Functional characterization of the mucus barrier on the Xenopus tropicalis skin surface

DOI:
10.1073/pnas.1713539115

Document Version
Accepted author manuscript

Link to publication record in Manchester Research Explorer

Citation for published version (APA):
Dubaisi, E., Rousseau, K., Hughes, G., Ridley, C., Grecis, R., Roberts, I., & Thornton, D. (2018). Functional characterization of the mucus barrier on the Xenopus tropicalis skin surface. Proceedings of the National Academy of Sciences. https://doi.org/10.1073/pnas.1713539115

Published in:
Proceedings of the National Academy of Sciences

Citing this paper
Please note that where the full-text provided on Manchester Research Explorer is the Author Accepted Manuscript or Proof version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version.

General rights
Copyright and moral rights for the publications made accessible in the Research Explorer are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Takedown policy
If you believe that this document breaches copyright please refer to the University of Manchester’s Takedown Procedures [http://man.ac.uk/04Y6Bo] or contact uml.scholarlycommunications@manchester.ac.uk providing relevant details, so we can investigate your claim.
SI Materials and Methods

Fluorescence in situ hybridization
Digoxigenin-labeled probes were generated using the clones TNeu027a13 and ThdA056k18, linearized and transcribed with the T7 RNA polymerase (Promega) to generate RNA probes. Fluorescent in situ hybridization was performed on tadpoles as described previously (31).

Morpholino design and injection
All morpholinos were purchased from Gene Tools. The otagl splice morpholino sequence is 5′-TAGAGTCATACATACCTCATC-3′. The control morpholino sequence is 5′- CCTCTACCTCAGTTACAATTATA-3′. Morpholinos were injected with a 15 ng dose at the one-cell stage.

Long template RT-PCR
RNA was obtained by Trizol (ThermoFisher) extraction and then reverse transcribed to generate cDNA using Superscript IV Reverse Transcriptase (ThermoFisher). Long template PCR was performed on the cDNA using Phusion High Fidelity DNA Polymerase (NEB). Primers used for PCR were as follows: otagl Fwd1 – 5′-TGGGCGTGGGGAATGTTATT-3′. otagl Rev1 – 5′-GTCCCAGTGGCGATTTGTAC-3′. PCR products were gel extracted and cloned into the pCR-XL-TOPO vector.

Single Molecule Real Time (SMRT) sequencing
pCR-XL-TOPO containing PCR product was linearized with NotI (NEB), purified and sequenced using Pacific Biosciences SMRT sequencing platform at the Earlham Institute, UK.

Collection of tadpole secretions and tadpole lysate
Secretions were collected over a 24 hr period from 5000 wild type tadpoles in low salt media (0.01x Marc’s Modified Ringer’s (MMR) solution). High purity GdmCl powder (Sigma) was added to the secretions (final concentration 4 M) and samples were concentrated using a VivaFlow apparatus with 10, 000 MW cutoff (Sartorius Stedim Biotech).

Tadpole lysate was collected in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5 with 0.5% (v/v) NP-40, 5 mM EDTA and 5 mM EGTA) and complete protease inhibitors (Roche) using 10 μl/ tadpole, incubated on ice for 10 min and centrifuged for 5 min to collect supernatant. Samples were
reduced (10 mM dithiothreitol, DTT, 37°C for 1 hr) and alkylated (25 mM iodoacetamide for 30 min in dark at room temperature) if required.

**Otogl purification by CsCl density gradient centrifugation**
Density gradient centrifugation was performed on skin secretions in a Beckman Ti70 rotor, at 40,000 rpm 68 hr 15°C, in 4 M GdmCl/CsCl at a starting density of 1.4g/ml. Tubes were unloaded from the top and fractions were analyzed for glycoproteins with biotin-labeled PNA (1:1000 dilution; Vector labs) followed by streptavidin labeled IRDye-680RD (1:10,000 dilution; Li-Cor) for detection on an Odyssey CLx Imaging System (Li-Cor).

**Rate zonal centrifugation**
Purified Otogl was analysed by rate-zonal centrifugation on 6-8 M GdmCl gradients as described previously (2). Samples were centrifuged in a SW40Ti swing-out rotor at 40,000 rpm for 5 hr.

**SEC-MALLS analysis**
Samples were applied to a Shodex SB-806 M HQ column (Waters corp.) in 25 mM Hepes, 150 mM NaCl pH 7.4. Column eluents passed through an inline DAWN EOS laser photometer and an Optilab rEX refractometer. Analysis was performed using ASTRA version 6 software (13).

