Fish and Shellfish Immunology 135 (2023) 108657

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Antimicrobial action of chromatin extracellular traps released by neutrophils of rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792)

Andre P. Van, James E. Bron, Andrew P. Desbois *

Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling, FK9 4LA, United Kingdom

ARTICLE INFO

Keywords:
Innate immunity
Neutrophil extracellular traps
Polymorphonuclear cells
Vibrio anguillarum

Chromatin extracellular traps (ETs) are released *in vitro* by certain fish polymorphonuclear leucocytes (PMNs), including neutrophils, in response to various chemical and biological stimuli such as bacterial cells and components like flagellin and lipopolysaccharide [1–4]. For mammals in particular, ETs have been shown to exert antimicrobial properties and function as part of the innate response [5,6]. However, there are few studies on the antimicrobial actions of ETs released by fish, with these properties reported for ETs in immune cell suspensions prepared from common carp (*Cyprinus carpio*) [7], turbot (*Scophthalmus maximus*) [8,9] and tongue sole (*Cynoglossus semilaevis*) [1]. In contrast, some studies failed to provide evidence for antibacterial activities, including those examining tongue sole ETs against *Edwardsiella tarda* [1] and Atlantic salmon (*Salmo salar*) ETs against *Aeromonas salmonicida* [4], indicating complexity in the interactions between bacteria and the actions of the ETs. This present study investigated whether ETs released by neutrophils of rainbow trout (*Oncorhynchus mykiss*) exert antibacterial activity against the important finfish pathogen, *Vibrio anguillarum*.

To achieve this, the abundance of *V. anguillarum* Vib87 was assessed by undertaking colony-forming unit (CFU) counts during incubation with rainbow trout PMNs that had been induced to release ETs and comparing these counts to controls where the ETs had been digested by addition of an exogenous DNase. First, PMN-enriched cell suspensions (ca. 60%) were prepared to 4×10^5 cells mL^{-1} in RPMI-1640 medium (supplemented with 1% fetal calf serum, 10,000 U mL^{-1} penicillin, 10 mg mL^{-1} streptomycin) for two fish according to Van et al. [2], and dispensed into flat-bottom 96-well microtitre plates (100 μL $well^{-1}$). Cells were allowed to settle (30 min, 15 °C) before ET release was induced by adding 20 μL of calcium ionophore in water (CaI; A23187, Thermo Fisher Scientific, Loughborough, UK) to give 5 μg mL^{-1} in the wells and then incubated for 3 h. To each well was added 16 μL DNase buffer (final well concentration of 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$; Thermo Fisher Scientific) and 24 μL distilled water (+ETs group); to digest away the ETs, wells in the −ETs group received 24 μL DNase-I (in distilled water; 10 U mL^{-1} final well concentration; Thermo Fisher Scientific) instead of the distilled water. A control group lacking PMNs contained bacteria only in medium supplemented with CaI and DNase (Bacteria + CaI + DNase). The plate was incubated for 10 min before adding 80 μL of bacterial suspension at ca. 1×10^3 CFU mL^{-1} in PBS to each well; the bacterium had been cultured and prepared according to McMillan et al. [10]. At this point, the entire contents of triplicated wells for each experimental group and each fish were plated on TSA supplemented with 1.5% NaCl and the agar plates incubated (24 h, 22 °C) to allow CFU to form ('pre-spin'). Then the 96-well plate was centrifuged (510×*g*, 10 min, 4 °C) to bring the bacteria to the bottom of the wells where they would make contact with any ETs present ('0 h') [1,8]. Immediately after centrifugation the contents of further triplicated wells for each experimental group and each fish were plated on TSA as previously, before the final wells from each group were collected and plated after 1 h incubation ('1 h'). Statistical analyses were performed with SPSS (v. 28.0.0.0; IBM, Armonk, New York, USA). CFUs were compared between groups at each time point by Kruskal-Wallis test with post-hoc pairwise comparisons. $P < 0.05$ indicated a statistically significant difference (adjusted by Bonferroni correction to account for multiple comparisons).

