Mucin glycans attenuate the virulence of Pseudomonas aeruginosa in infection

Kelsey M. Wheeler1,2, Gerardo Cárcamo-Oyarce3, Bradley S. Turner3, Sheri Dellos-Nolan4, Julia Y. Co1,2, Sylvain Lehoux5, Richard D. Cummings5, Daniel J. Wozniak4 and Katharina Ribbeck2,3*

A slimy, hydrated mucus gel lines all wet epithelia in the human body, including the eyes, lungs, and gastrointestinal and urogenital tracts. Mucus forms the first line of defense while housing trillions of microorganisms that constitute the microbiota. Rarely do these microorganisms cause infections in healthy mucus, suggesting that mechanisms exist in the mucus layer that regulate virulence. Using the bacterium Pseudomonas aeruginosa and a three-dimensional (3D) laboratory model of native mucus, we determined that exposure to mucus triggers downregulation of virulence genes that are involved in quorum sensing, siderophore biosynthesis and toxin secretion, and rapidly disintegrates biofilms—a hallmark of mucosal infections. This phenotypic switch is triggered by mucins, which are polymers that are densely coated with O-linked glycans that form the 3D scaffold inside mucus. Here, we show that isolated mucins act at various scales, suppressing distinct virulence pathways, promoting a planktonic lifestyle, reducing cytotoxicity to human epithelia in vitro and attenuating infection in a porcine burn model. Other viscous polymer solutions lack the same effect, indicating that the regulatory function of mucin does not result from its polymeric structure alone. We identify that interactions with P. aeruginosa are mediated by mucin-associated glycans (mucin glycans). By isolating glycans from the mucin backbone, we assessed the collective activity of hundreds of complex structures in solution. Similar to their grafted counterparts, free mucin glycans potently regulate bacterial phenotypes even at relatively low concentrations. This regulatory function is likely dependent on glycan complexity, as monosaccharides do not attenuate virulence. Thus, mucin glycans are potent host signals that ‘tame’ microorganisms, rendering them less harmful to the host.

To identify mechanisms in native mucus that control pathogens, we tested whether exposure to native intestinal mucus alters biofilm integrity. Our model organism was the opportunistic pathogen P. aeruginosa PA01, which is not pathogenic in healthy individuals (Fig. 1a), but can cause severe morbidity or death in people with compromised immunity, aberrant mucus production (Fig. 1b) or burn wounds4.5. Mature P. aeruginosa biofilms were exposed to buffer or native intestinal mucus. Although buffer alone did not affect biofilm integrity (Fig. 1c), biofilms exposed to mucus dissociated from the surface and 70% of cells shifted into the planktonic phase (Fig. 1d). As the growth rate was not altered in mucus relative to buffer alone (Supplementary Fig. 1), the shift to planktonic growth may arise from phenotypic regulation by mucus. Mucus-mediated biofilm dispersal is dependent on an intact flagellum (Fig. 1c), indicating that mucin triggers an active motility-driven escape, rather than mechanical disruption. To test whether mucin affects only biofilms or whether it more broadly regulates virulence traits, we measured the expression of genes that are important for establishing infection. This revealed that intestinal mucus transcriptionally suppresses quorum sensing (lasR), siderophore biosynthesis (pvdA) and type-3 secretion (pceV; Fig. 1e). Native gastric and salivary mucus similarly suppressed major infection-related genes (Fig. 1c), suggesting that virulence suppression is conserved across various mucosal surfaces. Together, these findings demonstrate that mucin contains factors that modulate bacterial behaviours at the levels of gene expression and phenotype.

To identify specific regulatory factors within complex mucus, we tested mucin fractions that were separated according to molecular weight. The primary bioactive component of whole mucus was larger than 100 kDa (Fig. 1f), indicating mucin polymers as possible candidates. Mucins are a major high-molecular-mass component of mucus that disperse cells in isolation. Adding purified mucins back into depleted mucus was sufficient to restore biofilm dispersal and suppression of virulence genes (Fig. 1f–h), providing strong evidence that mucin glycopolymers constitute key regulatory cues inside mucus. Here the use of natively purified mucins was critical because commercially available mucins harbour reduced chemical complexity owing to the harsh purification process.

