Supporting Information

Dubaissi et al. 10.1073/pnas.1713539115

SI Materials and Methods

Fluorescence in Situ Hybridization. Digoxygenin-labeled probes were generated using the clones TNeu027a13 and ThdA045k18, linearized, and transcribed with the T7 RNA polymerase (Promega) to generate RNA probes. Fluorescence in situ hybridization was performed on tadpoles as described previously (31).

Morpholino Design and Injection. All morpholinos were purchased from Gene Tools. The *otogl* splice morpholino sequence is 5′-TAGAGTCATACATACCTCCATCATC-3′. The control morpholino sequence is 5′-CCTCTTACCTCAGTTACAATTTATA-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: *otogl* Fwd1—5′-TGGGCGTGGGGAATGTTATT-3′ and *otogl* Rev1—5′-GTCCCAGTGGCGATTTGTAC-3′. PCR products were gel-extracted and cloned into the pCR-XL-TOPO vector.

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.

Collection of Tadpole Secretions and Tadpole Lysate. Secretions were collected over a 24-h period from 5,000 wild-type tadpoles in low-salt media (0.01× 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 a 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% (vol/vol) Nonidet P-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 DTT, 37 °C for 1 h) 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 × *g* 68 h 15 °C in 4 M GdmCl/CsCl at a starting density of 1.4 g/mL Tubes were unloaded from the top, and fractions were analyzed for glycoproteins with biotin-labeled PNA (1:1,000 dilution; Vector Laboratories) 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 analyzed by rate-zonal centrifugation on 6- to 8-M GdmCl gradients as described previously (2). Samples were centrifuged in a SW40Ti swing-out rotor at 40,000 × *g* for 5 h.

SEC-MALLS Analysis. Samples were applied onto a Shodex SB-806 M HQ column (Waters) 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 ddH_2O and applied on a glow-discharged carbon-coated 400-mesh copper grid (Electron Microscopy Sciences) and incubated for 30 s. Grids were then washed in ddH2O and subsequently negatively stained with 2% (wt/vol) 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 SC1000CCD 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 h, and temperature was gradually increased until it reached 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 h. 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% (wt/vol) agarose gels in 40 mM Tris–acetate, 1 mM EDTA, 0.1% (wt/vol) SDS, pH 8, at 30 V for 16 h 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 (1:1,000; custom made by Eurogentec) and biotin-labeled PNA (Vector Laboratories). 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 the manufacturer's 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 or 4 h. For removal of N-glycans, samples were denatured as before and then treated with PNGase F for 1 h 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 1:1,000. 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 1:1,000. 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 N_2 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 was performed as described previously (32). Mouse anti-GFP was used at a dilution of 1:1,000 and PNA-AlexaFluor-568 at a dilution of 1:1,000. 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 medium, supplemented with 100 μg/mL ampicillin, was centrifuged at 3,500 × *g* for 10 min, resuspended in 0.01× MMR, and incubated at room temperature with the control and

Otogl morphant tadpoles for 15 min before 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 h 30 min at 30 °C until $OD_{600\ nm}$ of ∼0.9 was reached. The culture was centrifuged at 3,500 × *g* for 10 min, washed, and resuspended in 0.01× MMR at the same volume as the starting culture. The culture was diluted 1:8 in 0.01× 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 1.5×10^8 cfu/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 h. 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 then cloned into the pCR2.1 plasmid (TOPO-TA cloning kit, 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 were selected on media supplemented with kanamycin at 50 μg/mL. For the infection time course, the same procedure was followed as described with the inclusion of kanamycin to maintain plasmid selection.

Mass Spectrometry. Tryptic peptides were separated by reverse-phase liquid chromatography and analyzed by tandem mass spectrometry using a NanoAcquity LC (Waters) coupled to a LTQ Velos mass spectrometer (Thermo Fisher Scientific). The data produced were searched using Mascot 2.5 (Matrix Science) software against a custom database that included the predicted Otogl protein sequence.

