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# Virulence factors of *Aeromonas salmonicida* subsp. *salmonicida* strains associated with infections in turbot *Psetta maxima*

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ABSTRACT: Virulence factors for *Aeromonas salmonicida* subsp. *salmonicida* (ASS) strains isolated from cultured turbot *Psetta maxima* L. are unknown with regard to this host. The presence of virulence genes associated with different stages of ASS infection in salmonids (*vapA, tapA, fla, ascV*, *ascC, aexT, satA* and *aspA*) was analysed using a polymerase chain reaction (PCR) technique in ASS strains isolated from turbot. Other ASS strains isolated from salmonids and environmental *A. salmonicida* (AS) strains were included for comparison. The presence of the genes was evaluated with respect to ASS virulence in turbot based on intraperitoneal and bath challenges. The genetic profile, including all of the genes studied, that was linked to virulent behaviour after intraperitoneal challenge was significantly more frequent in strains isolated from turbot than in those from salmonids or the environment. The data prove that it is not possible to predict the virulence of ASS in turbot based only on the presence of all genes tested. Moreover, the combined PCR results of *vapA*, *aexT*, *ascV* and *ascC* were useful for separating most of the ASS from environmental *A. salmonicida* strains. An association between virulence or genetic profile and the geographical or facility origin of the strains was not found.

KEY WORDS: *Aeromonas salmonicida* · Virulence · PCR · Turbot · Intraperitoneal challenge · Bath challenge



## INTRODUCTION

*Aeromonas salmonicida* subsp. *salmonicida* (ASS) is a pathogen mainly associated with salmonid aquaculture (reviewed by Austin & Austin 2007). Most previous ASS studies have focused on the identification of virulence factors in the strain A449 (Nash et al. 2006, Reith et al. 2008) and their association with virulence in salmonids (reviewed by Austin & Austin 2007, Boyd et al. 2008). Genes typically associated with different stages of ASS infection are *vapA, tapA, ascV*, *ascC, aexT, satA* and *aspA*. The *flaA* and *flaB* genes, which encode 2 subunits of the polar flagellum, have been associated with invasion and colonisation of motile *Aeromonas* on fish (Rabaan et al. 2001), but their role is unknown in ASS. The ability of ASS to cause infection in another economically important farmed fish, turbot *Psetta maxima*, has been shown (Nougayrede et al. 1990, Pedersen & Larsen 1996, Pérez et al. 1996, Toranzo et al. 2005, Najimi et al. 2008). Recently, we tracked virulent and avirulent strains (included in the present study) of ASS during infection of turbot, providing important information on the persistence of the pathogen in this host (Farto et al. 2011). Comparison of the genotypes of both salmonid and turbot ASS strains has shown variability (Nash et al. 2006). Thus, additional studies are needed to investigate virulence factors of ASS isolated from diseased turbot and provide a broader understanding of the virulence of ASS in this host.

Our study was focused on 14 ASS strains isolated from diseased cultured turbot (11 strains) and from

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undiseased turbot sampled during routine control testing of health hazards in aquatic animals (3 strains). Presence of the aforementioned 8 virulence genes was investigated to identify any difference among turbot strains and other salmonid and environmental strains that were included for comparative purposes. The present study will provide useful information on previously determined virulence factors of ASS in strains isolated from turbot.

## MATERIALS AND METHODS

### Bacterial strains

We used 14 ASS strains that were previously isolated from several turbot culture facilities in NW Spain and Portugal between 2002 and 2006. Reference strains provided by private and public collections were used for comparative purposes (Table 1). These strains were ASS isolated from salmonids and *Aeromonas salmonicida* (AS) isolated from other sources, which were grouped in this study under the term environmental. All the environmental AS strains, previously identified by Figueras et al. (2000), were provided by the Spanish Type Culture Collection (CECT, www.cect.org/english). All *Aeromonas* strains were grown on trypticase soy agar (TSA; Cultimed) and incubated at 18°C for 72 h. Positive identification of the ASS strains was confirmed in the present study using the pair of specific primers (MIY) and the polymerase chain reaction (PCR)-based assay previously developed to identify ASS strains (Byers et al. 2002). The PCR reactions were performed in triplicate with a colony from each isolate as a template. A phenotypic confirmation following the classic oxidase test, oxidative or fermentative glucose metabolism, motility and brown diffusible pigment production (detected in TSA) was also conducted for all tested strains.

