secretion of proteases was detected by growing the bacteria on skimmed milk agar. The skimmed milk agar medium consists of 10% skimmed milk and 1.2% of agar. The bacteria were first grown on furunculosis agar for 3 days at 18°C, streaked on skimmed milk agar, and then incubated at 18°C for 3-5 days. The proteolytic activity was observed by the formation of a clear zone around individual colonies (Rasch et al., 2007).

PCR genotyping

The PCR primers are listed in Table 2. Primers that were not found in the literature were designed using primer design software on the Integrated DNA Technologies (IDT) website (www.idtdna.com) using the pAsa5 sequence (GenBank: CP000646). Primer pairs for fourteen pAsa5 genes that were not redundant and were only found on the pAsa5 and not on other sequenced plasmids of *A. salmonicida* or on the chromosome were chosen. Primers were selected based on the sequence proposed by the IDT software.

DNA lysates were prepared by suspending three or four bacterial colonies of environmental strains or one bacterial colony of strains cultured at 25°C in the present study in 20 µl of lysis buffer solution containing 50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40, and 0.45% Tween 20. The suspensions were then heated at 95°C for 5 min. The PCR amplification mix for a 20 µl sample was as follows: 4 µl of 5x Go-Taq buffer (Promega, Madison, WI, USA), 1.6 µl of dNTP (2 mM), 1.3 µl of reverse primer (100 ng/µl), 1.3 µl of forward primer (100 ng/µl), 0.1 µl of GoTaq (5 units, Promega, Madison, Wisconsin, USA), 10.7 µl of H_2O, and 1 µl of DNA lysate (Charette and Cosson, 2004). The PCR conditions were as follows: 2 min 30 s at 94°C; 30 cycles of 30 s at 94°C, 30 s at 60°C; and 1 min 20 s at 68°C; with a 5 min final extension at 68°C (Charette and Cosson, 2004). The DNA samples were migrated on 1 or 1.5% agarose gels containing 0.5 µg/ml of ethidium bromide. The PCR analyses were performed at least twice for each set of primer pairs on each strain of *A. salmonicida*.

Virulence assay

D. discoideum amoeba strain DH1-10 was grown in a Petri dish at 21°C in HL5 liquid medium containing 15 µg/ml of tetracycline (Cornillon et al., 2000; Mercanti et al., 2006). Amoebae were harvested, centrifuged, and resuspended in HL5 without antibiotic at a concentration of 100 cells per 5 µl. *A. salmonicida* strains 01-B516 and 01-B526 as well as several strains lacking the TTSS region in pAsa5 due to plasmid rearrangement were initially grown on furunculosis agar for 3 days at 18°C and then resuspended in HL5 liquid medium. Suspensions (50 µl) of each strain were plated on HL5 agar in the wells of a 24-well plate. The dried bacterial lawns were spotted with 5-µl droplets of *D. discoideum* cell suspension. The plates were incubated at 18°C for 7 days and then examined for phagocytic plaques. The plaques, which are bacteria-free zones due to

amoebae growth, occur when amoebae are spotted on lawns of non-pathogen bacteria. However, they are not observed in the presence of pathogenic bacterial strains (Froquet et al., 2007; Froquet et al., 2009).

RESULTS

Absence of virulence factors in some environmental strains

The presence of the A-layer protein as well as the proteolytic and lipolytic activities of various environmental *A. salmonicida* strains obtained from veterinary microbiology services and reference centers was monitored to determine the effect of stressful growth conditions applied to these strains. As shown in Table 3, the majority of the strains presented the three tested elements. However, the HER strains which were the ones who were the most exposed to stressful growth conditions frequently do not possess the A-layer protein (6/7) nor the protease activity (3/7). The A449 strain for which the genome sequence has been determined (Reith et al., 2008) did not have the proteolytic activity. Finally, all the strains displayed lipolytic activity in our test. These results suggest a relatively high stability of the tested virulence factors to the growth in stressful conditions except in the case of HER strains which were repeatedly grown at 25°C over the years.