**TEM, Cryo-TEM and ESEM**
For EM, Otogl samples were diluted 1:100 in ddH2O and applied onto a glow-discharged carbon coated 400 mesh copper grid (Electron Microscopy Sciences) and incubated for 30 seconds. Grids were then washed in ddH2O and subsequently negatively stained with 2% (w/v) uranyl acetate (Agar Scientific) for 1 min. Using a Tecnai BioTwin at 100 Kv TEM data were recorded at 3.5 Å/pixel. Images were taken using a Gatan Orius SC1000 CCD camera. For cryo-TEM, tadpoles were frozen by immersion into liquid ethane, freeze substituted with 1% osmium tetroxide/ 0.5% uranyl acetate/ acetone solution over 48 hr, gradually increasing temperature, until room temperature. Specimens were washed with several changes of acetone and then infiltrated with TAAB LV resin and polymerized at 60°C in the oven over 24 hr. Ultrathin sections were cut with Reichert Ultracut ultramicrotome. EM images were collected as above. Surface ESEM imaging of live tadpoles was performed on an FEI Model Quanta 200 ESEM.

**Agarose gel electrophoresis, lectin blot and immunoblot**
Reduced samples were run on 0.7% (w/v) agarose gels in 40 mM Tris-acetate, 1 mM EDTA, 0.1% (w/v) SDS, pH 8) at 30 V for 16 hr at room temperature. Otogl was transferred to nitrocellulose via a vacuum blotter (45 mbar for 1 h 30 min). Blots were probed with rabbit polyclonal antibody to Otogl (1:1000; custom made by Eurogentec) and biotin-labeled PNA (Vector Labs). Otogl antibody was detected with goat anti-rabbit IRDye-800CW (Li-Cor) and biotin was detected with streptavidin labeled IRDye-680RD (Li-Cor). Dyes were detected simultaneously on an Odyssey CLx Imaging System (Li-Cor).

**Glycosidase treatment**

O-glycosidase, sialidase and PNGase F digestions were performed according to manufacturers instructions (NEB). Briefly, for O-glycosidase and/or sialidase treatment, samples were denatured at 100 °C for 10 min in denaturing buffer (NEB) and then digested at 37 °C for 2 hr or 4 hr. For removal of N-glycans, samples were denatured as before and then treated with PNGase F for 1 hr at 37 °C. In all cases, samples were reduced and alkylated after glycosidase treatment.

**Immuno-/lectin- fluorescence on whole tadpoles**

Immunofluorescence was carried out as described previously (32). Rabbit anti-Otogl antibody was used at a dilution of 1:1000. Goat anti-rabbit IgG-Alexa Fluor 488 (ThermoFisher Scientific) was used to detect anti-Otogl at a dilution of 1:500. PNA directly conjugated to the fluorophore AlexaFluor-568 (ThermoFisher Scientific) was used at a dilution of 1:1000. Membrane GFP mRNA was injected into embryos to mark cell boundaries as described previously (7). Mouse anti-GFP (ab1218, abcam) was used at a dilution of 1:500 and goat anti-mouse Alexa Fluor 647 (ThermoFisher Scientific) was used to detect anti-GFP at a dilution of 1:500.

**Cryopreservation and immuno-/lectin- fluorescence on sections**

To preserve a mucus barrier, tadpoles were snap-frozen in liquid N₂ and immediately transferred to optimal cutting temperature compound on a dry ice cooling bath (33). Blocks were sectioned (12 µm) on a Cryostat (Leica) and immunofluorescence performed as described previously (32). Mouse anti-
GFP was used at a dilution of 1:1000 and PNA-AlexaFluor-568 at a dilution of 1:1000. Goat anti-mouse Alexa Fluor-488 (1:500; ThermoFisher Scientific) was used as a secondary antibody for GFP.