MRVLPILVVTVACLVGTGAGDDGDCFYNDKIYKNGDTVKSNCQICECENGKIKDCARDVNCKIKRAVTPEQDEVPI
VYDDDPSVIESSEGKGNVRAKRAAVGVNKKNSAATKFSLGITGTDEDKEFLKTNVKNDKVNTNQNQGKGHDDSRKD
SKENSQEKGQENNKKFSKDSSEEKDDGSKEKWGNVKNDKVNTNQNQGKGHDDSRKDSKDNSQEKGQQNNKRFSKDS
SEEKDNASKEKSTFKKTYSVQTSQNQGKGHDDSSSESKENKKGQNKNGFSKDSSEEKDDGSKEKSAFKKTYSVQTS
QNQGKGHDDSSSESKENKKGQNKNGFSKDSSEEKDDGSKEKSGHGKNDKNKPNINKGNDHDDSSKESKENTDRQKG
QKNKNGWLKNSSHEKDDGSKEKEGHWKNGKGKPKQNKGKDQGDSSNESKSNSQEKGQNKDSSVEIVPTVSNENTGD
EEDNNVTPSVTPVVEQKRDQDISTDGPEEAVINSCIFNKESIADGETKDLSCSSCTCNKGKLTCMKNLNCPGVCSV
TGYQMIRTFDGSLYDSPGTCNYVLVKTSNFTISLSNKPCSGQDNGITNPDAVCIDTVDIYLPTIASIKILPDGSVL
SAGQQVDLPFFIAETITILRSSSTFLDVVTPMFSLQYDIKGNRLYVIVQTSYKDQTSGLCGTYNDNRLDDFRSSSD
ITETVSSLFSKSWKLQSQCSENVKTSDSPAQRVTADLSCTNSLQDSIFADCNLVIDTHNYQASCSNSIYFGKDNGF
CSAMADYAYRCALAGIYVPLSSAFPDCFVSCEGGMVPITESLFSQDDCAEHSSEILKFLIKLPLSEGCVCPDNLYY
DSTINQCVDGDVCPCYSNSHAYKIGENVTFSNGQSCTCERIMECTDNPELPTDEVPTCPEGEVFSDCLMGSGKSCE
PSCQNMAVIDQVCPLECEAGCICKYGTLRSNDGSCVPLQECPCVHGEDVYNPGETLAQDCNTCTCKDGKFSCTNNA
CNVVCNAYAGGQFFLFDNIWKTFNTGNCQIILVESQDGQVPKFRVVMQNTQTENLGGALATKKLFITFGGAYVELS
DSDPYLAQEPGSTTQVRVYRSGFYVVAHFLDGLAVYYDQHLDVIVQLQPQLQGKVQGMCGDADQSTTYELAISNMA
QYGAQFIIGKCDSPDVPQPPPPDNKHYTEKRCSQLFNEDFAECHIAVPVQPYYTACVEETEACREGESCLCFCTSL
AAYARACCRKGITLDWRNPDTCSSPCEYYNRDAGDGPYRLVMVNGQTLMADYDTQSVSLTPKDTPGNVKVSFMVTP
SLYKDPQYGRKLISLESALHRNFFIVQNADGTLGLKKWQPSASFRTQATFILREGRWINGYSTLESYSSRGQYLSF
DSNNDNLVMTRVKSANMLAMNFKLTEENFGLPSFSICTWKYRSCGPPCIPTCQDPQATQCTLTIPVEGCYPICAPG
MVFDEVTHRCVNKDDCVPTPTVPPTPVPPTPTQKPCTGVTCVEMNCKVGEKRVEIATTDPCCPRYKCEVIPTTPSP
PTSPNPCRGVTCNVPKCNTEQQLVQVTSTDPCCPKYTCQCLTTCSPPPVCSNGRPPIENFDPTSQCCAKYECLPET
TTPAPRTTTPGQCKGVTCEIVTCAEGEDKVAIVSTDPCCSRYVCVSSTTPTPIKPTTPESCTKVKCQVVTCSVGET