Rearrangement of the pAsa5 plasmid

Since it was reported that the pAsa5 plasmid might be affected by inappropriate growth conditions (e.g. cultivation at 25°C) (Stuber et al., 2003), the *A. salmonicida* strains were also assayed for the presence of fourteen genes covering the entire length of pAsa5. We observed seven different pAsa5 profiles (Figure 1). The plasmidic genes were present in all twenty *A. salmonicida* strains tested, except for HER1107 (Profile # 7, Figure 1), suggesting that this strain likely lost pAsa5 over the years. Of the other strains tested, 42% (8/19) had lost one or more of the fourteen pAsa5 genes (Profiles 2 to 6, Figure 1). Interestingly, the HER strains which are those that were the most exposed to stressful growth conditions are the ones presenting the more frequent pAsa5 alterations (6/7 compared to 3/13 for the other strains) (Table 3). These strains also displayed the most important alterations of the plasmid (Figure 1). These data suggest instability of pAsa5 to stressful growth conditions. However, they also indicate that this instability is not frequent except when the bacteria were repeatedly grown at 25°C over the years.

The plasmid gene loss profiles showed that the TTSS region was almost always affected when pAsa5 alterations are observed. While only the TTSS region was missing in strains 07-5957 and 07-7287 (Profile #3, Figure 1), other regions of pAsa5 were also affected, as shown by the profiles of the other strains, some of which had the same gene loss profile outside the TTSS region. For example, HER1084, HER1098, and HER1104 had all lost the same genes in addition to the six genes for the TTSS region (Profile # 4, Figure 1). These results confirmed that pAsa5 is instable (Stuber et al., 2003) and suggested that pAsa5 is altered by rearrangement mechanisms and is usually not completely lost. Moreover, the instability, mainly involved the TTSS region.

Frequency and variability of pAsa5 rearrangements

Since pAsa5 rearrangement was not documented previously, we attempted to reproduce this process by culturing A449, 01-B526, and 01-B516, all of which had intact pAsa5 plasmids in stressful conditions e.g. for two weeks at 25°C. The frequency and the nature of rearrangements in pAsa5 for these three strains were monitored by PCR analysis similar to those done on environmental strains. Four assays were performed for A449 and 01-B526, while six assays were done for 01-B516. A total of one hundred colonies were tested in the case of A449 and 01-B526, and 160 for 01-B516.

It appeared that pAsa5 instability occurred only at low frequency (Figure 2) even after a long period at 25°C. For the A449 and 01-B516 strains, rearrangements were infrequent, occurring in less than 5% of the colonies tested. Rearrangements were more common for *A. salmonicida* 01-B526, with 17% of the colonies displaying the loss of one or more pAsa5 genes. In the case of the 01-B526 strain, the majority of the colonies had lost only TTSS genes, but one strain had also lost the P5G011 gene in addition to the TTSS genes. For A449 strain, two colonies had lost genes upstream and within the TTSS region while three had only lost TTSS genes. Lastly, rearrangements in 01-B516 strain involved only the TTSS region. Interestingly, the frequency of rearrangement of pAsa5 was highly variable from trial to trial at 25°C. For example, the rearrangement frequency for 01-B526 ranged from 3 to 40%. On the other hand, when A449, 01-B526, and 01-B516 were cultivated at 18°C for two weeks in similar conditions, no loss of the TTSS was observed by PCR after analyzing 111, 106, and 144 colonies, respectively (data not shown).

These results confirmed that pAsa5 rearrangements exist and are induced by very stressful growth conditions. They also showed that rearrangements remain a relatively rare but significant

phenomenon under the conditions tested. There is some variability in the regions that are rearranged in pAsa5 but, in these experiments, the TTSS region was always affected when rearrangements occur. *A. salmonicida* A449 appeared more likely to lose other genes when a rearrangement occurred than 01-B516 and 01-B526, where the rearrangements occurred almost exclusively within the TTSS region.

