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Electron microscopical and bacteriological studies on epithelium-associated bacteria in the pyloric caeca of murrel, *Channa punctatus* (Bloch)

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Abstract
Scanning and transmission electron microscopy (SEM and TEM) were used to define the location of epithelium-associated bacteria in the pyloric caeca of the air-breathing fish, murrel (*Channa punctatus*). SEM of pyloric caeca revealed substantial association of coccoid bacterial cells on the apices and between microvilli of enterocytes. Endocytosis of bacteria by enterocytes was clearly visible in the pyloric caeca by TEM. Five isolated bacterial strains were evaluated for extracellular protease, amylase and cellulase production quantitatively. Out of five isolated bacterial strains, CPC2 exhibited the highest protease, amylase and cellulase activities. The most promising enzyme-producing adherent bacterial strain, CPC2 was identified as *Staphylococcus haemolyticus* (Accession No. JX656750) by 16S rRNA gene sequence analysis.

Keywords: Pyloric caeca, Murrel, Bacteria, SEM, TEM, Enzyme production, Identification, 16S rRNA.

1. Introduction
The pyloric caeca are finger-like blind-ended sphincterless pouches associated with the anterior intestine that extend outward from the pylorus close to the intestine [1, 2]. It has been estimated that 60% of known fish species possess pyloric caeca, which vary greatly in number (from 1 to 1000), length and diameter [3]. Pyloric caeca in fish have been reported to increase the surface area for digestion and absorption but do not have any role in fermentation or storage [1]. Although there is no clear correlation between the number or size of pyloric caeca and intestine length or feeding type [3, 4], the caeca are typically absent or much reduced in omnivorous and herbivorous species [5]. Compared to the numerous studies evaluating the finfish gut microbiota [6, 7, 8, 9, 10, 11, 12, 13, 14, 15], few studies have investigated the microbiota of pyloric caeca [16, 17, 18, 19, 20, 21].

The digestive tracts of fish possess specific microbiota consisting of aerobic, facultative anaerobic or obligate anaerobic bacteria [7]. It is already established that gastrointestinal (GI) tract of fish possess two types of bacteria, one is autochthonous (bacteria that can colonize) and allochthonous (transient) [22]. Till date, the exact role of microbiota in the caeca is not clearly known. Electron microscopic studies provide a good information about the bacterial association in the gut epithelium. A number of studies on various fresh- and saltwater fish have demonstrated bacteria in the intestinal lumen and associated with the intestinal epithelium of fish using transmission electron microscopy (TEM) and/or scanning electron microscopy (SEM) [2, 19, 23, 24, 25, 26]. To the authors' knowledge, no information is available on the mode of association of autochthonous bacteria in the pyloric caeca of Indian freshwater fish. Therefore, the primary aim of the present study was to detect autochthonous bacteria in the pyloric caeca of an Indian air-breathing fish, the murrel, *Channa punctatus* by SEM and TEM. The present study also investigated the extracellular protease, amylase and cellulase-producing capacity of selected bacterial strains isolated from the pyloric caeca of murrel through quantitative enzyme assay and identification of the most promising enzyme-producing strain by 16S rDNA gene sequencing.

2. Materials and methods

2.1. Test Fish

Adults of Indian air-breathing fish, the murrel, *Channa punctatus* (average weight: 96.5±2.20 g) were collected from the local fish farm near Santiniketan, West Bengal, India (23˚41′ 30″ N latitude and 87˚41′20″ E longitude). The feeding habits, average live weight, average fish length, average length and average weight of caeca of the test fish are presented in Table 1.

