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NOTE

# Effects of bacteria on the growth of an amoeba infecting the gills of turbot

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ABSTRACT: We analysed the influence of various bacteria on the *in vitro* growth of trophozoites of a *Platyamoeba* strain isolated from diseased gill tissues of cultured turbot. Little or no growth was shown by amoebae cultured in the presence of (1) the turbot-pathogenic bacteria *Vibrio anguillarum*, *Aeromonas salmonicida* or *Streptococcus* sp., (2) *Pasteurella piscicida* or *Vibrio vulnificus* (pathogenic for some fishes but not turbot), or (3) the non-pathogenic 'environmental' bacteria *Vibrio campbelli*, *Vibrio fluvialis* or *Pseudomonas dondorofii*. The only bacteria which were successfully utilized as food sources were *Aeromonas hydrophila* (pathogenic for some fishes but not turbot) and the non-pathogens *Vibrio natriegens*, *Pseudomonas nautica* and *Escherichia coli*. These results suggest that the colonization of the gills of cultured turbot by the epizoic amoeba *Platyamoeba* may be an indicator of faecal contamination.

KEY WORDS: *Platyamoeba* · Marine bacteria · Turbot



The majority of amoebae infecting marine fish are free-living forms that, under certain conditions, can become parasites (Lom & Dyková 1992); such species have been designated 'amphizoic amoebae' (Page 1974). In cultured turbot, infections by amoebae of this type may cause major mortalities (Dyková et al. 1995, 1998, 1999, Leiro et al. 1998). Like other free-living protozoans, the amoebae are basically bacteriophagous, and indeed they may have decisive effects on bacterial density in aquatic systems (Sawyer & Bodammer 1983, Güde 1986, Verity 1986, Nagata 1988, Iriberri et al. 1995). These effects of amoebae on bacterial density are of interest because pathogenic bacteria are a frequent cause of severe outbreaks of gill disease in fish cultivation (Munday et al. 1990). Additionally, in some cases gill disease may be caused by amoebae, whether alone or in mixed infections with bacteria (Bullock et al. 1994). Finally, free-living amoebae are increasingly recognized to serve as vehicles of dispersal for various bacterial pathogens and as hosts for a variety of obligate bacterial endocytobionts (Horn et al. 2000).

In this work, we analysed the influence of several fish pathogenic and non-pathogenic 'environmental' bacteria on the growth *in vitro* of trophozoites of a *Platyamoeba* species associated with for an amoebic gill disease (AGD) of cultured turbot (Leiro et al. 1998).

**Material and methods.** The amoebae were obtained in November 1996 from the gills of moribund farmed turbot (Leiro et al. 1998, Paniagua et al. 1998). The gills were washed several times with sterile filtered seawater (0.2 µm pore size) containing 80 mg $l^{-1}$ of gentamicin. The branchial lamellae were then disaggregated by rubbing them through a steel mesh, previously sterilized in an autoclave, into sterile seawater. After homogenization using a Pasteur pipette, a few drops of the mixture were placed onto agar plates (0.02 g of Difco Bactoagar $ml^{-1}$ distilled water containing 20 mg of NaCl and heat-killed *Escherichia coli*). The plates were then incubated at 21°C for 10 d, and examined daily with an inverted microscope to detect amoebae. Amoebae were cloned by removing a small square of agar containing trophozoites and transferring it to a fresh agar plate. Amoebae were identified on the basis of light- and electron-microscopic morphological criteria as previously described (Leiro et al. 1998).

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Amoeba trophozoite population dynamics were investigated in microcultures on microscope slides (Paniagua et al. 1998). Briefly, a few drops of agar medium (0.02 g of bactoagar $ml^{-1}$ distilled water containing 20 mg of NaCl) were placed onto a slide and cover-slipped. Once the agar had set, the cover-slip was removed, and the agar was trimmed to leave a $2 \times 2$ cm square. $6 \times 10^4$ viable trophozoites $ml^{-1}$, obtained as described above, and $10^8$ bacteria $ml^{-1}$ (see below) were pipetted onto the agar surface, which was then cover-slipped and sealed with Rondeau du Noyer lacquer. Microcultures were incubated at 15°C for 11 d, and counting was done daily as described previously (Paniagua et al. 1998). The species, strain and origin of the bacteria used in the present study are listed in Table 1. All bacteria were stored frozen at −75°C in tryptic soy broth (TSB, Difco) with 15% (v/v) glycerol, and working cultures were routinely grown in brain-heart infusion agar or broth (BHIA or BHIB, Oxoid) with 0.5% yeast extract added (Toranzo et al. 1995).

