Potential digestive function of bacteria in krill

Euphausia superba stomach

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ABSTRACT: Evidence is presented that bacteria in the stomach of live Antarctic krill Euphausia superba participate in the digestion of host dietary components. Total (AODC: Acridine Orange Direct Count) and culturable (CFU: Colony Forming Units) bacteria in this organ in fresh adult krill were compared in numerical and physiological terms with those in contemporaneously collected seawater samples in Admiralty Bay, King George Island, South Shetland Islands. On a per unit volume basis, AODC and CFU numbers in the stomach were greater than in the seawater, and higher than could be accounted for through selective prey filtration alone. CFU in the stomach and seawater averaged -4%, and <0.01% of the respective AODC, and in the stomach -6% of AODC were dividing, compared to less than 1% in seawater, observations suggesting that bacterial growth occurs in the stomach. Biovolume of cells in the stomach exceeded that of cells in seawater ($t_{21154} = -6.262$, $p < 0.001$), but the former were too small to have been selectively filtered. Maltose and gluconate assimilation, and lipase ($C_\text{L}$), 'trypsin'-like, and acid phosphatase activities were significantly more prominent in culturable bacteria from the stomach than seawater, further indicating selection. Conversely, $\alpha$-fucosidase production appears host derived. Yeasts isolated from the stomach were identified as Leucosporidium antarcticum and Metschnikowia australis.

KEY WORDS: Antarctic krill · Euphausia superba · Bacteria · Digestive enzyme · Yeast

INTRODUCTION

In the 1970s, Antarctic krill Euphausia superba stocks in the Southern Ocean were believed to constitute a protein source of a size that could feed the world. Although this belief is no longer tenable (e.g. Everson & Miller 1994) it did encourage studies on its bacterial flora (Espeche et al. 1979), particularly the role of bacteria in krill spoilage (Kelly et al. 1978). It is now clear, however, that the post mortem activities of this flora are secondary to krill’s autolytic processes which set in immediately after death (Fevolden & Eidsa 1981, Zdanowski 1981), although through their high numbers in the digestive tract bacteria do remain a potent force in krill spoilage.

Unfortunately, bacterial associations with marine animals such as those studied in other oceans (Fenical et al. 1991) have been largely overlooked in Antarctica, and have tended to concentrate on bacteria and krill (Zdanowski & Donachie 1993), and fish (e.g. Notothenia neglecta, cf. MacCormack & Fraile 1990, Donachie 1995). Addressing the former, Rakusa-Suszczewski (1988) and Rakusa-Suszczewski & Zdanowski (1989) suggested that bacteria in the stomach of live krill may participate in host digestive processes through the production of enzymes, and/or provide dietary co-factors, and Donachie et al. (1995) supported this with work on a related euphausiid, the Northern krill Meganyctiphanes norvegica in which bacteria were found to contribute to proteolytic, lipolytic, and chitinolytic enzyme pools.

To address how bacteria in the digestive tract of live Antarctic krill may interact with the host, we compared bacteria in its stomach with those in the water column; we described the total and culturable populations in this organ in live krill caught in Admiralty Bay, South Shetland Islands, and the Bransfield Strait, South Shetland Islands, and compared them in terms of num-
bers, cell size, biomass, biochemical, assimilatory, and constitutive enzyme activity with those in contemporaneously collected seawater samples. Fresh krill fecal pellets were harvested for similar studies. The results are discussed with respect to krill feeding ecology, and the role of metabolically active bacteria in terms of their likely contribution to the digestive enzyme pool.

**MATERIALS AND METHODS**

All krill used in this work were caught at a depth of 80 m between April and December 1990 with an RMT 4 (Rectangular Midwater Trawl, 2 x 2 m) in Admiralty Bay, King George Island, South Shetland Islands (Fig. 1), and in January 1991 ca 5 km south of the bay, in the Bransfield Strait. Only live adults were used. Those caught in the bay were sacrificed within 90 min, and those from the Bransfield Strait within 4 h. In total, there were 6 catches within the bay and 1 in the Bransfield Strait; newly caught krill were returned to the laboratory in a covered opaque plastic barrel containing fresh surface seawater.