KTEVPSADPCCPRYKCYQEPTTTLATTPELCKYVTCSTVTCADGETEMEVPSADPCCPRYTCVPETTVAPTPEEPC
RHVTCSDVTCAVGEIKTEVPSADPCCARYVCAPLPSTTPLPEPCQHVTCEDVTCADGETKMEGPQIDPCCARFVCV
PASSTTPQPELCKYVTCATVTCADGETEMEVPSADPCCPRYTCVPGTTIPTPEEPCSHVTCTDVTCAAGETRTEAP
SADPCCSRFVCVPLPTTTKLPEPCQYVTCPDVTCADGETKMEGPKIDPCCDRYVCVPESSTTPQPELCKYVTCATV
TCADGETQMEVPSADPCCPRYTCVPGTTIPTPEEPCSHVTCTDVTCAAGETRTEAPSADPCCSRFVCVPLQTTTEL
PEPCQYVTCSDVTCADGETKMEGPKIDPCCARYVCVPESSTTPQPELCKYVTCATVTCADGETQMEVPSADPCCPR
YTCVPGTTIPTPEEPCSHVTCTDVTCAAGETRTEAPSADPCCSRFVCVPLQTTTELPEPCQYVTCSDVTCADGETK
MEGPKIDPCCARYVCVPESSTTPQPELCKYVTCATVTCADGETEMKVPSADPCCPRYTCVPGTTIPTPEEPCSHVT
CTDVTCAAGETRTEAPSADPCCSRFVCVPLPTTTELPEPCQYVTCSDVTCADGETKMEGPKIDPCCARYVCVPESS
TTPQPELCKYVTCATVTCADGETQMEVPSADPCCPRYTCVPGTTIPTPEEPCSHVTCSDVTCAVGEFKTEAPSSDP
CCARFVCAPFPSTTPLPEPCQYVTCSDVTCTDGETKMEGPKIDPCCSRYVCVPQPNTTPLPTTTTTLPEVCQGVTC
NTVTCGDGEDMVALESSDPCCPHYACVKIATLPPFPTTPVVTQGVCSEVKCEVVKCKKEGETVVEVPSADPCCPRH
VCKCSSDCSLPPTCTDGRPPLKTIDPDTQCCPEYECRPVSETTPVPVTPSICSGVTCKTVTCKKEGEQVVEVPSSD
PCCPKFVCECAQSCSPPVPCVDGQPPVQITDAATKCCPTYECLPPIPTLPPNTTPEEPCKDVLCPVINCIKNGSSL
VSFMGKNPCCKVEYCDCLDPCPPPLTCKTGKPTMKIDPEAECCPTYTCPPEKVPEKTTTPYETQFTTTAPTPSSTT
LPECAGVKCYKDVTCRYNEDEIEEPNPFDPCCPRYRCERKPTPSPTPSPELTTTPRPPIITTPPECAGVTCSKELT
CAYNEIEVTKPNPLDPCCPLYYCEPKPTPSPELTTTPRPPIFTTPPECAGVTCSKELTCAYNEIEVAEPNPLDPCC
PIYHCEPKPTPSPELTTTPRPPIFTTPPECAGVTCYKDVTCRYNEDEIVEPNPFDPCCPRYRCEPKPTPSPELTTT
PRPPIFTTPPECAGVTCYKDVTCRYNEDEIEEPNPFDPCCPRYRCVPKPTPGPTPPPEVSTTPRRPDFTTPPECTG
VTCYKDVTCQYNEIEIAEPNPYDPCCPRYRCEPKITTIQPPSTIPITTKPECAGVTCNKKHFCAPNENEVEIPNPS
DPCCPNYYCEPKSTISPSGPPSTTRIISTTKIDCSKRICVKTSCRLSEKLIEVPNPYDPCCPIQKCECQCKTVPKC
RSDERLVPVSQNGQCCPKLKQERKRDECHPVRKTMLLKSGQCSASVELNVCSGYCHSKTEYTSFWQPITHCRCCSI
KTDRFMTFQLPCPDGTRTSLTVRNAVTCGCNTCSGPYEPGSGSGSGSGSGSGSGDGGSSDGSGFHEWSEFIRS