To directly test whether mucins trigger a global transcriptional response, we performed RNA sequencing (RNA-seq) on P. aeruginosa that were grown with or without (0.5% w/v) MUC5AC or MUC5B, which are the most abundant gel-forming mucins secreted in niches colonized by P. aeruginosa, including the nasal and oral cavities, respiratory tract, eyes and middle ear (Fig. 2a,b). Both mucins triggered a genome-wide response (Fig. 2c, Supplementary Table 1) and suppressed many virulence pathways, including type-1, -2, -3 and -6 secretion systems, siderophore biosynthesis (pyoverdine and pyochelin) and quorum sensing (Fig. 2d, Supplementary Table 2). Among the upregulated genes, we detected enrichment in the denitrification pathway (Supplementary Table 3), consistent with inverse regulation by quorum sensing. Several metabolic genes were also differentially regulated, including those associated with fumarate metabolism and amino acid and C5-carboxylate transport (Supplementary Table 1). These results are consistent with metabolic changes, which often correlate with changes in the denitrification pathway.
Virulence. Importantly, we found no enrichment in virulence pathways among upregulated genes (Supplementary Table 3). There were differences in the exact genes that were differentially regulated, as well as differences in the magnitudes of those changes after exposure to MUC5AC or MUC5B (Fig. 2c, Supplementary Fig. 2, Supplementary Table 4), suggesting differences in mucin biochemistry and specific regulatory function. We replicated this effect with various media, time points and bacterial strains (Supplementary Fig. 3, Supplementary Tables 5–7), demonstrating that mucin suppresses the expression of virulence genes across a range of experimental conditions.

We validated the regulation of virulence using quantitative PCR (qPCR; Supplementary Fig. 4). To confirm the phenotypic relevance of these changes, we combined functional biochemical assays and infection models (Fig. 2e,i). Protease activity and siderophore production were lower in supernatants from P. aeruginosa exposed to mucin than in supernatants from bacteria grown in medium alone (Supplementary Fig. 5). Consistent with the downregulation of
Fig. 2 | Mucins are sufficient to attenuate *P. aeruginosa* virulence in vitro and in vivo. a, Gene expression was evaluated in liquid culture with or without the native mucin network. b, The predominant gel-forming mucins secreted into mucosal niches throughout the body. The sources of mucin used in this study are highlighted. c, MUC5AC and MUCSB elicit global transcriptional responses in *P. aeruginosa* PAO1. A complete list of fold-change values and false discovery rate (FDR)-adjusted P values is provided in Supporting Table 1. Fold-change data are average measurements. FDR-adjusted P values were determined using the Benjamini–Hochberg P-value adjustment method. Data are from *n* = 6 (no mucin treatment), *n* = 3 (MUC5AC-treated) and *n* = 3 (MUCSB-treated) biologically independent replicates. Correspondence plots of the fold-change values are provided in Supplementary Fig. 2. Principal component analysis of expression data is provided in Supporting Fig. 13. The Venn diagrams contain the total number of genes that are differentially expressed (FDR-adjusted P < 0.05) after exposure to 0.5% w/v MUC5AC (purple) or MUCSB (orange). The significance of overlap was assessed using a hypergeometric test. Functional enrichment analysis of the non-overlapping regions of the Venn diagrams is provided Supporting Fig. 2. d, Functional enrichment analyses identify key virulence pathways among downregulated genes. Significance of enrichment was assessed using one-sided Mann–Whitney U-tests, for which ranking was calculated on the basis of mean log2-transformed fold changes from *n* = 6 (no mucin treatment), *n* = 3 (MUC5AC-treated) and *n* = 3 (MUCSB-treated) biologically independent replicates. Bars, FDR-adjusted P values. The red dashed line indicates FDR-adjusted P = 0.05. e, *P. aeruginosa* pathogenicity was evaluated in cell culture (containing a single human epithelial cell type, HT-29). f, Exposure to increasing MUC5AC concentrations inhibited *P. aeruginosa* attachment to HT-29 cells. The centre line indicates the median, the box limits indicate the upper and lower quartiles and the whiskers indicate 1.5x the interquartile range. Data are from *n* = 7 (no MUC5AC), *n* = 4 (0.01% MUC5AC), *n* = 4 (0.05% MUC5AC), *n* = 4 (0.1% MUC5AC) and *n* = 7 (0.5% MUC5AC) biologically independent replicates. Significance was assessed in relation to the medium-alone control using ordinary one-way analysis of variance (ANOVA), followed by Dunnett’s multiple comparisons test; ***P < 0.001. g, MUC5AC protects HT-29 epithelial cells from death in a concentration-dependent manner. Dotted lines indicate the 95% confidence interval for the dose-response curve. Data are based on bulk measurements of propidium iodide fluorescence 7.5 h after infection. Data are mean ± s.e.m.; *n* = 4 biologically independent replicates. IC50, half-maximum inhibitory concentration. h, MUC5AC maintains the intact epithelial cell monolayer and prevents the onset of HT-29 cellular rounding, bacterial attachment and HT-29 death. Representative confocal microscopy of HT-29 epithelial cells (bright field), GFP-expressing *P. aeruginosa* PA01 cells (green) and propidium iodide staining (red) after exposing HT-29 cells to *P. aeruginosa* for 5 h (top, medium alone) or 6 h (bottom, medium with MUC5AC) as indicated. Similar results were observed in different fields of view across three independent replicates. Scale bar, 20 μm. i, Bacterial viability was monitored in a live dermal wound model (containing living tissue, immune cells and secreted factors). j, Bacterial burden on porcine burn wounds decreases after treatment with 0.5% MUC5AC for 7 d. Symbols represent *P. aeruginosa* PA01 burden on six individual biopsies collected from two pigs following no treatment (circle), treatment with 0.05% MUC5AC (square) or treatment with 0.5% MUC5AC (triangle). The centre bars indicate the mean bacterial burden. Significance was assessed using Kruskal–Wallis tests followed by Dunn’s multiple comparisons test. k, MUC5AC in isolation does not alter *P. aeruginosa* viability relative to medium alone. Data are mean CFU ± s.e.m., *n* = 3 biologically independent replicates.
and less-aggregated planktonic-like state with cells still detectable at ≥50 μm from the glass surface (Supplementary Fig. 6). Collectively, these data highlight two facets of mucin regulatory function—one in which mucins induce a global transcriptional response that ‘dis-arms’ *P. aeruginosa* by downregulating important virulence genes, and another in which mucins suppress aggregation and bacterial attachment to surfaces. Mucin-mediated changes were sufficient to neutralize antagonistic interactions between *P. aeruginosa* and human cells. By monitoring the survival of human epithelial cells over time, we determined that MUC5AC reduced *P. aeruginosa*-mediated epithelial cell death in a dose-dependent manner (Fig. 2g) while maintaining the morphology and confluency of the epithelial cell monolayer (Fig. 2h).