Loss of virulence after pAsa5 rearrangement

Lastly, the effect of the pAsa5 rearrangement on the virulence of the bacterium was verified with an assay using the alternative host *D. discoideum* (Froquet et al., 2009). This organism makes it possible to evaluate the virulence of *A. salmonicida* as with fish cell lines, including the function of the TTSS (Dacanay et al., 2006; Froquet et al., 2007; Stuber et al., 2003). Using this approach, we confirmed that the loss of the TTSS region caused by the pAsa5 rearrangement led to a loss of virulence for both 01-B526 and 01-B516 strains (Figure 3). This is consistent with previously published results (Froquet et al., 2007; Stuber et al., 2003). The A-layer protein as well as the proteolytic and lipolytic activities was present in these rearranged strains tested with the *D. discoideum* model (Figure 3). It was not possible to test the effect of pAsa5 rearrangement on virulence in the case of the A449 strain since this strain was not virulent in the amoeba assay (data not shown) probably due to the absence of proteolytic activity (Table 3). These results showed that growth in stressful conditions leads to the attenuation of the virulence of *A. salmonicida* by specifically eliminating the region in pAsa5 that encodes the TTSS.

DISCUSSION

We demonstrated that routine cultivation of *A. salmonicida* strains at 25°C may affect the presence of virulence factors and can lead to rearrangements of the pAsa5 plasmid. These rearrangements have not been described previously and result in loss of the TTSS region and concomitant loss of virulence. We tested a number of *A. salmonicida* strains for the presence of 14 pAsa5 genes by PCR. Strains grown in the stressful conditions had a rearrangement of the pAsa5 plasmid with a low but significant frequency. Interestingly, the rearrangement of pAsa5 affected the TTSS region virtually all the time. Genes outside this region could also be lost, but this was a much less frequent occurrence. In addition, the rearrangement of pAsa5, while it was a phenomenon occurring at a low frequency, was variable, even within a same strain, both in terms of the frequency of rearrangement and the plasmid regions affected.

A previous study, which suggested that pAsa5 is completely lost when the bacterial host is grown at 25°C, targeted a single gene (*ascV*) from the TTSS region of pAsa5 (Stuber et al., 2003). As we show here, it is preferable to analyze multiple genes to determine whether the plasmid is intact, partly rearranged, or completely lost. Furthermore, the present study confirmed that it is important to work with isolated colonies and not with liquid cultures to study the thermostability of pAsa5. Of two previous studies investigating the thermostability of pAsa5, one used the isolated colony approach and reported that the *ascV* gene is lost after heating the bacteria at 25°C (Stuber et al., 2003) while the second used a liquid culture approach and reported that the *ascC* gene in the TTSS region is not lost after the heat treatment (Ebanks et al., 2006). Because of the low frequency of plasmid rearrangement, bacteria with intact pAsa5 plasmids will mask those with pAsa5 rearrangements in liquid cultures. Given that the liquid culture approach is unlikely to detect less frequent pAsa5 rearrangements, we developed the analytical method described in the present study where isolated colonies are systematically tested by PCR for various regions of

pAsa5. This may explain why pAsa5 rearrangement was not previously observed for A449 strain (Ebanks et al., 2006) while it was the case in this study.

In our hands, pAsa5 rearrangement is a sporadic phenomenon even for strains artificially grown in very stressful conditions. Stuber *et al.* reported that only 6 h at 25°C are required to force the loss of the *ascV* gene in all the bacterial cells tested at this temperature for the JF2267 strain (Stuber et al., 2003). Therefore, it appears that individual strains react differently to the incubation at 25°C. This was also shown in the present study by the difference in the rearrangement frequency of strains 01-B516 and 01-B526. It is possible that the complete plasmid instability seen for the JF2267 strain used by Stuber *et al* was idiosyncratic to the strain employed by those researchers. However, we used a different growth medium and formulation compared to Stuber *et al.* to grow *A. salmonicida* (Furunculosis agar versus Luria-Bertani broth) and one can not exclude the possibility that these parameters may also affect the stability of pAsa5. It will be interesting to determine the conditions that best promote the rearrangement of pAsa5.