Fig. S1. Full Otogl amino acid sequence translated from SMRT with the different domains highlighted in the colors 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.

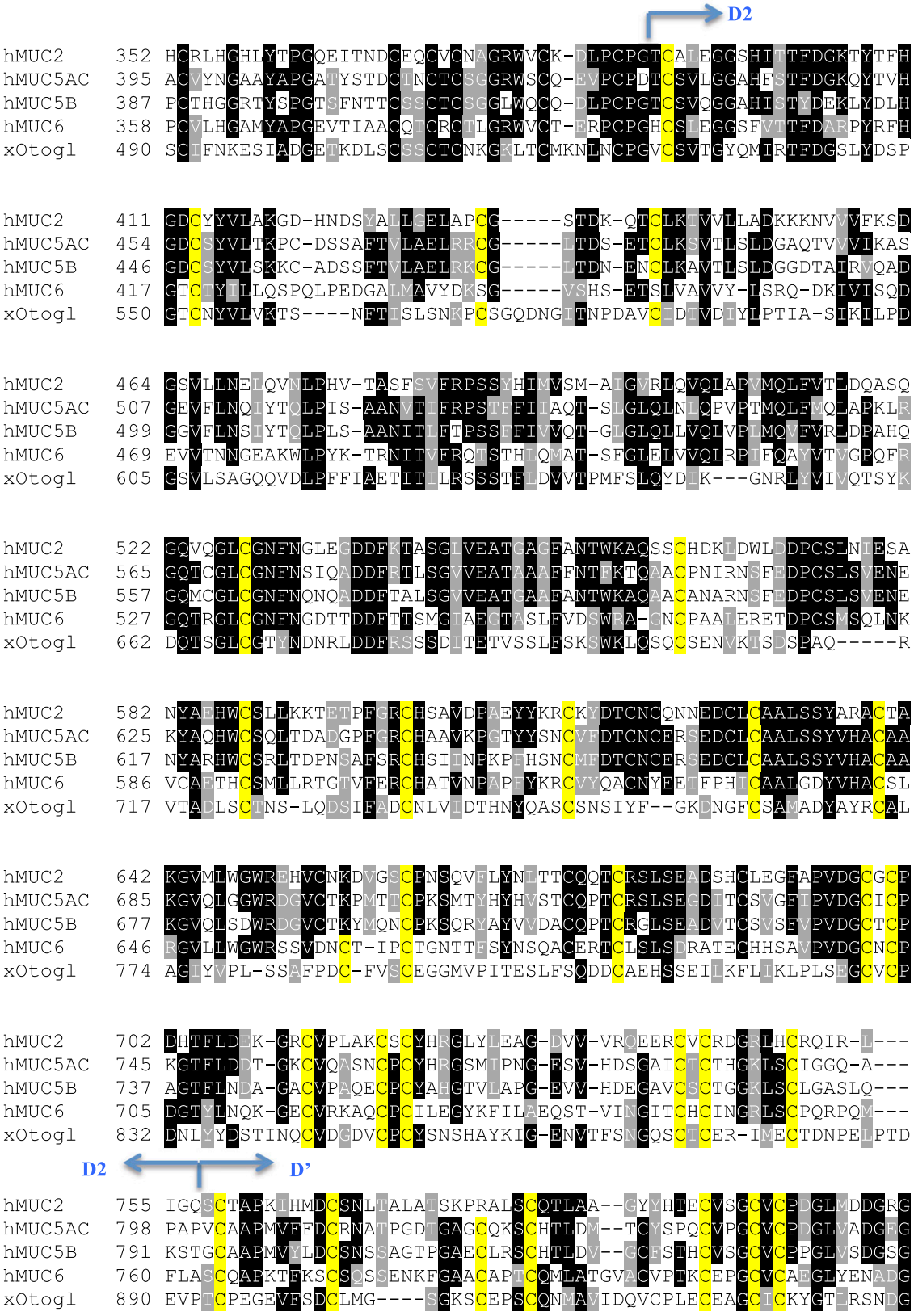

Fig. S2. (Continued)