### Detection of virulence genes using PCR

The presence of *vapA*, *tapA, fla, aexT*, *ascV, ascC, satA* and *aspA* was determined using PCR as described previously (Gustafson et al. 1992, Braun et al. 2002, Soler et al. 2002, Madetoja et al. 2003, Sen & Rodgers 2004, Burr et al. 2005, Ebanks et al. 2006, Nilsson et al. 2006). PCR reactions were performed in duplicate, using a colony from each isolate as a template. In addition, exponential phase cultures were denatured (94°C, 20 min) and used as a template in PCR reactions. ASS type strain CECT 894 was used as a positive control for each gene, apart from *ascV* and *ascC*, which did not amplify, as also reported by Burr & Frey (2009). The strain ACRp 43.1 was used as a positive control for *ascV* and *ascC*. Two *Aeromonas hydrophila* strains (CECT 398 and ATCC 7966) and a strain of each species of *A. sobria* (ATCC 9071), *Vibrio anguillarum* (CECT 522), *V. parahaemolyticus* (CECT 5306) and *Photobacterium damselae* subsp. *damselae* (CECT 626) were used as negative controls.

### Virulence assays

Healthy unvaccinated turbot (30 g ± 0.2 standard error [SE]) were used for all challenges. The fish were the offspring of turbot reared in an experimental fish farm, University of Vigo (ECIMAT, Marine Sciences Station of Toralla), with no history of disease. Prior to the challenge, 20 fish from stock were screened for bacterial pathogens according to routine diagnostic procedures. A liver sample from each fish was removed aseptically and weighed (200 mg). The samples from 5 fish were pooled, homogenised in 500 µl of phosphate buffered saline (PBS) and sampled for bacteria by streaking on TSA plates. Fish for each bacterial strain and experiment (intraperitoneal or bath challenges) were held for 2 wk in separate experimental tanks containing 20 l of sterile, 18°C seawater with a salinity of 33‰. Following the challenges, all fish were kept in tanks with non-circulating seawater. Both intraperitoneal and bath challenges were first tested at a dose of $\geq 10^7$ colony-forming units (cfu) per fish or cfu $ml^{-1}$, respectively, and in the event of detecting mortality, a one order of magnitude lower dose was tested for each strain. This sequence was repeated to determine the minimal lethal dose and defined as the minimal dose of bacterial suspension that causes any mortality of fish. Virulent strains were defined as those showing any mortality after intraperitoneal or bath challenges at doses $\leq 10^6$ cfu per fish or cfu $ml^{-1}$, respectively. All animal experiments were performed according to practices approved by the Spanish Ethical Committee (Royal Decree 1201/2005, 10 October, on the protection of animals for experimentation and other scientific purposes; BOE 21 October 2005, pp. 34367–34391; www.boe.es/diario_boe/index.php?id=en).

#### Intraperitoneal challenge

A preliminary intraperitoneal test of strains was selected due to the usually faster development of

Table 1. *Aeromonas salmonicida* subsp. *salmonicida* and environmental *A. salmonicida* strains. Source, genetic profile based on PCR (Table 2) and virulence. Donors: USC: Departamento de Microbiología y Parasitología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain; CETGA: Centro Tecnológico Gallego de Acuicultura, Riveira, Spain; CECT: Spanish Type Culture Collection, Valencia, Spain; LMG: Belgian Co-ordinated Collections of Micro-Organisms, Brussels, Belgium. Outbreak: strains isolated from disease outbreaks; RCT: strains isolated from undiseased fish sampled during routine control testing of health hazards in aquatic animals; IP: intraperitoneal injection; *type strain; nd: no data; nt: not tested