Our findings demonstrate the importance of culturing the various strains of *A. salmonicida* at temperatures under 20°C to preserve the expression of virulence factors and of plasmid genes such as those coding for the TTSS. This is best illustrated by the alteration in the pAsa5 plasmid of three strains recently isolated from fish in the wild (07-9324, 07-5957 and 07-7287) that were likely induced by growing them in unfavorable conditions only one or two times after they were isolated. The situation is even worse when strains are routinely grown at 25°C as was the case for the HER strains. This indicates that not growing *A. salmonicida* at optimal conditions (e.g. <20°C) may lead to plasmid rearrangements and other alterations such as the loss of the A-layer

protein or secreted proteolytic activity. Moreover, the profiles observed for the HER strains are more complex and it is possible that they are the result of many different rearrangements. Therefore, even if these alterations are not so frequent, veterinary microbiology services and reference centers should pay attention to the way they isolate and grow *A. salmonicida* to preserve their complete set of virulence factors. This is essential for research on the virulence of this bacterium.

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FIGURE LEGENDS

Figure 1. Rearrangement profile of the pAsa5 plasmid in various *A. salmonicida* strains. For simplicity sake, the pAsa5 plasmid is shown as a linear representation. Black rectangles indicate positive PCR signals for the corresponding genes while white rectangles indicate negative signals. PCR analyses were performed at least twice for each target gene in every strain tested. Seven profiles were obtained. Many strains had the same pAsa5 rearrangement profile. The strains had the following profiles: A449, 01-B516, 01-B522, 01-B526, 07-7817, 07-7346, 08-2647, 08-2783, 08-4188, 09-0167, and HER1085 (Profile 1); 07-9324 (Profile 2); 07-5957 and 07-7287 (Profile 3); HER1084, HER1098, and HER1104 (Profile 4); HER1110 (Profile 5); HER1108 (Profile 6); and HER1107 (Profile 7).

Figure 2. pAsa5 rearrangements of *A. salmonicida* strains grown at 25°C. Strains A449 and 01-B526 (four experiments), and 01-B516 (six experiments) were grown at 25°C, and isolated colonies (n) were analyzed by PCR as described in Figure 1 to determine the presence or absence of the fourteen target sequences of the pAsa5 plasmid. Black rectangles indicate a positive PCR signal while white rectangles indicate a negative signal. The genes are in the same order as in Figure 1. The line above the central rectangles indicates the position of the TTSS region. The italic numbers to the left of the plasmid representations indicate the number of times the profiles were observed. The rearrangement percentages for each experiment are indicated.

Figure 3. The pAsa5 rearrangement resulted in a loss of virulence. Bacterial lawns of *A. salmonicida* parental strains 01-B516 and 01-B526, as well as several strains derived from these parental strains that had lost the TTSS region of pAsa5 due to a plasmid rearrangement (ΔTTSS), were spotted with 5-µl droplets containing 100 *D. discoideum* cells. The plates were examined for phagocytic plaques after a 7-day incubation at 18°C. Phagocytic plaques were only observed with strains lacking the TTSS region, suggesting that they were non-virulent. The presence of the A-layer protein as well as secreted proteases and lipases in each strain was assessed (see Material and Methods section). The HER1098 strain, which lacks the TTSS region on pAsa5 (Figure 1) and the A-layer protein was used as an avirulent control in this assay. This experiment was performed three times and similar results were obtained each time.