**Infection of tadpoles with E. coli DH5α(pCOC2)-GFP and A. hydrophila**

An overnight culture of *E. coli* DH5α(pCOC2)-GFP (19) in Luria-Bertani (LB) medium, supplemented with 100 μg/ml ampicillin, was centrifuged at 3500 rpm for 10 min, resuspended in 0.01x MMR and incubated at room temperature with the control and Otogl morphant tadpoles for 15 min prior to snap freezing. For the infection time course, a 10 ml overnight culture of *A. hydrophila* (ATCC7966) was diluted 1:100 in Tryptic Soy Broth (Sigma) and cultured for 3 hr 30 mins at 30 °C until OD$_{600}$ nm of ~0.9. The culture was centrifuged at 3500 rpm for 10 minutes, washed and resuspended in 0.01x MMR at the same volume as the starting culture. The culture was diluted 1:8 in 0.01x MMR based on a preliminary experiment to determine the dose at which wild type tadpoles are able to survive. This dilution was determined to correspond to 1.5 x 10$^8$ c.f.u./ml based on Miles and Misra counting (34). MOC and Otogl MO injected tadpoles (20 of each) were added to 4 ml of the diluted culture and survival frequency was recorded over 48 hr. The criteria for survival were active swimming and response to touch. Mortality was associated with skin peeling/blistering.

To generate GFP-expressing *A. hydrophila*, the DNA sequence corresponding to *gfpmut1* was amplified by PCR from pNF8 plasmid (35) using Taq polymerase (NEB) and TA-cloned into the pCR2.1 plasmid (ThermoFisher Scientific) so that *gfpmut1* would be under the control of the *plac* promoter. The pCR2.1-*gfpmut1* construct was transformed into *A. hydrophila* by calcium chloride-mediated transformation (36) and transformants selected on media supplemented with kanamycin at 50 μg/ml. For the infection time course, the same procedure as described with the inclusion of kanamycin to maintain plasmid selection.

**Mass spectrometry**

Tryptic peptides were separated by reverse phase liquid chromatography (LC) and analyzed by tandem mass spectrometry (LC-MS/MS) using a NanoAcquity LC (Waters, Manchester, UK) coupled to a LTQ Velos mass
spectrometer (Thermo Fisher Scientific). The data produced were searched using Mascot 2.5 (Matrix Science, UK) software against a custom database that included the predicted OtoGl protein sequence.
**Supplementary Figure S1**
Full Otogl amino acid sequence translated from single molecule real time sequencing with the different domains highlighted in the colours shown in the key. Highlighted in yellow are the peptides identified by mass spectrometry of purified secreted Otogl. Underlined is the peptide (repeated four times) used to generate an antibody to Otogl.

**Supplementary Figure S2**
Alignment of Otogl with human gel-forming mucins (MUC2, MUC5AC, MUC5B and MUC6) shows conservation of protein sequence in D2, D’, D3 and CK domains. Clustal Omega analysis, indicates 30.57 % identity of Otogl with human MUC5B in region from D2 to end of D3 (44 % similarity (positives)). There is 30 % identity of human MUC5B and Otogl in the CK domain (45 % similarity), with complete conservation of the position of cysteine residues. D2, D’, D3 and CK domains are annotated. Conserved cysteine residues are highlighted in yellow, black boxes are identical amino acids and grey boxes represent similar amino acids.

**Supplementary Figure S3** Alignment of repeats in the mucin domain
Alignment of sequence repeats in the Otogl mucin domain. Proline, threonine and serine (PTS) residues are highlighted in blue. Cysteine residues are highlighted in red. The consensus sequence (see right of alignment) CCxxxxC forms the basis for the alignment, whilst three other cysteine residues are also well conserved. These are named Cys-rich regions (highlighted with **). Through this alignment, PTS-rich regions are also evident (highlighted with *).

Overall, there are 39 Cys-rich regions and 34 PTS-rich regions in the mucin domain. Peptides identified in the mucin domain by mass spectrometry are underlined, with the vast majority identified in the Cys-rich regions.

**Supplementary Figure S4** Otogl morphants have a depleted ‘mucus’ surface layer
Representative environmental scanning electron micrographs of the skin surfaces of live MOC and Otogl MO injected tadpoles. The boxes highlight globular features that are more abundant in MOC than Otogl MO injected tadpoles. Scale bars represent 50 µm.