**Results and discussion.** It is generally thought that amphizoic amoebae typically only colonize the gills of immunocompromised fishes, or individuals showing an existing bacterial infection providing a food source (i.e. the bacteria) (Lom & Dyková 1992). However, gill pathologies may be associated with any of several physical and biological agents, including bacteria (bacterial gill disease, BGD) and various protozoa including amoebae (amoebic gill disease, AGD). Identifying the primary aetiological agent of a given infection is thus often difficult (Sawyer et al. 1975, Daoust & Ferguson 1985). Recent studies have shown that amoebae may act as protective hosts for certain bacteria (Baker & Brown 1994), and indeed that amoebae may increase the effective thermotolerance, invasiveness and antibiotic-resistance of pathogenic bacteria (Walochnik et al. 1999).

*Vibrio anguillarum* is a virulent pathogen in fisheries and aquaculture systems, causing heavy mortalities and correspondingly high economic losses (Fouz et al. 1990). In northwest Spain, *V. anguillarum* is an important cause of epizootic infections in freshwater, estuarine and marine fishes including farmed trout, salmon and turbot (Toranzo et al. 1987). *V. vulnificus* is an estuarine/marine bacterium occurring in large numbers in fish and molluscan shellfish (Dalsgaard 1998), and causing disease outbreaks in some fish species (Austin & Cross 1998, Dalsgaard et al. 1999), notably the European eel; it may also cause primary bacteraemia due to consumption of raw shellfish (Dowdy et al. 1999) and has been associated with severe and often fatal disease in people who have recently handled fresh whole fish (Bisharat et al. 1999). *Aeromonas hydrophila*, like *V. anguillarum*, is a virulent fish pathogen causing severe losses in aquaculture (McGarey et al. 1991); this bacterium is a normal inhabitant of aquatic environments, and is an opportunistic pathogen of a variety of fishes and terrestrial animals, including humans (Sam & Amy 1994). The bacterium *Pasteurella piscicida*, the causative agent of fish pasteurellosis, has recently been reclassified as *Photobacterium damselae* subsp. *piscicida*, on the basis of 16S rRNA gene sequence comparisons (Osorio et al. 1999) (though in the present report we will use the name *Pasteurella piscicida*); this disease affects many species of marine fish (Evelyn 1996).

Table 1. Bacteria used in the present study. All amoeba were dead by Day 1; these results are not shown in Fig. 1

| Species | Strain | Source |
|---|---|---|
| **Pathogenic species** | | |
| *Streptococcus* sp.[a] | $RA_{103.1}$ | Turbot |
| *Aeromonas salmonicida*, subsp. *salmonicida* | $RSP_{23.1}$ | Turbot |
| *Aeromonas hydrophila* | $PO_{x.24}$ | Rainbow trout |
| *Pasteurella piscicida* | $DI_{2.1}$ | Gilthead sea bream |
| *Vibrio vulnificus* biotype 2 | ATCC 27562 | European eel |
| *Vibrio anguillarum* | $RQ_{80.1}$ | Turbot |
| **Environmental species** | | |
| *Vibrio campbelli* | ATCC 25920 | |
| *Vibrio natriegens* | ATCC 14084 | |
| *Vibrio aestuarinus*[a] | ATCC 35048 | |
| *Vibrio fluvialis* | ATCC 33812 | |
| *Pseudomonas dondorofii* | NCMB 1965 | |
| *Pseudomonas nautica* | DSM 50418 | |
| *Escherichia coli* | TG.1 | |

[a]In cultures with *Streptoccous* sp. and *Vibrio aestuarinus*

In *Platyamoeba* trophozoite cultures with bacteria that are pathogenic for turbot, namely *Vibrio anguillarum*, *Aeromonas salmonicida* and *Streptococcus* sp., all amoebae died within at most 3 d of culture (Fig. 1A). In cultures with *Pasteurella piscicida* and *V. vulnificus* (pathogenic for some fishes but not for turbot), all amoebae again died within at most 4 d of culture (Fig. 1B). In cultures with *A. hydrophila* (likewise pathogenic for some fishes but not for turbot), there was a clear initial increase in the number of amoebae, which peaked between Days 4 and 7; however, the population then rapidly declined, and by Day 9 all trophozoites were dead. In cultures with the non-pathogenic 'environmental' bacteria *V. natriegens* and *V. fluvialis*, there was a clear increase in the number of amoebae, in contrast to cultures with the pathogenic bacteria *V. anguillarum* and *V. vulnificus*: most notably, cultures with *V. natriegens* showed strong growth of amoebae for more than 11 d