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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)

159 **Table 2. Primer sequences used to detect genes of the *A. salmonicida* pAsa5 plasmid**

Target	Sequence (5'-3') Fw/Rev	PCR products (pb)	References
P5G011	TTGTCTGACTCTGCATCCAGCGAA	295	This study
	AGGTGCCTGAATTACCACCAGTGA		
traE	ATGGTCGCTATCGTCATTGTCGCA	338	This study
	ACCTCGTTGCGCTTTATTTGCTGG		
traC	TGCACTATCCCCAGCTATCC	424	This study
	TCGGTAATCGCGGTCTTGTC		
traD	ATTCATGACCAATCCGCCGACCTA	408	This study
	TCGAGAAATTTGCGCATGAGCGTC		
ati2	TTGACCTGTGGTCAGGTTAGCAGT	550	This study
	ACACGATGATACGCACCTAGCCAA		
ascC	GCATTGGAGCAACAGTCCCA	476	(Ebanks et al., 2006)
	CCTTCAATCCCCTTGCGAT		
exsD	AGAAGTGATCCTGACCCAAGGCAA	631	This study
	TTGCAACGACTGTTGCCAAGAACC		
acrV	GTAAAGGGTTGCGGGATGAG	416	(Ebanks et al., 2006)
	GCCGCTCTTCTTCAGGTCAC		
aopN	GCATCAGCGAGATCGAGG	430	(Ebanks et al., 2006)
	CGGCTGTGAATATCGC		
ascU	GCTGGTCATCTACATCAAGC	722	This study
	TAGTGTTCGAAGGCGTAGTC		
aopO	TCCACAACGACATCAAACCGGGTA	681	This study
	TGATCTGACCCTGATCGGCAAACT		
resD	TCAGAAACTTGGCCATCGCTCACA	504	This study
	TGATGTGCAGATTTCCCTGGAGCA		
traM	AAGGTAATCGGAAGCACCTGCCAT	331	This study
	TTCTCGACACCAACGTGTTGCTCA		
tniR	TGACACAGATCACCGACCTTGGTT	261	This study
	TGGCATTGGCCTCAAAGTGTTGAC		

160

161

Table 3. Presence of virulence factors in environmental *A. salmonicida* strains

Strain	A-layer[a]	Proteolytic activity[a]	Lipolytic activity[a]	pAsa5 integrity[b]
A449	P	A	P	Y
HER1084	A	A	P	N
HER1085	A	P	P	Y
HER1098	A	P	P	N
HER1104	A	P	P	N
HER1107	P	P	P	N
HER1108	A	A	P	N
HER1110	A	A	P	N
07-5957	P	P	P	N
07-7287	P	P	P	N
07-7346	P	P	P	Y
07-7817	P	P	P	Y
07-9324	P	P	P	N
08-2647	P	P	P	Y
08-2783	P	P	P	Y
08-4188	P	P	P	Y
09-0167	P	P	P	Y
01-B516	P	P	P	Y
01-B522	P	P	P	Y
01-B526	P	P	P	Y