To better understand the relevance of the regulatory function of mucin in a complex biological system of multiple cell types and an active immune system, we exposed *P. aeruginosa*-infected porcine burn wounds to a wound dressing containing MUC5AC and quantified the bacterial burden over time by counting colony-forming units (CFU; Fig. 2i, Supplementary Fig. 8). Exposure to MUC5AC resulted in two-log reductions in CFU in wounds one week after infection, compared with no reduction in the mucin-free mock treatment (Fig. 2i). The sustained clearance of *P. aeruginosa* detected here is probably not due to direct killing by MUC5AC, as viability was not inhibited by mucins in isolation (Fig. 2k). Rather, mucin likely mediates bacterial clearance through the regulation of microbial phenotypes, which attenuates pathogens and thereby facilitates host-mediated clearance.

It is tempting to speculate that mucin triggers this phenotypic switch by maintaining cells in the planktonic non-aggregate form. To test this hypothesis, we evaluated whether mucins still suppressed virulence genes in mutants lacking flagellar motility (ΔmotABCD) or the ability to aggregate (Δpsipel; Fig. 3a). Both mutants responded to mucins (Fig. 3b), indicating that the virulence-attenuating function of mucin is not a downstream consequence of changes to motility or aggregation, but rather a parallel effect. Integrating our RNA-seq results with the major genetic components of the *P. aeruginosa* virulence network9 (Fig. 3c) revealed that the broad virulence-attenuating effect of mucin probably involves multiple regulatory systems. Polymer solutions such as mucin have complex structural and biochemical properties that could directly or indirectly trigger signalling events through many sensory systems in *P. aeruginosa*. One possibility is that mucins trigger a general response through their electrostatic or hydrophobic properties or by creating geometric constraints. An alternative hypothesis is that the observed response is triggered by sensing specific biochemical moieties that are presented on secreted mucins. To distinguish between these two possibilities, we tested whether carboxymethylcellulose (CMC)—a well-established mucin mimic with charge and viscoelastic properties that are similar to those of native mucins10—could elicit changes in *P. aeruginosa* virulence. Importantly, exposure to CMC...
Fig. 4 | Complex O-linked glycans are the major regulatory component of MUC5AC. a. Oligosaccharides released by alkaline p-elimination were resolved using capillary electrophoresis. The mucin glycan pool includes extended chains consisting of >7 residues (bottom). Top, glucose (Glc) polymer standards. b. Monosaccharide composition of mucin glycan was assessed using capillary electrophoresis (bottom). These mucin oligosaccharides are predominantly O-linked, as evidenced by the ratio of mannose (Man; N-linked) to N-acetylgalactosamine (GalNAc; O-linked). The red labels indicate the quantification standard maltose (Malt) and the migration standard galacturonic acid (GalA). Migration times for monosaccharides standards (top) were as follows: N-acetylgalactosamine, 4.5 min; N-acetylmannosamine (ManNAc) from N-acetylaceuraminic acid, 4.95 min; N-acetylgalactosamine (GlcNAc), 5.26 min; mannose, 6.08 min; glucose (Glc), 6.33 min; xylose (Xyl), 6.99 min; fucose (Fuc), 7.29 min; and galactose (Gal), 7.67 min. c. MALDI-TOF spectrum of O-linked glycans from MUC5AC. The complete list of glycan structures with experimental and theoretical masses is provided in Supplementary Table B. For a–c, similar results were observed in three independent replicates. d. Representative images of GFP-expressing P. aeruginosa biofilms after a 3 h treatment with medium