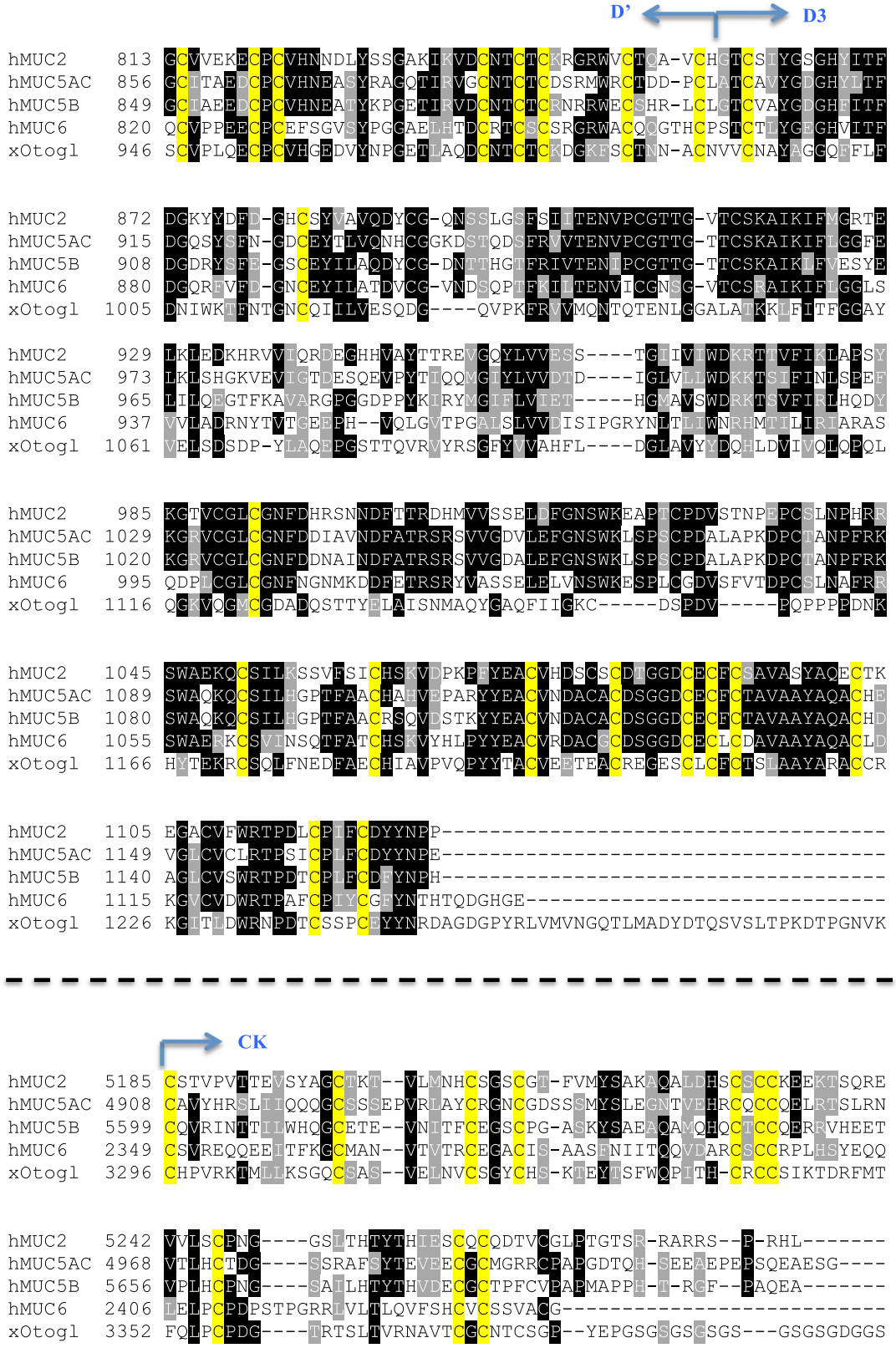

Fig. S2. Alignment of Otogl with human gel-forming mucins (MUC2, MUC5AC, MUC5B, and MUC6) shows conservation of protein sequence in D2, D′, D3, and CK domains. Clustal Omega analysis indicates 30.57% identity of Otogl with human MUC5B in the region from D2 to the end of D3 [44% similarity (positives)]. 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 gray boxes represent similar amino acids.

Fig. 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-aligned sequences) CCxxxxC forms the basis for the alignment, while three other cysteine residues are also