| Strain | Donor | Source (Area, Facility, Year etc.) | Genetic profile | Minimum lethal dose (% mortality) IP challenge (cfu fish$^{-1}$) | Bath challenge (cfu ml$^{-1}$) |
|---|---|---|---|---|---|
| ***Aeromonas salmonicida* subsp. *salmonicida*** | | | | | |
| Turbot outbreak | | | | | |
| ACRp 43.1 | USC | Galicia. A. 2002 | T-I | $1.6 \times 10^6$ (100) | $1.3 \times 10^8$ (11) |
| ACR 173.1 | USC | Galicia. A. 2003 | T-I | $3.0 \times 10^5$ (66) | $6.6 \times 10^7$ (44) |
| RM 266.1 | USC | Galicia. C. 2003 | T-I | $1.9 \times 10^6$ (100) | $1.6 \times 10^6$ (66) |
| RM 274.1 | USC | Galicia. C. 2004 | T-I | $3.5 \times 10^6$ (100) | $9.6 \times 10^7$ (100) |
| RO 11.1 | USC | Galicia. D. 2003 | T-I | $2.0 \times 10^6$ (33) | nt |
| RI 161.2 | USC | Galicia. E. 2003 | T-I | $2.1 \times 10^6$ (100) | nt |
| RIM 37.1 | USC | Galicia. G. 2004 | T-I | $3.9 \times 10^7$ (0) | nt |
| RIM 47.1 | USC | Galicia. G. 2005 | T-I | $3.5 \times 10^8$ (100) | nt |
| RIM 33.1 | USC | Galicia. G. 2004 | T-I | $2.2 \times 10^4$ (100) | $4.2 \times 10^6$ (100) |
| PC 884.1 | USC | Galicia. L. 2004 | T-I | $1.5 \times 10^8$ (100) | nt |
| RSP 74.1 | USC | Portugal. N. 2002 | T-I | $4.6 \times 10^8$ (66) | $1.7 \times 10^8$ (0) |
| Turbot RCT | | | | | |
| SF 3.1/02 | USC | Galicia. B. 2002 | T-I | $4.1 \times 10^6$ (100) | nt |
| 1.44 | CETGA | Galicia. H. nd | T-I | $3.5 \times 10^6$ (100) | $2.0 \times 10^8$ (100) |
| RSP 83.1 | USC | Portugal. N. 2004 | T-I | $3.2 \times 10^7$ (0) | nt |
| Salmonids RCT | | | | | |
| EO 0303 | USC | Galicia. F. 2004 | T-I | $2.9 \times 10^6$ (66) | nt |
| X32r | USC | Galicia. K. 2006 | T-I | nt | nt |
| 11 | USC | Galicia. F. 2004 | T-II | $4.7 \times 10^7$ (0) | nt |
| EO 2103 | USC | Galicia. F. 2004 | T-II | nt | nt |
| EO 1603B | USC | Galicia. I. 2004 | T-II | nt | nt |
| EO 1603R | USC | Galicia. I. 2004 | T-II | nt | nt |
| EO 02.05 | USC | Galicia. J. 2005 | T-II | $6.4 \times 10^8$ (100) | nt |
| Salmonids outbreak | | | | | |
| CECT 894* | CECT | Dead salmon | T-II | $3.0 \times 10^8$ (0) | nt |
| CECT 4237 | CECT | UK. Trout | T-II | $6.0 \times 10^8$ (0) | nt |
| 08/01/03 | USC | Galicia. M. 2003 | T-II | $1.5 \times 10^7$ (0) | nt |
| LMG 3776 | LMG | UK. Diseased salmon | T-III | $5.6 \times 10^8$ (100) | nt |
| LMG 3778 | LMG | Diseased salmon | T-III | $5.3 \times 10^8$ (0) | nt |
| LMG 3780 | LMG | UK. Dead salmon | T-III | $4.0 \times 10^8$ (0) | nt |
| NF-19-1 | USC | Spain. Trout. nd | T-III | $3.1 \times 10^8$ (0) | nt |
| SCO 4.1 | USC | Spain. Trout. nd | T-III | $4.6 \times 10^6$ (0)[a] | nt |
| CECT 4235 | CECT | Diseased trout | T-IV | $3.3 \times 10^8$ (0) | nt |
| **Environmental *Aeromonas salmonicida*** | | | | | |
| CECT 5234 | CECT | Spain. Seawater | E-I | $3.9 \times 10^8$ (100) | nt |
| CECT 5220 | CECT | Spain. Seawater | E-II | nt | nt |
| CECT 5229 | CECT | Spain. Well water | E-II | nt | nt |
| CECT 5230 | CECT | Spain. Well water | E-II | nt | nt |
| CECT 5231 | CECT | Spain. Pipe water | E-II | $1.1 \times 10^6$ (0)[a] | nt |
| CECT 5232 | CECT | Spain. Spring water | E-II | nt | nt |
| CECT 5227 | CECT | Spain. Mussel | E-II | $3.6 \times 10^6$ (0)[a] | nt |
| CECT 5238 | CECT | Spain. Shaft water | E-III | $3.5 \times 10^8$ (100) | nt |
| CECT 5218 | CECT | Spain. Cake | E-IV | nt | nt |
| CECT 5221 | CECT | Spain. Cake | E-IV | $2.8 \times 10^8$ (66) | nt |
| CECT 5225 | CECT | Spain. Mussel | E-IV | $3.0 \times 10^6$ (0)[a] | nt |
| CECT 5249 | CECT | Spain. River water | E-V | nt | nt |