alone, 0.01% mucin glycan or a 0.01% pool of monosaccharides. Glycan solutions reduce biofilm biomass in the WT strain, but not the flagellar mutant (ΔfliD). Similar results were observed in different fields of view across three independent replicates. Scale bar, 20 μm. e. Mucin glycan, but not monosaccharides, disperse biofilm biomass into the planktonic state for the WT strain, but not the flagellar mutant (ΔfliD). Data are mean ± s.e.m.; n = 3 biologically independent replicates. Significance was assessed in relation to the medium-alone control using one-way ANOVA followed by Dunnett’s multiple comparisons test. f. Mucin glycan inhibit bacterial attachment to human epithelial HT-29 cells. The centre line indicates the median, the box limits indicate the upper and lower quartiles and the whiskers indicate 1.5 times the interquartile range. Data are from n = 6 (medium alone), n = 2 (0.01% mucin polyaccharides) and n = 6 (monosaccharides) biologically independent replicates. Significance was assessed in relation to the medium-alone control using one-way ANOVA, followed by Dunnett’s multiple comparisons test. g. Relative size distributions of aggregates were identified using live 3D confocal microscopy and analysed using IMARIS. In medium alone and medium with monosaccharides, P. aeruginosa biomass is concentrated in large surface-associated aggregates, whereas MUC5AC glycan suppress the formation of aggregates. The centre line indicates the median, the box limits indicate the upper and lower quartiles and the whiskers indicate 1.5 times the interquartile range. Data are average aggregate sizes compiled from six separate z-stacks. Significance was assessed using the Kruskal-Wallis test followed by Dunn’s multiple comparisons test. h. Low concentrations of MUC5AC glycan elicit a transcriptional response that positively correlates with transcriptional changes elicited by whole MUC5AC. Fold-change data are average measurements from three biologically independent replicates. Significance was assessed using a regression slope test. i. Free glycan suppresses the same virulence pathways as whole mucin. The significance of enrichment was assessed using Mann–Whitney U-tests, for which ranking was calculated on the basis of mean log2-transformed fold changes from three biologically independent replicates. j. Growth is not altered by the presence of the monosaccharide components in mucin glycan. Data are mean optical density at 600 nm (OD600) ± s.e.m.; n = 3 biologically independent replicates. k. Complex mucin glycan, but not their monosaccharide components, induce expression changes in signature virulence genes. Data are qPCR measurements of relative gene expression ± s.e.m., n = 3 biologically independent replicates. l. Bacterial burden on porcine burn wounds decreases after treatment with 0.1% mucin glycan for 7 d. Symbols represent the burden of P. aeruginosa PA01 on individual biopsies collected from burn wounds following no treatment (circle, n = 6) or treatment with 0.1% MUC5AC glycan (square, n = 3) or treatment with 0.1% monosaccharides (triangle, n = 3). The centre bar indicates the mean bacterial burden. Significance was assessed in relation to the no-treatment control using Kruskal-Wallis tests followed by Dunn’s multiple comparisons test.
did not differentially regulate signature virulence genes (Fig. 3d), prevent surface attachment (Supplementary Fig. 9) or protect epithelial cells from \( P. \ aeruginosa \)-induced death (Supplementary Fig. 9). These results suggest that the polymeric structure of mucin is not sufficient to mediate these virulence-attenuating effects. Rather, specific biochemistry that is present in mucins, but not in CMC, is necessary to attenuate \( P. \ aeruginosa \) virulence.