a. P = presence, A = absence

b. Y = yes, N = No

Figure 1

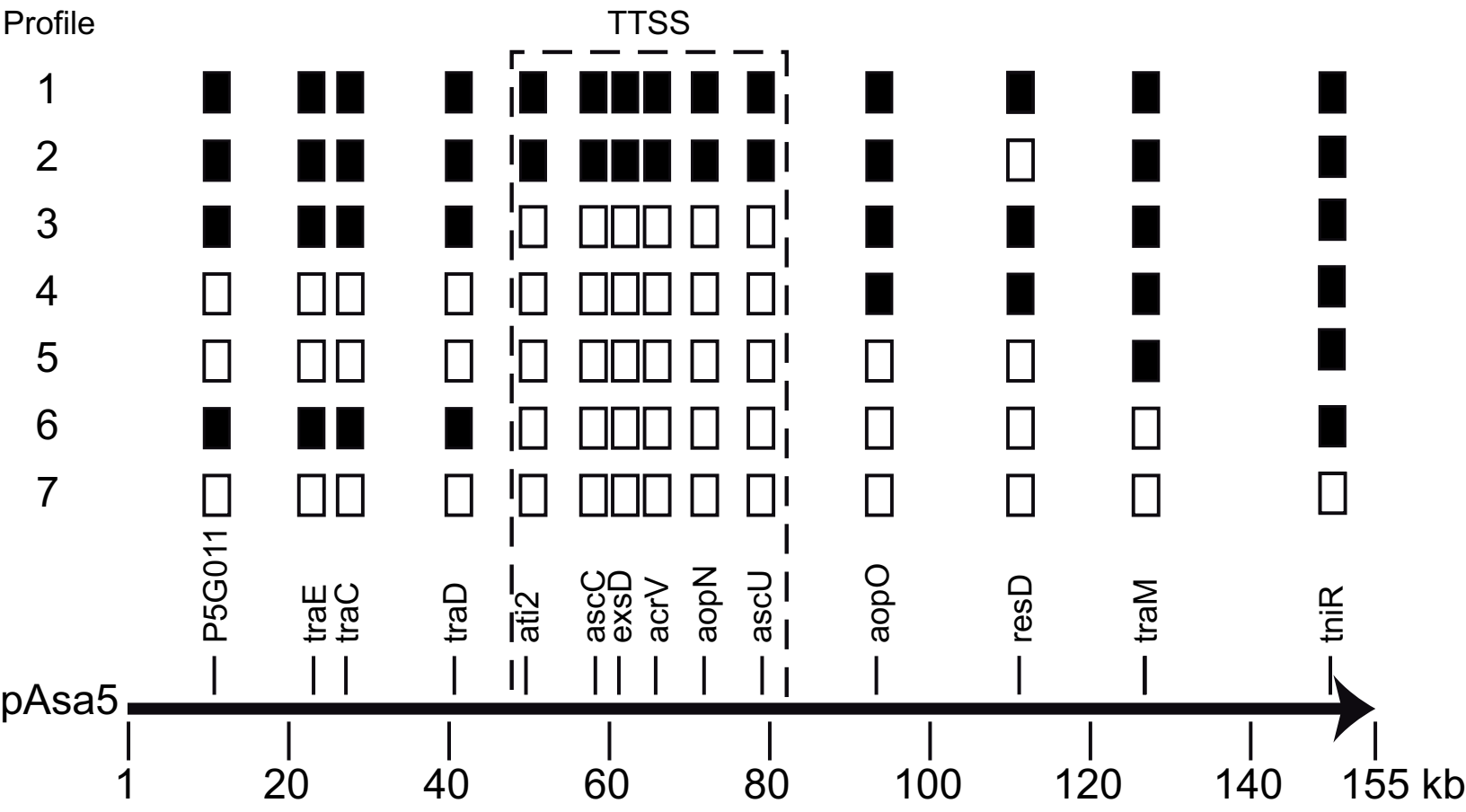
Profile
TTSS
1
2
3
4
5
6
7
P5G011
traE
traC
traD
ati2
ascC
exsD
acrV
aopN
ascU
aopO
resD
traM
tniR
pAsa5
1
20
40
60
80
100
120
140
155 kb