Table 1: Food habit, average live weight, fish length, length and weight of pyloric caeca of *Channa punctatus*

Food habit	Average live weight (g) (SD)	Average fish length (cm) (SD)	Average length of caeca (cm) (SD)	Average weight of caeca (mg) (SD)
Insectivorous	96.5 (2.20)	15.4 (0.5)	1.36 (0.15)	71 (4.35)

SD- standard deviation

2.2. Isolation of epithelium associated bacterial flora

Isolation of autochthonous bacteria from pyloric caeca was done following the method of Banerjee *et al*. [14]. Briefly, caeca were dissected out carefully, homogenized in phosphate buffer and serial dilution was made up to five times. 100 µl sample from each test tube was taken, spread on TSA agar plate and incubated at 37 ˚C for 24 h. The well separated colonies with different colony morphology were selected and pure culture was done by repeated streaking method.

2.3. Quantitative assay of extracellular enzyme producing ability

For assessing extracellular enzyme producing capacity of the bacterial strains, they were cultured in broth (peptone-gelatin, starch and CMC broth for protease, amylase and cellulase, respectively) medium. For protease, amylase and cellulase enzyme production, the bacterial isolates were cultured in peptone-gelatin, starch and carboxymethylcellulose medium, respectively in a shaker incubator. After incubation, the broth was centrifuged at 8000 × g and the supernatant was collected for enzyme assay. Quantitative enzyme assay for protease, amylase and cellulase was done according to Walter [27], Bernfeld [28] and Denison and Kohen [29], respectively. Protein content of the supernatant was estimated by the method of Lowry *et al*. [30]. The specific enzyme activity was expressed as unit (U).

2.4. Phenotypic characterization of selected bacterial strains

The selected bacterial strains were cultured in TSA plates and colony morphology (configuration, elevation, margin, surface, density and pigment) were determined visually. Physiological characterization was done in tryptone soya broth (pH 7.0) at different temperature, pH and NaCl concentration. Biochemical characterization was done using HiMedia biochemical kits (KB002 and KB009).

2.5. Identification of the promising bacterial strain by 16S rRNA gene sequence analysis

The bacterial strain showing highest enzyme producing capacity was identified by 16S rRNA gene sequence analysis following the method described by Ringø *et al*. [22]. Briefly, genomic DNA was extracted and amplified by forward primer 27F (AGAGTTTGA TCMTGGCTCAG) and reverse primer 1491R (GGTTACCTTGTTACGACTT). All sequences were aligned and analyzed using bioinformatics tool (Codon-code and Mega 4.0) for finding the closest homolog of the microbes using a combination of NCBI (National Centre for Biotechnology Information) GenBank and RDP (Ribosomal Database Project) database. The final sequence was deposited in NCBI and phylogenetic tree was prepared using Mega 4.0 software.

2.6. Tissue preparation for electron microscopy

2.6.1. Scanning Electron Microscopy

Sample preparation for scanning electron microscope was done according to Ghosh *et al*. [26] with little modification. Briefly, fresh pyloric caeca were dissected out, incised longitudinally, cut into small pieces and fixed in 0.1 M cacodylate buffer containing 2.5% glutaraldehyde for 2 h. In order to remove the water, the tissue was dehydrated with graded ethanol as follows: 50% (30 min), 70% (40 min), 90% (1 h) and absolute alcohol (1 h). Thereafter, three consecutive changes were made in ethanol and amyl acetate in three different ratios 3:1, 2:2 and 1:3 (each for 30 min) and finally, tissues were kept in pure amyl acetate for 12 h. Critical point drying (CPD) was done in liquid nitrogen. The dry samples were then mounted in stub, coated with gold particle and observed under scanning electron microscope (Model Hitachi S530) at an accelerating voltage of 20 kV.

2.6.2. Transmission Electron Microscopy

For transmission electron microscope, pyloric caeca were dissected out and fixed in sodium cacodylate buffer containing 2.5% glutaraldehyde for 5 h at 4 ˚C. To remove the excess fixative, tissues were washed in fresh 0.1 M sodium cacodylate buffer for three times and again fixed in secondary fixatve (1% osmium tetroxide) for 1 h. Dehydration was done in ascending concentration of acetone 30%, 50%, 70%, 90%, 100% and finally in dry acetone for 15 min at 4 ˚C. Tissues were then rinsed in xylene, embedded in resin block and ultrathin sections were prepared using ultra microtome. The silver coloured sections floating on water were collected, stretched in chloroform vapour, put on a grid, stained with uranyl acetate and lead citrate and finally visualized under transmission electron microscope (JEOL JSM 6360) at an accelerating voltage of 20 kV.