**Bacterial numbers.** Ten randomly selected live krill were taken from the barrel in a minimum of water into a sterile 1 l glass beaker, held in ethanol rinsed, air dried, arm length plastic gloves. They were then aseptically transferred with blunt, ethanol flamed forceps to a sterile beaker containing 1 l of 0.2 pm filtered and autoclaved seawater (to remove external debris) in an ice bath, prior to a second transfer to sterile plastic Petri dishes (1 per krill) for dissection under a binocular microscope. Each krill was laid on its ventral surface and held laterally across the carapace with ethanol flamed, cooled forceps. An ethanol flamed mounted needle was placed immediately behind the eyes. A firm downward movement severed the head, which when pulled from the thorax often drew the stomach from the thoracic cavity (Zdanowski 1981). This step also rapidly and cleanly separated the stomach from the hepatopancreas and digestive tract. Each stomach was collected with sterile forceps, and weighed on sterile pre-weighed foil strips (Cahn 25 micro-balance) before aseptic transfer to an ice-bath maintained hand-held sterile Potter homogeniser containing 1 ml of 0.2 pm filtered and autoclaved seawater per stomach. The suspension was homogenised gently with a sterile Teflon shaft for 30 s.

Total bacteria were counted in all 7 pooled stomach samples after Zimmerman & Meyer-Reil (1974) with acridine orange (Acridine Orange Direct Count, AODC) under a Carl Zeiss Jena FLUOVAL 2 microscope fitted with an apochromat H 100/1.32; 160/0.17 objective with oil immersion. Undiluted stomach homogenates were fixed with 0.2 pm filtered and buffered formalin to a final concentration of 1%, stored at 4°C in sealed glass ampoules, and counted within 6 mo.

Culturable bacteria (Colony Forming Units, CFU) were determined by the spread plate method (0.1 ml) from a decimal series of the homogenate to 10⁻⁴ in 0.2 pm filtered and autoclaved ‘aged’ seawater, on nutrient agar (Oxoid) prepared on an ‘aged’ seawater base (Zdanowski 1982, Zdanowski & Donachie 1993), referred to as NASW, after parallel incubation at 1 and 15°C for 15 d.

Bacterial numbers are described in terms of AODC or CFU per g wet wt of stomach tissue. The latter are based on the means of the 1 and 15°C incubations since similar counts were derived at each temperature. Procedures and results of AODC and CFU determinations in seawater during this work are given in Donachie (1995, 1996), the results of which are described here for comparative purposes.

**Bacterial cell sizes and morphology.** During epifluorescence microscopy, 1255 randomly selected bacterial cells in 3 stomach samples were described in terms of cell morphology, dimensions, and bacterial carbon according to the conversion factor of 220 fg C pm⁻³ (Bratbak & Dundas 1984). These cells were also compared in the same terms with 1636 cells from the upper 100 m of the bay. During microscope work the frequency of dividing cells (FDC) in the stomach and seawater was also recorded, and those in seawater that were attached to particles were counted.
Bacterial numbers in *Euphausia superba* fecal pellets. Three freshly caught krill were rinsed (3 × 30 s) in 0.2 μm filtered and autoclaved seawater, manipulated aseptically through each transfer, and placed in sterile glass beakers containing 1 l of filtered and autoclaved seawater. Each beaker was fitted with an ethanol rinsed and flamed foil cap and maintained in a walk-in ‘cold room’ (-0°C). AODC and CFU were counted in water samples taken aseptically from each beaker prior to introduction of the krill, and again immediately before removal of fecal pellets.