[a]Only a dose of 106 cfu fish$^{-1}$ was tested

Table 2. *Aeromonas salmonicida* strain profiles, based on possession of various genes. T-I isolated from turbot and salmonids, and T-II, T-III and T-IV isolated from salmonids (see Table 1). GP = genetic profile

| GP | Gene | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *vapA*[a] | *tapA* | *fla*[a] | *aexT*[a] | *ascV*[a,b] | *ascC*[a,b] | *satA* | *aspA* |
| **Isolated from fish** | | | | | | | | |
| T-I[b] | + | + | + | + | + | + | + | + |
| T-II | + | + | + | + | − | − | + | + |
| T-III | + | + | + | + | + | − | + | + |
| T-IV | − | + | + | + | − | − | + | + |
| **Isolated from environmental samples** | | | | | | | | |
| E-I | − | + | + | + | + | + | + | + |
| E-II | − | + | + | − | − | − | + | + |
| E-III | − | − | + | − | + | − | + | + |
| E-IV | − | + | − | − | − | − | + | + |
| E-V | − | + | + | − | − | − | + | − |

[a]Significantly higher presence of *vapA, aexT* ($p < 0.0001$), *fla* ($p = 0.0192$), *ascV* ($p = 0.0024$) and *ascC* ($p = 0.0129$) in strains derived from fish than in environmental strains

[b]Significantly higher presence of T-I profile, *ascV* and *ascC* ($p < 0.0001$) in turbot strains than in salmonid or environmental strains

disease, and associated mortality using this method compared with that of the bath challenge. Furthermore, this method was useful for reducing the number of fish tested by bath challenge, because of the low probability of finding a virulent behaviour after bath challenge in strains identified as avirulent based on intraperitoneal challenge. We used the 14 ASS strains isolated from turbot. Further representative ASS (12) and environmental AS (6) strains were randomly selected from each genetic profile and tested for comparison (Table 1). All strains were grown in shaking TSB (18°C, 24 h), and cells were harvested and resuspended in PBS to an optical density ($OD_{590}$) of 0.5 ($10^9$ cfu $ml^{-1}$). Serial dilutions in PBS were prepared to obtain the cell concentration required, and abundances (cfu $ml^{-1}$) were determined on TSA plates. The bacterial suspension (0.1 ml) was inoculated in turbot (n = 9) by injection into the abdominal cavity, with doses of $10^3$ to $10^8$ cfu per fish, and monitored for 14 d. The surviving fishes were euthanised by an overdose of tricaine methanesulfonate (MS-222; Sigma). From all moribund, dead and euthanised turbots, the liver and kidney tissues were sampled for bacteria by streaking on TSA plates, and identification of suspicious re-isolated colonies was carried out as above. Fish intraperitoneally injected (0.1 ml) with PBS were used as negative controls and sampled as described above.

### Bath challenge

Based on intraperitoneal challenge results, 6 virulent and 1 avirulent ASS strains isolated from turbot were chosen to perform bath challenges (Table 1). The bacterial inoculum of the strains was prepared by growth in shaking TSB (300 to 600 ml; 18°C, 24 to 48 h) then added for 1 h to each tank, containing 9 fish, with non-circulating seawater to a final concentration of $10^5$ to $10^8$ cfu $ml^{-1}$ (adjusted to an $OD_{590}$ of 0.3 to 0.4). The starting bacterial numbers were confirmed by determining cfu $ml^{-1}$ of each tank at Time 0. Each fish group was monitored for 20 d and sampled as described above. Fish exposed to PBS and maintained under the same conditions were used as negative controls.