Native mucins display a plethora of complex glycan structures that are covalently linked to serine and threonine\(^{11-19} \), creating a wealth of biochemical information with the potential to influence microbial gene expression. \( P. \ aeruginosa \) uses many strategies to sense and respond to host signals, enabling it to coordinate the switch between its pathogenic and host-compatible states\(^{20} \). The sensing of signature mucin-glycan motifs may be an effective mechanism to limit the production of metabolically costly virulence factors in niches in which virulence would not be advantageous. The potential of complex mucin glycans to regulate microbial behaviour has been largely overlooked owing to fundamental technical limitations, including the difficulty of purifying intact mucins, non-standard methods for isolating O-linked glycans and the analytical complexity of predicting glycan structures\(^{16} \). To determine whether glycans contribute to the virulence-neutralizing capability of mucin, we assessed the degree to which glycans isolated from the MUC5AC glycans contribute to the virulence-neutralizing capability of mucin, specifically glycans in our library were predominantly built on core-1 (Gal\(^1\)-3(GlcNAc\(^β\)_1-6)GalNAc) (Fig. 4d,e), prevent attachment to glass (Supplementary Fig. 11) and yielded bacterial aggregates smaller than those formed in mature biofilms (Fig. 4d, Supplementary Fig. 10) into the planktonic medium alone or with monosaccharides (Fig. 4g). To determine the mucin-regulated pathways that were specifically altered in response to mucin glycans, we exposed \( P. \ aeruginosa \) to a pool of potential glycan cues and monitored changes in gene expression. RNA-seq revealed that \( P. \ aeruginosa \) that was exposed to relatively low concentrations (0.01% w/v) of free MUC5AC glycans undergoes a transcriptional response that mirrors that associated with exposure to 0.5% (w/v) whole native mucin that comprised up to 0.4% (w/v) grafted glycans (Fig. 4h). Functional enrichment analyses confirmed that the same virulence pathways suppressed by intact MUC5AC were also suppressed by MUC5AC glycans (Fig. 4i), further indicating that an integral part of mucin, rather than another mucus-associated factor, is the primary virulence-neutralizing agent in mucus. We anticipate that increasing the glycan concentration to levels present in mucin will improve the dynamic range of the transcriptional response. Importantly, the viability of \( P. \ aeruginosa \) was not altered by the presence of monosaccharides that are present in mucin glycans (Fig. 4j). Exposure of \( P. \ aeruginosa \) to a pool of these monosaccharides did not trigger dispersal (Fig. 4d,e), prevent attachment to glass (Supplementary Fig. 11) or human cells (Fig. 4f), or differentially regulate quorum sensing (IasR), siderophore production (pvdA) or type-3 secretion (pcrV) genes (Fig. 4k). Monosaccharide exposure also did not suppress the production of virulence factors, even with increasing concentrations of monosaccharides (Supplementary Fig. 12), although a non-significant effect on bacterial burden in vivo was detected (Fig. 4l). On the basis of these data, we conclude that the complex arrangement and particular stereochemistry of these sugar residues are critical to their function as regulatory signals.

The diversity of O-linked glycans on mucins exceeds even that on tissue surfaces\(^{16-18} \), and their complexity makes them ideal for encoding biological information with a high degree of specificity. In this way, mucins present and retain a myriad of potential regulatory cues. We propose that microorganisms probably evolved mechanisms to recognize, process, uptake and respond to specific moieties within the complex array of mucin glycans\(^{26} \). The question remains as to how mucin glycans interact with, and are sensed by, \( P. \ aeruginosa \) at the molecular level. One possibility is that glycans directly serve as a signal through a carbohydrate binding site in a global regulatory system, such as those affecting the secondary messenger c-di-GMP or the non-coding RNAs rsmY and rsmZ\(^{26} \). Potential glycan sensors have been identified in \( P. \ aeruginosa \) that are thought to feed into regulatory virulence pathways and have annotated carbohydrate binding sites, such as the two-component sensor LadS and the diguanylate cyclase NicD\(^{15-17} \). Mucin glycans may also trigger metabolic changes by serving as a nutritional substrate, or regulate signalling pathways through interactions with specific \( P. \ aeruginosa \) lectins or surface adhesins. We speculate that the structural diversity of mucins and mucin-associated glycans enables them to interact with several bacterial receptors and mediate distinct functions.