well conserved. These are named Cys-rich regions and are highlighted with double asterisks (**). Through this alignment, PTS-rich regions are also evident and highlighted with a single asterisk (*). 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.

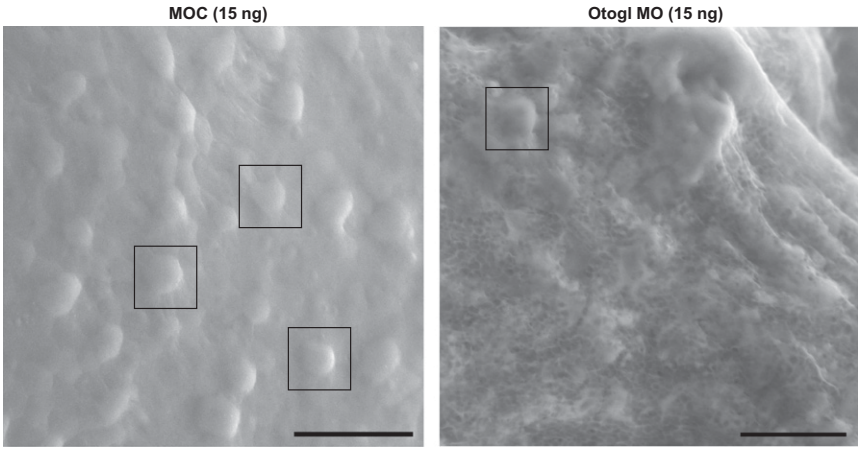

Fig. S4. Otogl morphants have a depleted mucus surface layer. Representative environmental scanning electron micrographs of the skin surfaces of live MOC- and Otogl MO-injected tadpoles. The boxes highlight globular features that are more abundant in MOC- than in Otogl MO-injected tadpoles. (Scale bar: 50 μm.)