Figure 2

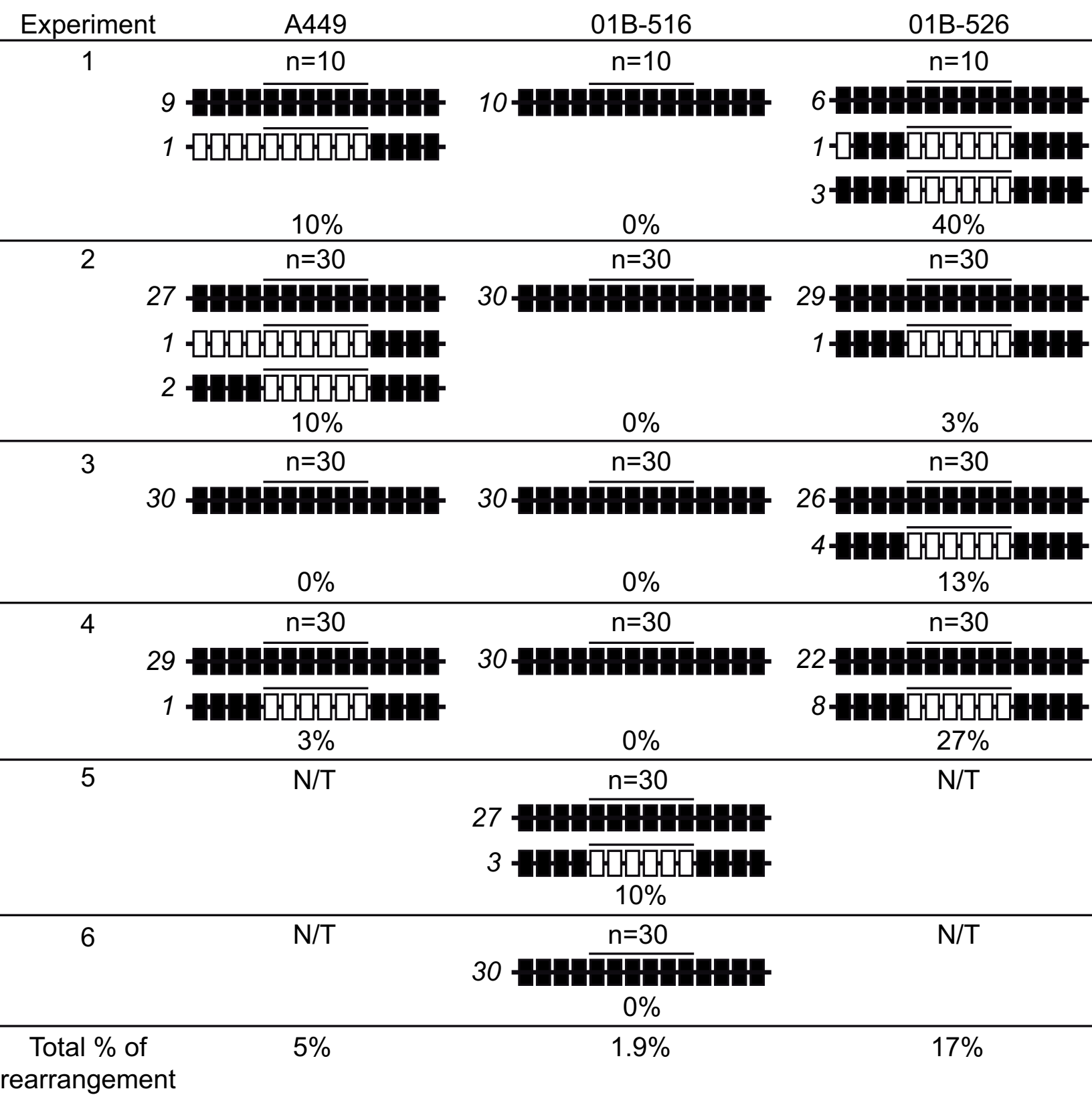
Experiment
A449
01B-516
01B-526
1
n=10
9
1
10%
n=10
10
0%
n=10
6
1
3
40%
2
n=30
27
1
2
10%
n=30
30
0%
n=30
29
1
3%
3
n=30
30
0%
n=30
30
0%
n=30
26
4
13%
4
n=30
29
1
3%
n=30
30
0%
n=30
22
8
27%
5
N/T
n=30
27
3
10%
N/T
6
N/T
n=30
30
0%
N/T
Total % of rearrangement
5%
1.9%
17%