After ~24 h a number of fecal pellets in the beaker were harvested aseptically with a sterile Pasteur pipette. AODC were counted in typical pellets fixed in 1% buffered formalin. CFU were counted in pellets homogenised in 0.2 μm filtered and autoclaved ‘aged’ seawater in a hand-held Potter homogeniser with a sterile Teflon shaft, maintained in an ice bath; spread plates (0.1 ml) were prepared on NASW followed by parallel incubation at 1 and 15°C.

Biochemical, assimilatory, and constitutive enzyme activity of culturable bacteria. Over 700 colonies on NASW from all krill stomach samples, fecal pellets, and seawater were purified (1 to 3 passages on NASW, 15°C) (Donachie 1995). Of these, 80 from krill stomach, 78 from seawater, and 11 from the fecal pellets were passed through the API 20NE system (API bioMerieux Ltd, Basingstoke, UK). This system allows considerable descriptive data regarding the nutritional requirements of each isolate to be generated quickly and economically, and it has performed reliably when applied to Antarctic bacteria (Tearle & Richard 1987, Zdanowski & Donachie 1993, Donachie 1995, Zdanowski 1995). A second system, API Zym, which permits the activities of 19 constitutive enzymes in an isolate to be determined, was applied to 30 isolates from krill stomach, 31 from seawater, and 8 from fecal pellets. This has also been used with Antarctic marine bacteria (Zdanowski & Donachie 1993), and boreal marine bacteria (Donachie et al. 1995). Both systems were used as described by Donachie (1995), with incubations at 15°C. Responses in each system of bacteria from krill stomachs and the water column were compared by chi-square tests.

Within API 20NE and API Zym the following tests can be conducted: API 20NE — reduction of nitrates to nitrites, reduction of nitrates to nitrogen, indole production, fermentation of glucose, arginine dihydrolase production, urease production, esculin hydrolysis, gelatin hydrolysis, α-galactosidase production, assimilation of glucose, arabinose, mannose, mannotol, N-acetyl-β-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate, and phenyl acetate, and the production of cytochrome oxidase; API Zym — presence of alkaline phosphatase, esterase (C₁₄), esterase lipase (C₄), lipase (C₁₄), leucine, valine, and cysteine arylamidases, trypsin, chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase.

Constitutive enzyme activities in krill stomachs were determined in 3 undiluted stomach homogenates inoculated aseptically through each transfer, and placed in sterile glass beakers containing 1 l of filtered and autoclaved seawater. Yeasts were purified (1 to 3 passages on NASW, 15°C). Their responses in a range of oxidation and fermentation tests in the API 20C system incubated at 15°C, and their morphologies, were compared to those of type strains in the literature (Barnett et al. 1990). Tests employed in this API system are as follows: fermentation of galactose, maltose, sucrose, lactose, raffinose, trehalose, melibiose; oxidation of inositol, glucose, galactose, maltose, sucrose, lactose, raffinose, trehalose, melibiose, cellobiose, and resistance to actidione (cycloheximide).

**RESULTS**

Bacterial numbers in *Euphausia superba* stomachs

AODC and CFU in krill stomachs numbered 3.28 × 10⁸ g⁻¹ (SD = 2.51 × 10⁸, n = 7) and 1.09 × 10⁸ g⁻¹ (SD = 1.93 × 10⁸, n = 7) wet wt respectively (Fig. 2). Assuming that 1 ml of tissue has a mass of 1.1 g, these outnumbered parallel counts in seawater (10⁷ and 10 ml⁻¹ respectively) on a per unit volume basis by several orders of magnitude. CFU in the stomach comprised on average a greater fraction of AODC than parallel counts in seawater (Table 1). AODC in the upper 100 m rarely exceeded 10⁷ ml⁻¹ from May to December, but attained 10⁶ ml⁻¹ in January; CFU averaged 3.5 ml⁻¹, but exceeded 30 ml⁻¹ by mid January.