To observe the interaction between the ETs and bacteria by fluorescence microscopy, PMN-enriched cell suspension was prepared and

* Corresponding author.
E-mail address: andrew.desbois@stir.ac.uk (A.P. Desbois).

https://doi.org/10.1016/j.fsi.2023.108657
Received 6 October 2022; Received in revised form 14 February 2023; Accepted 28 February 2023
Available online 1 March 2023

induced to release ETs exactly as before. Meanwhile, the membrane-permeable fluorescent DNA stain, 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific), was added to a *V. anguillarum* Vib87 culture in exponential growth phase to 1 μM, before incubation for 30 min in the dark on ice. Following this, the bacteria were centrifuged (3000×*g*, 10 min, 4 °C) and washed in 5 mL PBS three times to remove any unbound stain. The bacteria were resuspended to 1×10^8 CFU/mL in PBS and 80 μL of suspension was added into triplicate wells containing the PMNs. The plate was centrifuged (510×*g*, 10 min, 4 °C) and then incubated (1 h, 15 °C). Extracellular DNA in the wells (i.e., ETs) was stained for 5 min by addition of 20 μL of SYTOX Green (Thermo Fisher Scientific) to give a final well concentration of 5 μM. The well contents were observed using fluorescence microscopy (Olympus IX-70 inverted microscope; Olympus, Essex, UK) and representative images collected in DAPI and fluorescein isothiocyanate (FITC) wavelength channels with an Axiocam MRC camera paired with the Axiovision imaging software (v.4.8; Zeiss, Cambridge, UK). Subsequently, images from the channels were merged using ImageJ software (v.1.50i; National Institutes of Health, USA).

CFUs determined immediately after the bacteria were added to each well at the start of the experiment showed there to be no significant differences between the experimental groups ('pre-spin'; $p > 0.05$; Fig. 1). However, after the 5-min centrifugation to bring the bacteria and ETs into contact (0 h), significantly fewer CFUs were recovered from wells containing rainbow trout ETs compared to control wells containing bacteria but no PMNs, and this was also the case at 1 h (Fig. 1). There were no significant differences in the CFUs recovered from control wells containing bacteria and the wells treated with DNase to degrade the ETs,