3. Results

The bacterial population in the pyloric caeca i.e., colony forming unit (CFU) was expressed in term of log total viable count (TVC) /g caeca tissue. The bacterial population on TSA plate was recorded to be 3.71 /g caeca tissue. Cellulolytic bacterial population was recorded to be highest (log TVC = 3.61 /g caeca tissue) followed by proteolytic (log TVC = 3.30 /g caeca tissue) and amylolytic population (log TVC = 3.07 /g caeca tissue).

Results of quantitative enzyme activity exhibited by the bacterial isolates are presented in the Table 2. None of the bacterial isolates were good extracellular enzyme producers. The bacterial strain CPC2 exhibited the highest protease (0.358 ± 0.007 U), amylase (0.625 ± 0.066 U) and cellulase

(2.081 ± 0.025 U) activities.

Table 2: Quantitative extracellular enzyme activities of the selected bacterial strains

Strain designation	Protease (U)[1]	Amylase (U)[2]	Cellulase (U)[3]
CPC1	0.237 (± 0.01)	0.434 (± 0.011)	1.818 (± 0.059)
CPC2	0.358 (± 0.007)	0.625 (± 0.066)	2.081(± 0.025)
CPC3	0.136 (± 0.004)	0.322 (± 0.02)	1.154(± 0.03)
CPC4	0.273 (± 0.009)	0.512 (± 0.008)	0.890 (± 0.023)
CPC5	0.219 (± 0.02)	0.472 (± 0.03)	1.712 (± 0.037)

±- SE of three determinations

[1]-µg of tyrosine liberated/mg protein/ml of culture filtrate

[2]- µg of maltose liberated/mg protein/ml of culture filtrate

[3]- µg of glucose liberated/mg protein/ml of culture filtrate

Phenotypic characterization of most promising bacterial strain CPC2 showed that it was coccoid shaped and able to tolerate high temperature (50 ˚C), pH (9.0) and NaCl (9.5%). The selected bacterial strain CPC2 showed positive reaction for citrate utilization, nitrate reduction, ortho-nitro phenol galactoside (ONPG) utilization and catalase production, but negative for oxidase and urease production. Finally, the bacterial strain CPC2 was identified as *Staphylococcus haemolyticus* (Genbank Accession No. JX656750) by 16S rRNA gene sequence analysis. The phylogenetic relationship of CPC2 with its closest relatives is shown in the dendrogram (Figure 1).