### Statistical analysis

Statistical differences in the occurrence of a given gene, the genetic profile T-I or virulence by intraperitoneal challenge among ASS isolated from turbot, salmonids and environmental AS were analysed. Strains from each of those groups were also compared to identify statistical differences when the occurrence of the genetic profile T-I and virulence (based on intraperitoneal challenge) were analysed together. Differences between the strains isolated from disease outbreaks and strains isolated from undiseased fish sampled during routine control testing of health hazards in aquatic animals were also evaluated. All of the results were analysed by applying Fisher exact tests using Yate's continuity correction (significant differences $p < 0.05$). All tests were calculated using GraphPad Instat® version 3.0. (GraphPad Software).

## RESULTS

### Confirmation of *Aeromonas salmonicida* subsp. *salmonicida*

All ASS strains isolated from turbot and salmonids and the positive control, ASS type strain CECT 894, were correctly identified by ASS-specific PCR. None of the strains used as negative controls, 2 *Aeromonas hydrophila* strains (CECT 398 and ATCC 7966) and an *A. sobria* strain (ATCC 9071), were amplified. Nor was any environmental AS strain, as expected. The phenotypic assays indicated that all of the ASS were positive for all tests except motility. Conversely, the

environmental AS strains showed a positive result for motility and were unable to produce pigment, as previously described (Béaz-Hidalgo et al. 2010).

### Detection of virulence genes using PCR

A homogeneous distribution of *tapA, satA* and *aspA* was found among ASS and environmental AS strains. The *fla* and *aexT* genes were detected in 100% of ASS strains (from turbot and salmon) and *vapA*, *ascV* and *ascC* were found in 97%, 70% and 53%, respectively. However, in environmental AS strains, the detection was lower, showing significant ($p < 0.02$; Table 2) differences when compared to ASS strains (*fla* 75%, *ascV* 17%, *aexT* and *ascC* 8% and *vapA* 0%). We found 9 genetic profiles, which showed an apparent absence of association with the geographical areas from which the *Aeromonas* strains originated. Each profile was given an arbitrary number (Table 2). All of the strains isolated from turbot were included in profile T-I, which was the only profile with ASS strains isolated from turbot and salmonids. This genetic profile comprised the 8 virulence genes tested, and its presence was significantly ($p < 0.0001$, Table 2) more frequent in turbot (100%) than in salmonid or environmental strains. The remaining genetic profiles obtained lacked one or more of the virulence genes tested and were found in strains isolated from salmonids or the environment. In addition, the strains isolated from turbot formed a homogeneous group. The group included strains isolated from disease outbreaks from the same facility in different years, strains isolated from undiseased fish sampled during routine control testing and strains isolated from different geographical areas. However, the strains isolated from salmonids showed more variability, mostly due to the absence of the *ascV* and/or *ascC* genes. Variability in salmonid ASS did not appear to be linked to the origin of the isolate nor associated with isolation during disease outbreaks versus during routine control testing (Table 1).

### Virulence assays

#### Intraperitoneal challenge

We inoculated 32 strains of *Aeromonas*, and 10 were virulent for turbot at doses ranging from $10^4$ to $10^6$ cfu per fish. The ASS strains originally isolated from turbot were significantly more virulent in turbot than those isolated from salmonids ($p = 0.0097$) or the environment ($p = 0.00108$). The virulent strains were 7 strains of 11 isolated from disease outbreaks of turbot as well as 2 of 3 strains isolated from routine control testing of turbot. The strain EO 0303 was also isolated from routine control testing of salmon and was virulent for turbot. All displayed the genetic profile T-I, which was significantly more prevalent in turbot strains than in those isolated from salmonids ($p = 0.0016$) or from fish than from the environment ($p = 0.0006$). The other strains (5 ASS from turbot, 11 ASS from salmonids and 6 environmental AS) were avirulent under the current experimental conditions because no mortality or mortality only at doses $\geq 10^7$ cfu per fish was detected (Table 1). Interestingly, all of the virulent strains were isolated from Galicia; however, other strains from the same area were avirulent for turbot. There was no association between the facility from which strains were isolated and virulence (Table 1). All of the virulent ASS strains could be recovered in pure culture from all of the fish (a positive identification by PCR with 100% reproducibility was obtained). Bacteria were never recovered using a culture from the liver of euthanised fish infected with avirulent ASS or environmental AS strains or from uninfected control fish. The lethal effect was detected ≤5 d after inoculation. Clinical signs included anorexia, abnormal swimming behaviour, haemorrhagic lesions on the liver and intestine or pale liver and ascetic fluid in a few individuals.