Cell sizes and morphology of bacteria in *Euphausia superba* stomachs

Both mean bacterial cell length and biomass (μg C cell⁻¹) in the stomach exceeded those of bacteria in the upper 100 m of the water column (Table 1). In the stomach, bacterial cell biovolume was significantly greater than that of bacteria from seawater (2-sample t-test, t2634 = -6.262, p < 0.001), and the frequency of dividing bacterial cells in the stomach exceeded by
Gram negative rods dominated the culturable bacteria in both habitats (krill stomach, 78%; seawater, 70%); most of the remaining cells were Gram-negative cocci.

Bacterial numbers in *Euphausia superba* fecal pellets

Fecal pellets averaged $0.95 \pm 0.31$ mm in length ($n = 19$), and $0.21 \pm 0.05$ mm in width. Total bacterial counts based on a pellet density of 1.1 g ml$^{-1}$ were of the order of $1.25 \times 10^{11}$ g$^{-1}$ wet wt and consisted almost solely of well stained regular rods; 1 spiral form was noted, along with unidentified protists (Fig. 3). CFU counts were precluded due to rapidly attained confluent growth on NASW. AODC and CFU counts in the beaker prior to introducing the krill confirmed the water was sterile; after 24 h these counts had attained $3.8 \times 10^{4}$ ml$^{-1}$ and $2.5 \times 10^{4}$ ml$^{-1}$ respectively.

Biochemical, assimilatory, and constitutive enzyme activity of culturable bacteria

The application of the API 20NE and API Zym systems to pure cultures of culturable bacteria showed that those from krill stomach and seawater varied significantly in 5 tests (Table 2). Maltose and gluconate assimilation, and the enzymes lipase (C14), 'trypsin'-like, and acid phosphatase were most prominent in bacteria from krill stomach. None produced indole (TRP), or showed $\alpha$-fucosidase activity ($\alpha$-Fu). An identical bacterial strain was found in all krill stomach samples taken in the bay, krill fecal pellets, seawater that was sterile prior to the introduction of krill, in the stomach of a related euphausiid caught in the bay during this study, *Thysanoessa macrura* (Donachie 1995), and in seawater.

Constitutive enzymes in krill stomach

Constitutive enzyme activities differed in each krill stomach sample. All but $\alpha$-mannosidase and $\alpha$-fucosidase were detected in krill caught on 4 April. These and $\alpha$-galactosidase and $\beta$-glucuronidase were, however, absent from the 4 May sample. Activities of all 19 enzymes in API Zym were recorded in stomachs from 3 October, i.e. $\alpha$-fucosidase activity was recorded only in this sample.

Yeasts in *Euphausia superba* stomach and seawater

Nine pure yeast strains were prepared from krill stomach samples. One was identified as *Leucosporid-
and January 1991. Our results show that these populations differ in terms of number per unit volume, with those in krill stomach and fecal pellets exceeding those in the water column by several orders of magnitude. Bacterial cell sizes and biomass, the size of the culturable fraction (CFU) with respect to the total bacteria (AODC), and the frequency of dividing cells were all greater in the stomach samples. Furthermore, a number of assimilatory and enzyme activities were significantly more prominent in culturable bacteria from this organ. These findings support the thesis that bacteria in the stomach are metabolically active.

DISCUSSION

In this paper we have compared the total bacteria (AODC) and its culturable fraction (CFU) in stomachs excised from live Euphausia superba with those in seawater in Admiralty Bay, collected between April 1990 and January 1991. The differences in bacterial numbers and cell sizes between krill stomach and seawater cannot be attributed to the selective filtration of specific bacterial size classes. The filtration rate of $357 \text{ ml h}^{-1}$ (Quetin & Ross 1985) determined through the clearance rate of cells from unialgal cultures is too low to account for the bacterial numbers in the stomach, particularly as the animal can only feed inefficiently on small cells (McClatchie & Boyd 1983, Fig. 11; Quetin & Ross 1985). In this respect, over $98\%$ of the almost 2900 bacteria measured (including epibacteria, and all the cocci in the stomach and seawater) were too small ($<2 \mu\text{m}$, cf. Boyd et al. 1984) to have been selectively grazed. Furthermore, bacterial aggregates of a size that may have been selected were extremely rare, and paired cells or chains of bacteria were also unusual in the water column. Considering that less than $1\%$ of the AODC in seawater were dividing, none of which were epibacteria, the high numbers of bacteria in the stomach can also not be accounted for through the ingestion of groups of dividing bacterial cells, bacterial aggregates, or colonised particles of over $2 \mu\text{m}$. A mechanism that would give rise to such differences between these populations is bacterial growth in the stomach. The evidence for this is manifold, and includes the higher number of dividing cells in the stomach compared to the seawater. In its absence, cell sizes and FDC in this organ and seawater would be similar. This was not the case. All cocci in the stomach were too small to have been grazed, but their average volume exceeded by some $80\%$ that of cocci in seawater.