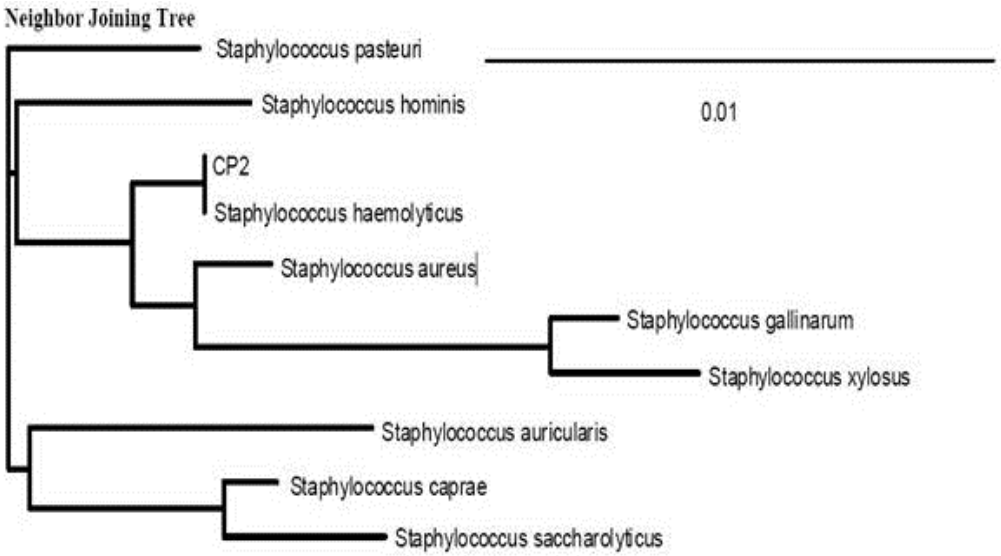

Fig 1: Dendrogram showing phylogenetic relationships of the strain CPC2 with other close homologs available in NCBI database

SEM evaluation indicated that bacteria associated with the epithelium of pyloric caeca of *C. punctatus* are coccoid-shaped (Figures 2 and 3). Substantial association of coccoid bacterial cells on the apices and between microvilli of enterocytes could also be revealed by SEM (Figure 4). In all the cases, the bacterial profiles are seen to be contained in internalized, membrane-bound endocytic vacuoles. TEM elevations of pyloric caeca of *C. punctatus* are presented in Figures 5 and 6. Numerous round shaped bacteria were detected in the microvilli border (Figure 5) and within the microvilli (Figure 6).

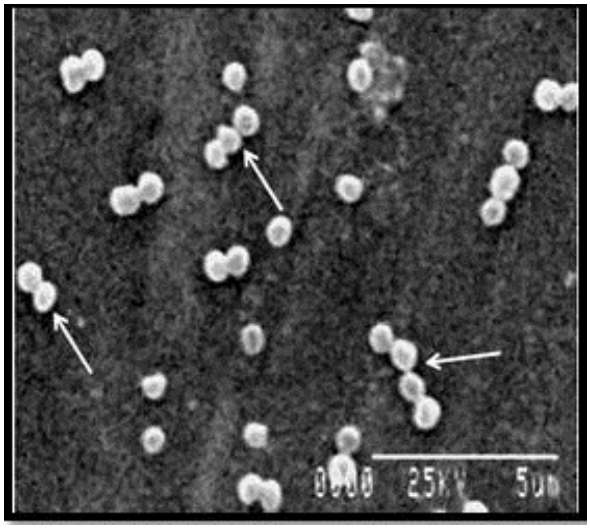

Fig 2 (× 8000)

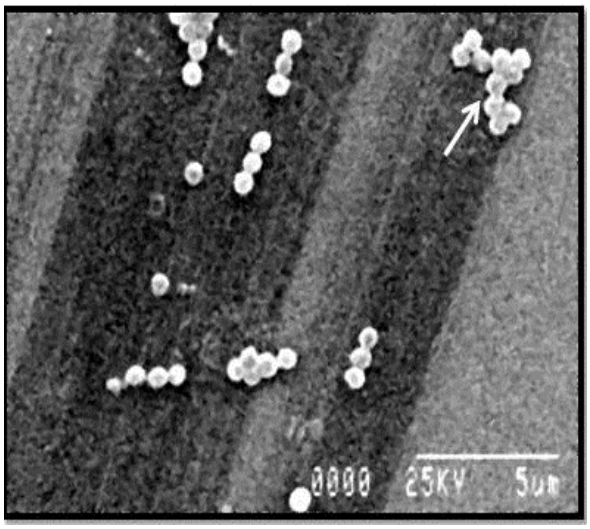

Fig 3 (× 6000)