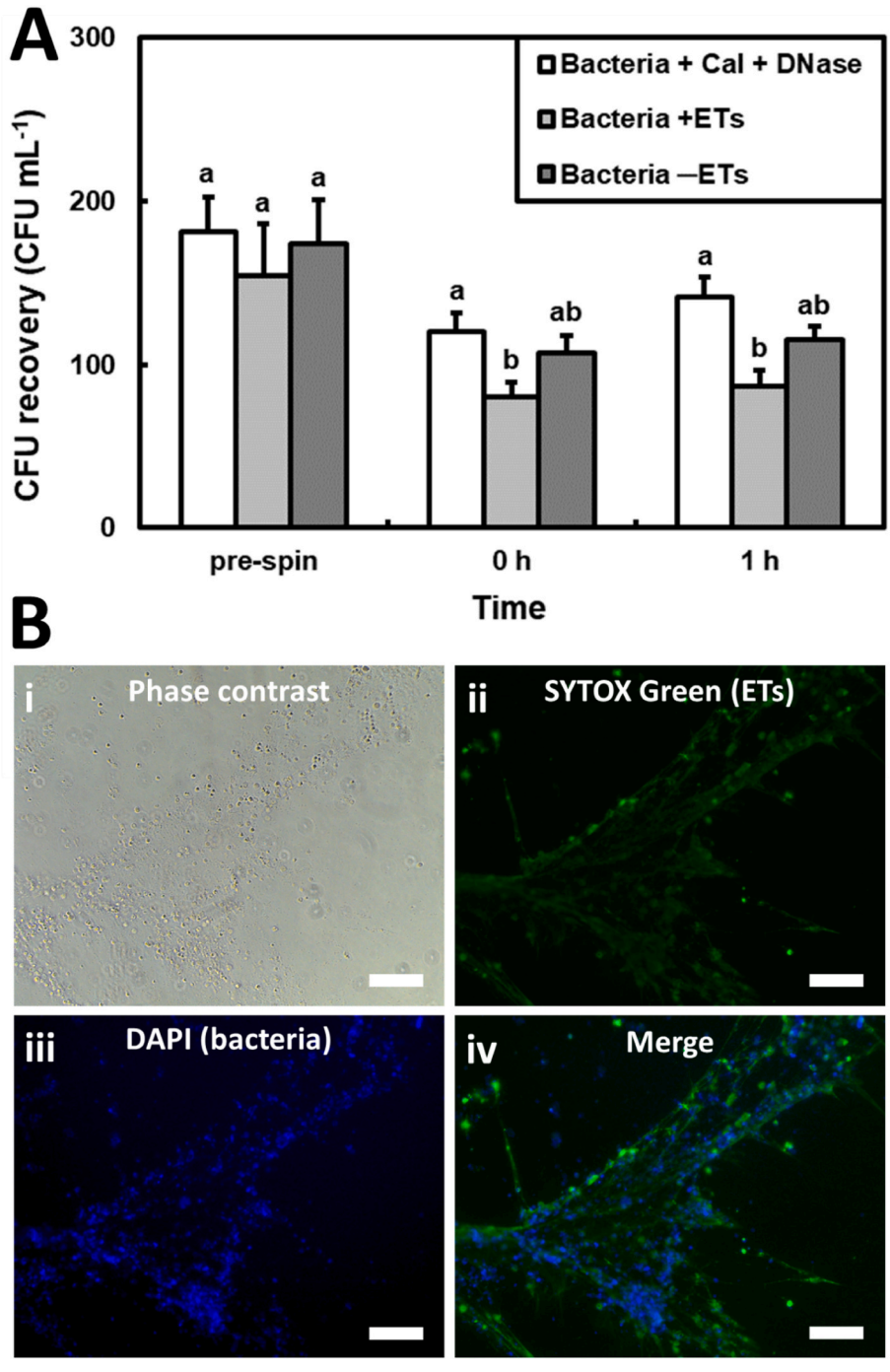

Fig. 1. (A) Bar graph showing the abundance of *V. anguillarum* Vib87 recovered from wells containing 4×10^4 cells of a suspension enriched for polymorphonucleocytes (PMNs) in RPMI-1640 and induced to release chromatin extracellular traps (ETs) by 5 μg mL^{-1} calcium ionophone (+ETs), or where the ETs were digested subsequently with 10 U mL^{-1} DNase-I (−ETs); control wells lacked PMNs and contained just the bacteria, medium and chemical reagents (Bacteria + CaI + DNase). CFU counts were determined immediately after addition of the bacteria (pre-spin); once the centrifugation had brought the bacteria and any ETs into contact (0 h); and after 1 h incubation at 15 °C. Kruskal-Wallis tests were performed to assess differences in CFUs between groups at each sampling time, with significant differences detected at 0 h ($\chi^2_{(2)} = 9.106$, $p = 0.028$) and 1 h ($\chi^2_{(2)} = 9.718$, $p = 0.021$) but not pre-spin ($\chi^2_{(2)} = 1.722$, $p = 0.632$). Post-hoc pairwise comparisons were performed and significant differences between groups at each sampling time are signified by different letters above the bars (error bars are standard error of mean; $n = 6$). (B) Representative phase contrast and fluorescent images of the same field of view showing co-localisation of *V. anguillarum* Vib87 (stained blue by 1 μM DAPI) with rainbow trout ETs (stained green with 5 μM SYTOX Green) after incubation of 4×10^7 CFU/mL bacteria with CaI-induced PMN-enriched cell suspension (4×10^5 cells mL^{-1}) for 1 h at 15 °C. (i) PMNs and bacteria under phase contrast; (ii) FITC channel showing a mesh of ETs; (iii) DAPI channel showing bacteria; (iv) merged DAPI and FITC channels showing co-localisation of bacteria and ETs. Scale bar = 100 μm; all images acquired with a × 40 objective lens.

both immediately after centrifugation and at 1 h, suggesting the DNase concentration was sufficient to degrade the ETs and prevent bacterial trapping (Fig. 1). Though not statistically significant, at both 0 h and 1 h, fewer CFUs were recovered from wells containing ETs compared to those where the ETs had been degraded with DNase (Fig. 1).

After the bacteria and ETs were subject to fluorescence labelling, the bacteria were observed by fluorescence microscopy to co-localise with the chromatin fibres of the ETs, suggesting close interaction (Fig. 1). Notably, the blue-stained bacteria were visibly more abundant in areas overlapping the green-stained ETs compared to elsewhere in the field of view, perhaps indicating attachment to these structures (Fig. 1).

This present study is the first to confirm that ETs released by rainbow trout PMNs can exert antibacterial activity, thereby implicating an antimicrobial function in immunity. ETs reduced the abundance of CFUs of *V. anguillarum* during co-incubation and this effect was reduced by addition of DNase-I that degraded the chromatin fibres of ETs and disrupted their structural integrity. Fluorescence and phase contrast microscopy demonstrated a close association between the bacteria and the ETs. Collectively, these observations suggest trapping and/or bactericidal action for the rainbow trout ETs.