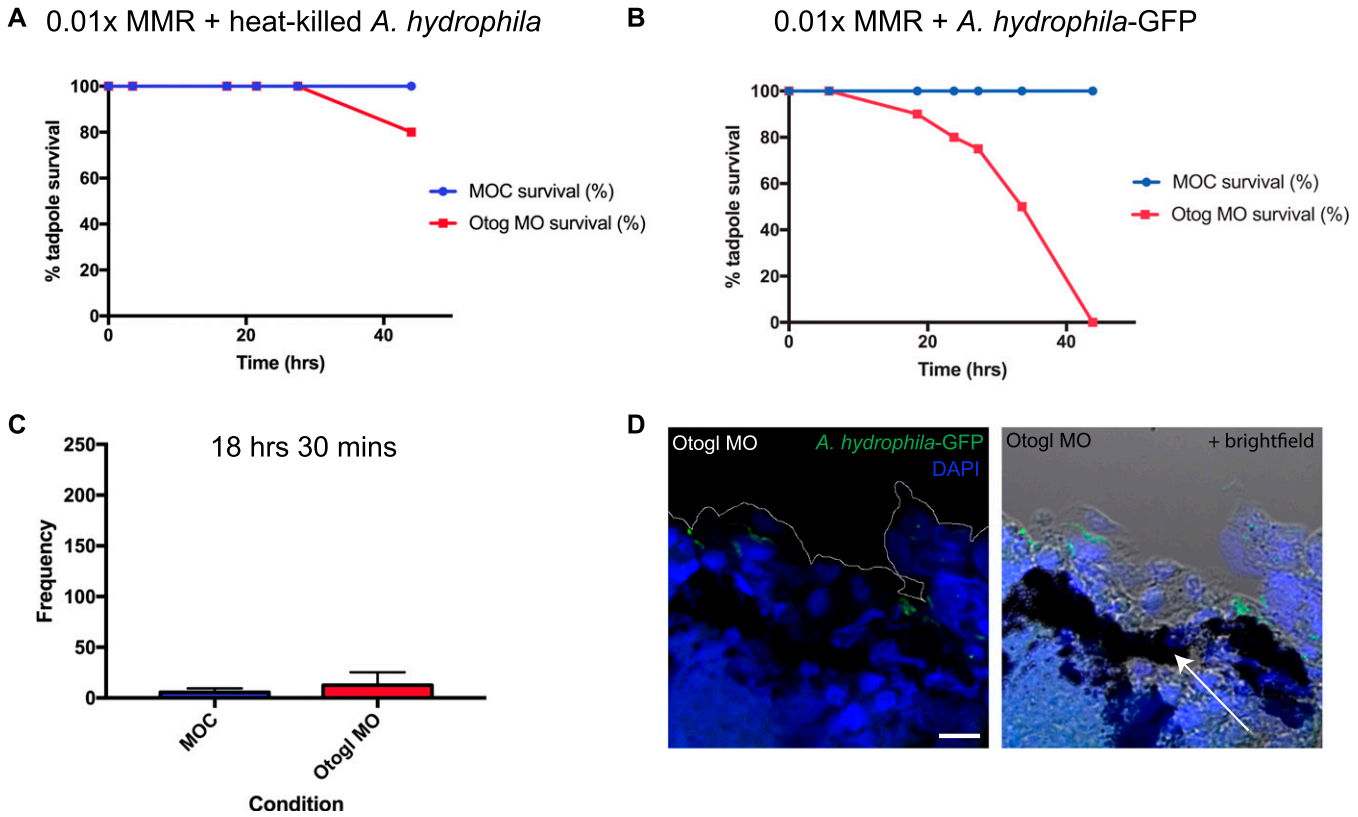

Fig. S5. Infection studies with heat-treated *A. hydrophila* and live *A. hydrophila*-GFP. (*A*) Survival time course of MOC- and Otogl MO-injected tadpoles in 0.01× MMR containing 1.5×10^8 cfu/mL of *A. hydrophila*, heat-killed at 65 °C for 30 min before infection (at time point 0 h). (*B*) Survival time course of MOC- and Otogl MO-injected tadpoles in 0.01× MMR containing 1.5×10^8 cfu/mL of GFP-expressing *A. hydrophila* (at time point 0 h). (*C*) Chart showing frequency of bacteria located inside MOC- and Otogl MO-injected tadpoles at time point 18 h 30 min. Bars represent mean number of bacteria found within MOC (n = 3 tadpoles)- and Otogl MO (n = 3 tadpoles)-injected tadpoles. Error bars represent SEM. (*D*) Representative image of an Otogl morphant tadpole section at 34-h time point showing internally localized GFP-expressing *A. hydrophila* bacteria. White line on *Left* image represents the apical surface membrane from brightfield images (*Right*). Note the presence of a highly pigmented region (white arrow). (Scale bar: 10 μm.)