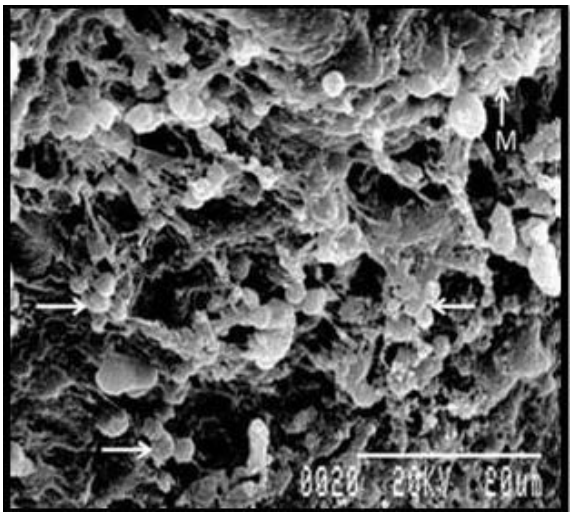

Fig 4 (× 6000)

Fig 2-4: Scanning electron micrograph of bacteria isolated from pyloric caeca of *C. punctatus* showing grape-shaped or *Staphyloccus* pattern (Fig 2 and 3). Fig 4 represents SEM of pyloric caeca showing coccoid-shaped bacteria (arrows) associated with enterocytes

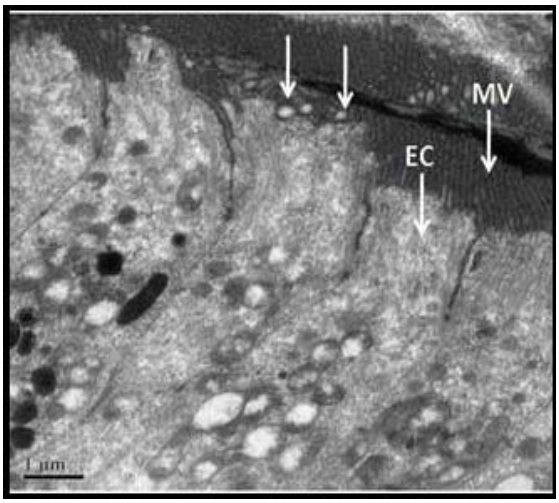

Fig 5 (Bar= 1.0 µm)

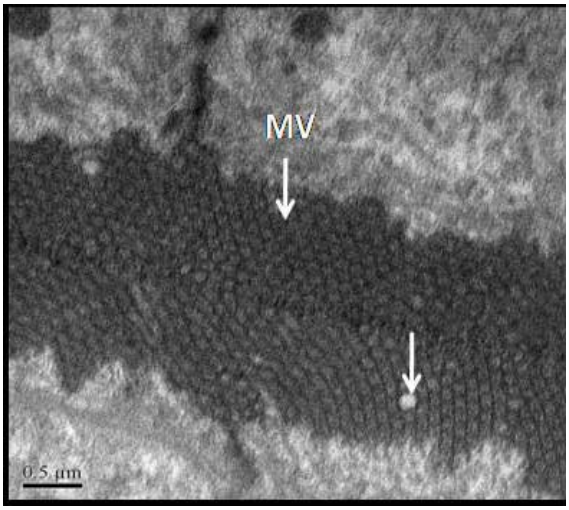

Fig 6 (Bar= 0.5 µm)

Fig 5 and 6: Transmission electron micrographs of pyloric caeca of *C. punctatus* showing coccoid-shaped bacteria associated with the enterocyte microvilli (arrows). In Fig 6, an internalized bacterium is clearly visible (arrow). MV- Microvilli

4. Discussion

Generally, bacteria are abundant in the environment in which fish live and it is therefore, rather impossible to avoid them being a component of their diet [31, 32]. Though pyloric caeca is not the direct part of digestive tract, there are possibilities that it may take part in digestion in carnivorous fish due to their short intestine. The extra cellular enzyme producing ability of the caecum microbial flora was investigated in this study. None of the bacterial isolates showed good proteolytic and amylolytic activity, but it was very surprising that their cellulolytic activity was moderately good. The bacterial strain CPC2 isolated from pyloric caeca of *C. punctatus* showed the highest cellulolytic activity.