**Supplementary Figure S5** Infection studies with heat-treated *A. hydrophila* and live *A. hydrophila*-GFP
(A) Survival time course of MOC and Otogl MO-injected tadpoles in 0.01x MMR containing 1.5 x 10^8 c.f.u./ml of *A. hydrophila*, heat-killed at 65 °C for 30 mins prior to infection (at time point 0 hr). (B) Survival time course of MOC and Otogl MO-injected tadpoles in 0.01x MMR containing 1.5 x 10^8 c.f.u./ml of GFP-expressing *A. hydrophila* (at time point 0 hr). (C) Chart showing frequency of bacteria located inside MOC and Otogl MO-injected tadpoles at timepoint 18 hr 30 mins. Bars represent mean number of bacteria found within MOC (n = 3 tadpoles) and Otogl MO (n = 3 tadpoles) injected tadpoles. Error bars represent standard error of the mean. (D) Representative image of an Otogl morphant tadpole section at 34 hr timepoint showing internally localized GFP-expressing *A. hydrophila* bacteria. White line on image to the left represents the apical surface membrane from brightfield images (right). Note the presence of a highly pigmented region (white arrow). Scale bar; 10 µm.
** PTPVPTPQKCTGVCVEMNCVKGEKRVEIAITTDPCCPRYKC
EVITPSPTSPNPCRGVCNVPTCNETQQLVQVTSDDCPCKYTC
QCLTCSPPPVCSDQRPPIIEFDTSQCCARKYEC
LPETTAPRVTTPQCGVCVEVTCEAEEDKVAIYSTDCCSRYVC
VSSSTTPTPAEPSTPSCTKVCKQVVCVSGETKTEVPSADCPPRYKC
YQFPTTPILATTPCLCKYVCTSVTVCADGETEMEVSADCCPRYTC
VEPTVAPTPEEPCRHVCSDTCAVGEIKTEVPSSADCCARYVC
APLPSTTPLPEPQHVTCEVDTCADGETKMEPFQDPCCPRYVC
VPAASSTTPQPELCKYVCTAVTADGETEMEVPSADCCPRYTC
VPFTTTAEPESPSTVCATDVCAAGETRTEAPSDPCCSRYVC
VPLPTTTKLPFPQYCTDPADGETKMEPKIDCPDDRYVC
VPESSTTPQPELCKYVCTAVTADGETQMVPSADCCPRYTC
VPFTTITPEEPESHTCVTDVCAAGETRTEAPSDPCCSRYVC
VPLQTETELPEPQYCTSDTVCADGETKMEPKIDCPCCARYVC
VPESSSTTPQPELCKYVCTAVTADGETQMEVPSADCCPRYTC
VPFTTIPEEPESHTCTDVCAAGETRTEAPSDPCCSRYVC
VPLQTETELPEPQYCTSDTVCADGETKMEPKIDCPCCARYVC
VPESSSTTPQPELCKYVCTAVTADGETQMEVPSADCCPRYTC
VPFTTIPEEPESHTCTDVCAAGETRTEAPSDPCCSRYVC
VPLQTETELPEPQYCTSDTVCADGETKMEPKIDCPCCARYVC
VPESSSTTPQPELCKYVCTAVTADGETQMEVPSADCCPRYTC
APFPSTTPLPEPQYCTSDTCTDGETKMEPKIDCPCCSRYVC
VPQNTTQTLPTTTTLPFVEQGVCNTVTGCDGDMVALEDSDPCPHYAC
VIATLPPFTTPVTQVCSGVCKCSEVVCCKKEGETVEVESADPCPRHVC
KCSDDSLPPTTDGRPLKTDPTOCFCYEKC
RPVSETTPVPVTPISCQSGVCTCCKKEEQVQVESPSDPCPKFVC
ECAGSCSPPVPCVDQPQVQIDTAAATCCTPYEC
LPPIPTLPPNTTPEEPCKDVLCPVINICINGSSLV8FMKRPCCOKYEV
LCLDPCPPPLCTKGTTPKIDPPEACCPPYTC
PPEKVEKTTTPYETQFTTTAAPTSPSTTLPECAGVCKYKDVTCRYNEDEEIEEPFPPDCP PRYRC
ERKPTPSPTSPELTTTPRPPITTTPEECAGVCTSKELTCAYNEIETKPNLDPCCPYLC
EPKPTPSPTSPELTTTPRPPITTTPEECAGVCTSKELTCAYNEIETKPNLDPCCPYLC
EPKPTPSPTSPELTTTPRPPITTTPEECAGVCTSKELTCAYNEIETKPNLDPCCPYLC
EPKPTPSPTSPELTTTPRPPITTTPEECAGVCTSKELTCAYNEIETKPNLDPCCPYLC
VPKPTPQPTPPEVSTTPRPPDTTPPECTGVTCYKDVCQYNEIEAEPNPYPDCP PRYRC
EPEKTIQTQSPSTIPTKTPCAAGVTCNNKHFCAPNENEVIEPNPSDCPCP NYYC
EPKSTISPSGPSTSTRIISTTKIDCKRICVTKTCSRLSEKLLIEVNPYDPCCP1QKC
EQCKTVPKCRSSERLVFVSQMGCCPKLKC