Table 2. Significant differences between pure bacterial cultures from krill stomachs and those from seawater in Admiralty Bay, South Shetland Islands, were recorded in terms of their responses to each of 5 assimilatory and constitutive enzyme tests in the API ZONE and API Zym systems. Positive responses were most prominent in those from the stomach

<table>
<thead>
<tr>
<th>Test</th>
<th>$\chi^2$</th>
<th>p</th>
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<tbody>
<tr>
<td>Maltose assimilation</td>
<td>7.549</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Gluconate assimilation</td>
<td>5.918</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Lipase ($C_{14}$)</td>
<td>3.976</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Trypsin'-like</td>
<td>5.074</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>4.504</td>
<td>&lt;0.05</td>
</tr>
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results, viz. the higher incidence in these bacteria of maltose and gluconate assimilation, and the prominence of a number of enzymes. Sugars, proteins, and lipids released by the mechanical destruction (Ulrich et al. 1991) of the ingesta, e.g. diatoms, encourage in situ bacterial growth, but whether or not bacterial activities benefit the host is unclear. According to Sieburth (1979) and Prieur (1991), however, the bacterial flora in a marine animal’s gut reflects its diet, an observation that infers viability, with an herbivorous or carnivorous diet respectively selecting for saccharolytic or proteolytic bacteria. In this respect our work supports field observations of omnivory in krill (Price et al. 1988). It is a versatile feeder, grazing selectively (Nordhausen et al. 1992) on copepods in the presence of high phytoplankton concentrations (Price et al. 1988, Granelli et al. 1993), consuming its exuviae, cohorts, diatoms, and its own fecal pellets (Smetacek pers. comm.). Through coprophagy, enteric bacteria will be reintroduced to the krill digestive tract or other zooplankton, e.g. Thysanoessa macrura although this will not be the sole mechanism. Bacteria that are too small to be selectively filtered will also be ingested either as epibacteria or in seawater that will accompany the food pellet as it is passed from the krill’s feeding basket to the mouth. The presence of bacteria in the ‘aquarium’ after introduction of the krill, however, testifies to the animal’s role in introducing bacteria to the pelagia.

With respect to bacterial production of enzymes in the digestive tract, Donachie et al. (1995) showed overlapping chitinase FPLC (Fast Protein Liquid Chromatography) elution profiles from Meganyctiphanes norvegica and bacteria from its stomach and hepatopancreas, with fewer peaks in antibiotic treated animals. The activities of lipase and protease were also significantly reduced compared to non-treated controls. The same authors (unpubl.) also isolated chitinolytic bacteria from Euphausia superba stomach and hepatopancreas. In this respect, Mayzaud et al. (1987) contrasted 7 ‘trypsin’-like fractions in the E. superba cephalothorax with studies that determined only 3 to 4, and suggested that spatial variations in trypsin activity are related to physiological and environmental factors. Considering that in this work ~90% of cultivable bacteria from the krill stomach produced trypsin, it would be pertinent to consider the activities of an incident bacterial flora in enzyme studies that include the stomach in an analysis.