Staphylococcoi are catalase producing Gram-positive bacteria, appears in grapes like pattern. Padilla *et al*. [33] reported the disease producing *Staphylococcus xylosus* isolated from red porgy (*Pagrus pagrus*). However, a research group in Norway stated that *Staphylococcus* and *Micrococcus* are predominant microbial flora in *Salvelinus alpines* [34]. Whereas, Laukova *et al*. [34] isolated and characterized five different species of *Staphylococcus* (*S. warneri, S. haemolyticus, S. epidermidis*, *S. hominis, S. pasteuri*) isolated from gastrointestinal tract of *Salmo gairdnerii* and *Salmo trutta* East Slovakian water sources. In the present study, partial 16s rRNA gene sequence analysis of the strain CPC2 showed that it has close relation with *Staphylococcus haemolyticus* (GenBank Accession No. EU867334.1) and *Staphylococcus hominis* (GenBank Accession No. HE578786.1). Occurrence of *Staphylococcoi* in nature is very common, but in case of animal these are host specific and may be due to human activities [34]. Staphylococci are widespread microbiota but some species observed in some animals appear more rarely on more distantly related host species [34]. Laukovă *et al*. [34] opined that human influence on environment including rivers should be taken into account to discuss the occurrence of these species.

Electron microscopy study has been established as an important tool for investigating the microbial ecology. Compared to numerous studies evaluating the finfish gut microbiota [6, 7, 8, 9, 10, 11, 12, 13, 35], few studies have investigated the microbiota of pyloric caeca [16, 17, 20, 21, 36]. In the present study, the SEM elevation of pyloric caeca of *C. punctatus* demonstrated coccoid-shaped bacteria associated with the surface of enterocytes. Navarrete *et al*. [20], however, reported that pyloric caeca, stomach and intestine in juvenile farmed Atlantic salmon, *Salmo salar* have similar bacterial compositions. Ringø *et al*. [19] reported the existence of bacteria at the tips of the microvilli as well as between adjacent microvilli in the pyloric caeca of Arctic charr, *S. alpinus*. Buddington and Diamond [1] however, rejected the fermentative function of pyloric caeca in fish due to lack of specific resident bacteria. Whether the caeca in fish have a fermentative function linked to their associated microbiota is not clearly known [37]. TEM of sectioned material of GI tract is used primarily to provide high resolution information about the mechanism of interaction between bacteria and the enterocyte cell surface [2]. Compared to SEM, few published reports are available on the microbial diversity and their interaction with the host using TEM [9, 19, 27, 32, 38, 39,]. These studies confirmed the classical microbiology and light microscopical findings with respect to distribution of bacteria along the pyloric caeca. Endocytosis of bacteria in the GI tract is highly relevant as the GI tract is considered as the potential port of entry of pathogens [2]. The results of the present study clearly indicated endocytosis of bacterial cells in the pyloric

caeca of *C. punctatus*. This indicates that this region is involved in bacterial endocytosis. Endocytosis of bacteria by enterocytes has also been reported to occur in the epithelial border of the GI tract of various fish species [13, 19, 32, 40, 41, 42]. Although some TEM studies have demonstrated translocation of bacterial cells by endocytosis in the GI tract of larvae and adult fish as well as uptake of bacterial antigen [2], such information are lacking in Indian fishes.

5. Conclusion

The results of the present study provide evidence that autochthonous bacteria exist in the pyloric caeca of freshwater air-breathing fish. The investigation further confirms that there is also a distinct microbial source of digestive enzymes (cellulase, amylase, and protease) in the pyloric caeca. The presence of coccoid shaped bacteria has been reported for the first time in the pyloric caeca of Indian freshwater fish using SEM and TEM. However, the mechanisms involved in bacterial translocation must be investigated in greater details to evaluate differences between the indigenous and pathogenic microflora.

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