#### Bath challenge

The ability of 7 ASS strains to cause disease in turbot was also tested by bath challenge (Table 1). The strains RM 266.1 and RIM 33.1 were virulent at doses of $10^6$ cfu $ml^{-1}$. Both could be recovered in pure culture from all fish, and their identification was confirmed by PCR, whereas the remaining 5 ASS strains tested were avirulent. Bacteria were never recovered from cultures of the liver of euthanised fish infected with avirulent ASS or the uninfected control group. The lethal effect was detected between 6 and 7 d after inoculation. The clinical signs observed in the diseased turbot were the same as those described above.

## DISCUSSION

Comparisons between colonised turbot tissues by virulent and avirulent ASS strains during an infection

have shown significant differences in how these strain types persisted in this host (Farto et al. 2011). For the first time, ASS isolated from cultured diseased turbot was investigated to detect the presence of 8 genes (most of them typically associated with different stages of infection in salmonids) and their association with virulence in the turbot assessed. The presence of several of these genes had previously been studied in ASS strains isolated from salmonids (reviewed by Austin & Austin 2007, Boyd et al. 2008), but not the gene combination studied here. Interestingly, all of the strains isolated from turbot were included in the main profile (T-I) of our study, which gave a positive result for all genes tested, whereas the remaining strains (salmonid ASS and environmental AS) showed variable profiles, mostly due to the absence of the *ascV* and/or *ascC* genes, both located on pAsa5 plasmid (Reith et al. 2008). This fact suggests that these 2 genes are not always linked, as previously noted for *ascV* and *aopO* or *aopH,* which are located in the same plasmid (Burr & Frey 2007, Reith et al. 2008). Single gene or operon gene loss can be frequent in ASS (Najimi et al. 2009). The absence of amplification could also be due to the absence of sequence similarity or assay failure. In addition to *ascV* and *ascC*, the presence of *vapA* and *aexT* was significantly more frequent in ASS than in the environmental AS strains studied. This obvious difference in their distribution showed the usefulness of applying PCR for these genes in separating most of these ASS from environmental AS strains.

To determine if any specific genetic profile would be useful to predict the virulence of the ASS strains for turbot, conventional infection assays (intraperitoneal and bath challenges) using turbot were performed. Previous studies showed that in virulent ASS isolated from salmonids, the products encoded by *vapA, tapA, aexT, satA* and *aspA* appeared to be insufficient as sole virulence determinants in salmonids (Vipond et al. 1998, Masada et al. 2002, Dacanay et al. 2006, Austin & Austin 2007, Boyd et al. 2008). However, the lack of *ascV* and *ascC* was associated with the absence of mortality in salmonids after intraperitoneal challenge (Burr et al. 2005) and after both intraperitoneal and bath challenges (Dacanay et al. 2006). In the present study, both virulent and avirulent ASS strains isolated from turbot possessed all of these virulence factors. In a previous study, we found differences in the interactions of virulent and avirulent ASS strains with turbot: the avirulent strain ACRp 43.1 was cleared from turbot 4 d after a bath challenge, whereas the virulent strain RIM 33.1 persisted until death occurred in turbot (Farto et al. 2011). These 2 strains both showed a T-I gene profile. Therefore, the presence of the genes tested is insufficient to differentiate these strain types. Quantifications of the expression of the genes tested and the dynamics of their expression could contribute to clarifying if there are differences between these virulent and avirulent ASS strains.

The present data prove that all ASS strains isolated from turbot possess known virulence genes typically associated with different stages of infection in salmonids, but even in the case of a positive detection, it is not possible to predict the virulence of ASS in turbot.

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