In contrast to the likely bacterial digestion of proteins and lipids in Euphausia superba, no isolate expressed α-fucosidase. The same applied to 25 isolates from Antarctic seawater and sea-ice (Zdanowski & Donachie 1993). Its activity here in Euphausia superba stomach and Meganyctiphanes norvegica (Donachie et al. 1995) must have originated with the host, and/or bacteria capable of utilising the substrate employed were not encountered. If they were indeed not tested, then on the basis of the number of bacteria considered, the enzyme is unlikely to be widespread among culturable bacteria in these habitats.

Pseudomonas species appear to be associated with Euphausia superba, or indeed euphausiids, as one bacterial strain considered identical in all krill from the bay, a krill aquarium, seawater from the bay, Thysanoessa macrura stomach (Donachie 1995), and the stomach and hepatopancreas of krill caught in the bay 1 yr after this study ended (Donachie et al. unpubl.) was an identical Pseudomonas sp. Moreover, Zdanowski & Donachie (1993) isolated a Pseudomonas sp. from the stomachs of krill caught off Elephant Island in 1988/89, and from sea-ice and underlying water. Such an association may be driven by the ingestion of particular food material (sensu Sieburth 1979), or the differential survival of ingested bacteria (Harris 1993) may ensure these species are principally associated with the alimentary tract (Turkiewicz et al. 1982), rather than the tissue as a whole. They may invade the tissue and participate in its breakdown after krill death (cf. Zdanowski 1981, 1988).

No work on Euphausia superba microbiology to date has referred to either Leucosporidium or Metschnikowia yeasts, but Turkiewicz et al. (1982) isolated a white budding yeast from the krill’s alimentary canal that may correspond to any of those in this study. In light of Fell’s (1976) suggestion that most yeasts in the sea are saprophytic and that evidence for other relationships is weak, however, those described may be derived from ingested particles, e.g. fecal pellets. Metschnikowia species found in the krill stomach and seawater are nearshore species previously isolated around the South Shetlands (Fell & Hunter 1968), but unlike Leucosporidium are not restricted to Antarctica. The former have been reported as parasitic on copepods (Fize et al. 1970) and were originally described after isolation from Daphnia (Metschnikoff 1884). Yeast numbers in seawater were within the range given by Fell (1974).

In conclusion, bacterial growth in krill stomach is inferred by their high numbers relative to the surrounding seawater, by their larger cell biovolumes and biomasses, and by the population structure, viz. the large culturable fraction of the total bacteria and higher frequency of dividing cells. Such differences between these populations cannot be explained through krill selectively filtering the bacteria described. Culturable bacteria in the stomach may participate in the breakdown of the ingesta through production of enzymes such as trypsin, lipase, and acid phosphatase. Other requirements such as vitamins (Dempsey & Kitting
1987, Rakusa-Suszczewski & Zdanowski (1989) may also be provided. In light of our findings, the bacterial role in krill’s post mortem breakdown, and the fact that krill may be a major source of chitin in the sea (Jeuniaux et al. 1993, Nicol & Hosie 1993), Tanner (1985) showed some foresight in suggesting that krill-bacteria associations may be important in Southern Ocean nutrient cycles.

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LITERATURE CITED


Donachie SP (1995) Ecophysiological description of marine bacteria from Admiralty Bay (Antarctica), and the digestive tracts of selected euphausiidae. In: Rakusa-Suszczewski S, Donachie SP (eds) Microbiology of Antarctic marine environments and krill intestine, its decomposi-
tion and digestive enzymes. Department of Antarctic Biology, Polish Academy of Sciences, Warsaw, p 101–196


Espeche ME, Sanchez J, Fraile ER (1979) Counting and recovery of viable aerobic bacteria from Antarctic krill. Contribu-


Fell JW, Hunter IL (1968) Leucosporidium gen. n., the heterobasidiomycetous stage of several yeast of the genus Candida. Antonie Leeuwenhoek 34:365–376


Sieburth JMcN (1979) Sea microbes. OUP, New York


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