Figure 3

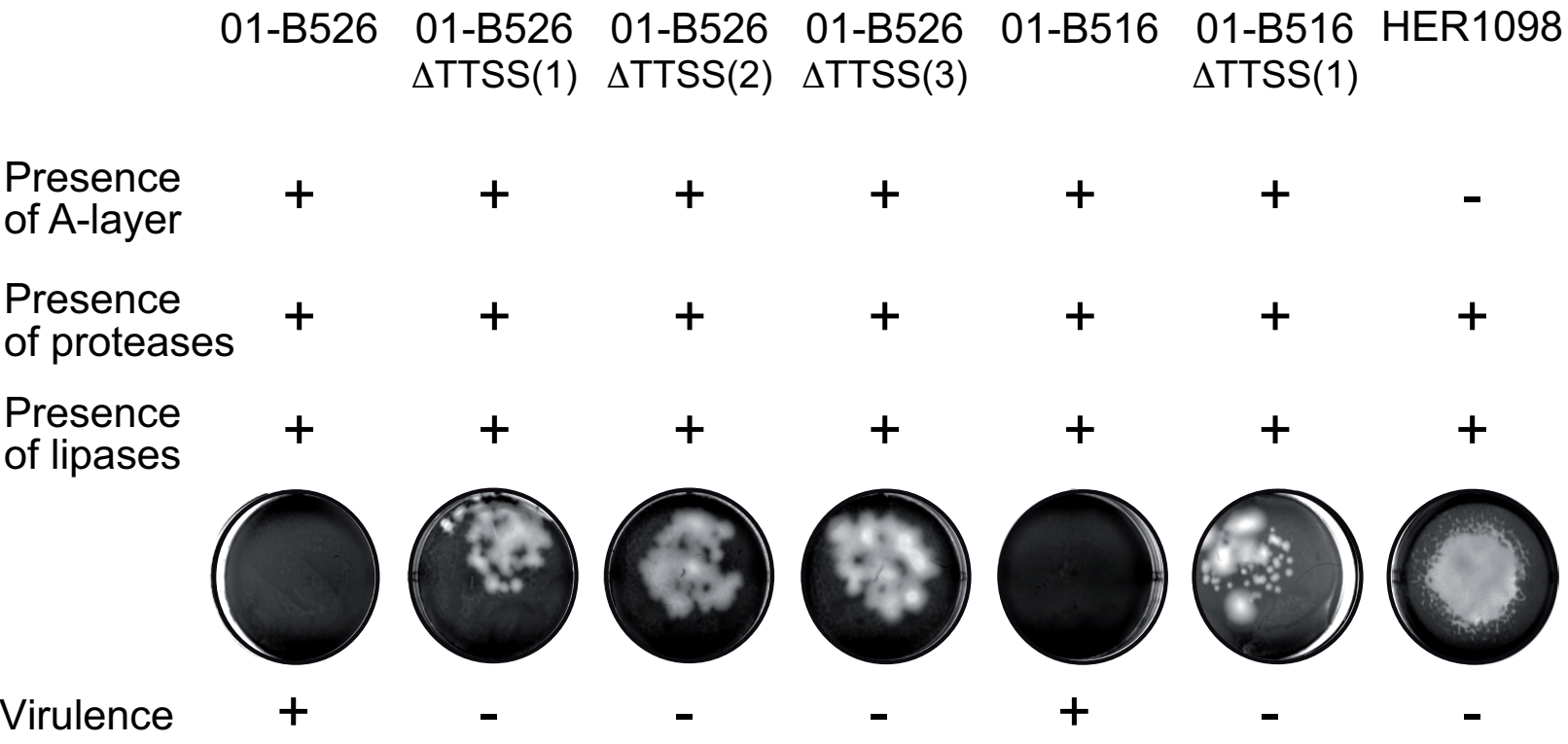

Figure 3