The reduction in CFUs was detected immediately after the centrifugation step to bring the ETs and bacteria into contact, which suggests rapid action. Additionally, although the methodology used in this present study is unable to distinguish definitively trapping from bactericidal action, the lack of a further reduction in CFU after an additional 1 h incubation suggests that trapping is the main mechanism, as the antimicrobial components of the ETs (e.g., histone fragments and neutrophil elastase) typically exert their activity quickly [6,11,12]. The trapping of the bacteria on the chromatin mesh, as observed in the microscopy images, would bring the bacteria into closer proximity, potentially even in clumps, that may explain the formation of fewer CFUs when plated out on agar. Other studies have suggested trapping rather than bactericidal action for ETs, and differences between findings may be explained by bacterial, host and other methodological factors [5]. By trapping bacteria, the ETs prevent their effective dissemination around the host, thus buying time for other immune cells to migrate to the area to counter the threat through the production of humoral components and engulfing the aftermath of ET release and the microbes in the vicinity [5].

Refinement of the described methodology to include the addition of exogenous DNase prior to plating out the contents of wells containing ETs, in order to liberate trapped bacteria from the structures, may provide more conclusive evidence to distinguish trapping from killing. Nevertheless, experiments to determine the action of ETs against microbes are challenging and a very low abundance of bacteria was used in this present study to obtain the sensitivity required to observe an antimicrobial effect, which is consistent with other studies [1]. Even then, qualitative data such as the imaging of ET-bacteria interactions presented here and elsewhere provide necessary additional support for concluding a trapping ability for ETs [1,8,13]. Still, the full potential of ETs to trap bacteria may not be reflected wholly in the CFU data, which showed only modest reductions in this present study.

Much remains to be learnt of ET release in fish, not least the differential effects of ETs on different bacterial species and why it is that certain pathogens resist trapping or killing [6]. Álvarez de Haro et al. [4] reported ETs released by Atlantic salmon to reduce CFUs of *A. salmonicida* but this reduction was not statistically significant, whilst Chi and Sun [8] observed that replication of *Escherichia coli* and *Pseudomonas fluorescens* was inhibited significantly in the presence of tongue sole ETs. Moreover, Zhao et al. [1] observed that *E. tarda* resisted the effects of tongue sole ETs despite the ETs inhibiting the replication of two other fish pathogens, *Ps. fluorescens* and *Vibrio harveyi*. This present study provides new data for another host-pathogen interaction, specifically the effects of rainbow trout ETs on *V. anguillarum*, and further studies on the interactions between bacteria and ETs, particularly *in vivo* investigations, will help to clarify the role of ETs in immunity.

Several studies have demonstrated a role for bacterial nucleases in escaping trapping by ETs [14] and, even in fish, the nucleases produced by the fish pathogen, *Aeromonas hydrophila*, can degrade ETs released by carp in PMN-enriched cell suspensions [15]. Interestingly, a nuclease-deficient mutant of *A. hydrophila* was more susceptible to killing by blunt-snout bream (*Megalobrama amblycephala*) head kidney leukocyte suspensions and was less virulent in the fish than the parent strain [16]. *V. anguillarum* Vib87 was selected for this present study because it lacks detectable DNase activity *in vitro* (Supplementary Fig. 1), which could counteract the trapping action of ETs. Notably, we observed no significant reduction in the abundance of CFUs in the presence of ETs when repeating our experiment with the nuclease-producing strain *V. anguillarum* Vib6 (Supplementary Fig. 1). Still, bacteria are likely to employ other mechanisms in addition to nucleases to counter the antimicrobial effects of ETs and more research of this subject is warranted.

In conclusion, this study is the first to show that ETs released by rainbow trout PMNs can exert antibacterial activity, implicating a role in the innate response against microbial threats. Improved understanding of ETs and their role in defending fish against infection opens up possibilities to manipulate this response for fish health and welfare benefits during production.

Ethics statement

This study was approved by the Institute of Aquaculture Ethics Committee.

CRediT authorship contribution statement

Andre P. Van: Investigation, Data curation, Writing – original draft, Methodology, Formal analysis, Writing – review & editing, Visualization. **James E. Bron:** Supervision, Methodology, Formal analysis, Writing – review & editing, Visualization. **Andrew P. Desbois:** Conceptualization, Supervision, Data curation, Writing – original draft, Methodology, Formal analysis, Writing – review & editing, Visualization.

Declaration of competing interest

The authors confirm they have no known conflicts of interest.

Data availability

Data will be made available on request.

Acknowledgements

The authors are grateful to Dawnfresh Farming and the staff at the Frandy fishery for supplying the fish and to Dr Dawn Austin (Heriot Watt University, UK) for kindly gifting the bacterial strains.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2023.108657.

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