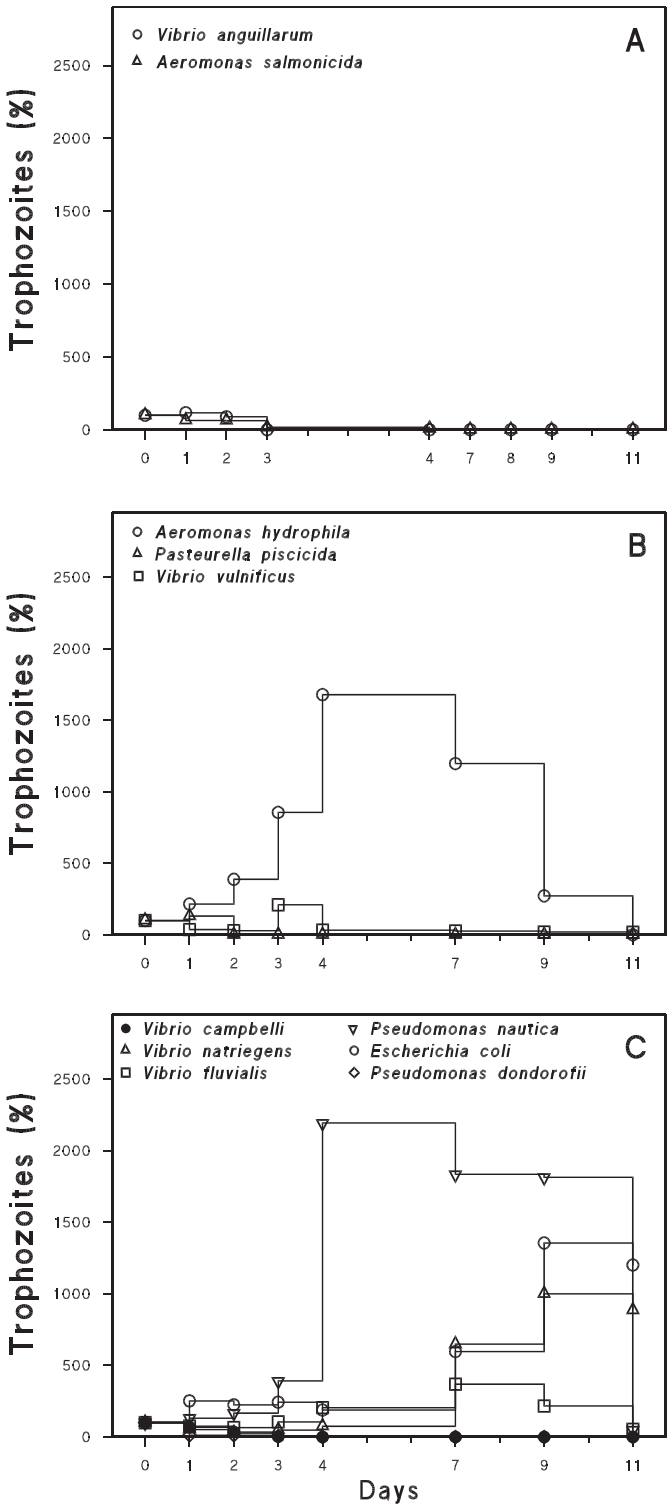


Fig. 1. Time courses of amoeba population size in microcultures containing (A) bacteria pathogenic for turbot, (B) bacteria pathogenic for other fish species but not turbot, and (C) non-pathogenic 'environmental' bacteria. Values shown are percentages of original (Day 0) population size ($10^2$ viable trophozoites per microculture); all values are means for 5 microcultures

(Fig. 1C). Results with non-pathogenic bacteria of the genus *Pseudomonas* were non-uniform. Cultures with *P. nautica* showed very strong growth, with numbers peaking between Days 7 and 9, and then declining rapidly from Day 11 onwards; in cultures with *P. dondorofii*, by contrast, all amoebae died very rapidly (Fig. 1C). Finally, cultures with *Escherichia coli* showed very strong growth, which continued even after 11 d of culture (Fig. 1C).

These results thus suggest that, of the bacteria tested, the only taxa utilizable as a food source by this *Platyamoeba* strain are *Aeromonas hydrophila* (pathogenic for some fishes but not turbot) and the non-pathogenic *Vibrio natriegens*, *Pseudomonas nautica* and *Escherichia coli*. The turbot-pathogenic bacteria *V. anguillarum* and *A. salmonicida* were not utilizable. It should be stressed that results obtained *in vitro* using microculture systems of this type may not be fully representative of amoebic and bacterial growth *in vivo* (Paniagua et al. 1998). Notably, this is a closed culture system, so that growth will eventually be restricted by nutrient limitations or the accumulation of toxic products: this is apparent from our results, which show death of all trophozoites after 9 to 11 d of culture (Fig. 1B,C). Nevertheless, the fact that the culture has a limited lifespan does not reduce the validity of results obtained before this stage.

In conclusion, the results of the present study indicate that the euryhaline amoeba *Platyamoeba* is not able to exploit *in vitro* either *Vibrio anguillarum* or *Aeromonas salmonicida* (the bacteria most commonly associated with gill infections in farmed turbot) as a food source. Additionally, the apparent suitability of *E. coli* as a food source suggests that the presence of *Platyamoeba* may be an indirect indication of faecal contamination of the waters feeding the fish farm.

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