Collectively, our findings reveal a previously unrecognized role for mucin glycans as potent host-derived regulators of a bacterial phenotype that has broad implications for how the body prevents mucosal infections while maintaining a diverse microbiota. The reason why diseased mucus no longer retains the ability to attenuate virulence\(^{25-28} \) remains an open question. Based on our findings that glycans trigger a switch in bacterial phenotype, we postulate that changes to mucin glycosylation patterns in disease, such as increased sialylation\(^{44} \), will alter both the binding properties of mucin with microorganisms and its protective function. By isolating a mucin-glycan library, we have established the conceptual and technical framework to systematically address these open questions about the glycan regulatory code. We posit that cracking this code will explain the influence of glycans on the virulence of microorganisms that interact with the host through mucosal surfaces. Identification of specific bioactive glycans will likely reveal a class of therapeutics for treating intractable bacterial infections and may inspire treatment strategies that tune host glycan signals to attenuate virulence and stabilize the healthy microbiota.

**Methods**

**Strains and growth conditions.** Batch cultivation of \( P. \ aeruginosa \) strains (Supplementary Table 9) was carried out under shaking at 37 °C in Luria–Bertani broth (LB; Difco). Gentamicin (30 μg ml\(^{-1} \)) was added to the medium for strains with gentamicin-resistant plasmids.

For whole-mucus experiments, overnight cultures of PAO1 were diluted tenfold in ABTGC (ABT minimal medium, described previously\(^{37} \), supplemented with 5 g glucose and 5 g casamino acids) and grown for 4 h. Then, 75 μl of these cultures was exposed to 75 μl of solubilized mucus or solubilization buffer (described in the ‘Preparation of whole mucus’ section) for 1 h at 37 °C in a static 96-well microtitre plate (Cellstar.

For experiments involving liquid culture, overnight cultures of PAO1 were diluted to an OD\(_{600}\) of 0.01 in 150 μl ABTGC with or without MUC5AC (0.01%–0.5%), MUC5B (0.5%), CMC (0.5%; Sigma), MUC5AC glycan (0.01%) or monosaccharide mixture (0.01%), and grown for 5 h at 37 °C in a static 96-well microtitre plate. Note that, on the basis of the data shown in Fig. 1b, the monosaccharide mixture contained equal weights of galactose, N-acetylgalactosamine, N-acetylgalactosamine, fucose and sialic acid (all of the sugars were obtained from Sigma). The concentration of glycans was selected on
the basis of the mucin dose response curves shown in Fig. 2; in these experiments, we found that 0.01% was the lowest mucin concentration with measurable effects (Fig. 2). Owing to the technical challenges of preparative-scale purification of mucins, we used the minimal inhibitory concentration in this research.

**Collection of human saliva.** Submandibular saliva was collected from human volunteers using a custom vacuum pump, pooled, centrifuged at 2,500g for 5 min and protease inhibitors were added as previously described. Samples of human saliva were collected after explaining the nature and possible consequences of the studies, obtaining informed consent and receiving approval from the institutional review board and Massachusetts Institute of Technology’s Committee on the Use of Humans as Experimental Subjects under protocol no. 1312006096.

**Preparation of whole mucus.** Mucus was scraped from fresh pig stomachs and intestines and solubilized (1 g scrapings in 5 ml) in 0.2 M sodium chloride buffer with protease inhibitors (5 mM benzamidine hydrochloride, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride and 5 mM EDTA, pH 7) and 0.04% sodium azide (Sigma). Cellular debris and food waste was removed using low-speed centrifugation at 8,000g (7,000 r.p.m., Sorvall GS-3 rotor) for 30 min at 4°C.

**Mucin purification.** This study used native porcine gastric mucins (MUC5AC), porcine intestinal mucins (MUC2) and human salivary mucins (MUC5B), which differ from industrially purified mucins in their rheological properties and bioactivity. Human mucins eluted from preparative gels were purified using repeated centrifugal evaporation.

In brief, mucus was scraped from fresh pig stomachs and intestines, solubilized in sodium chloride buffer (described above) and insoluble material was removed by ultracentrifugation at 190,000g RCF for 1 h at 4°C (40,000 r.p.m., Beckman 50.2 Ti rotor with polycarbonate bottles). Submandibular saliva was collected from human volunteers using a custom vacuum pump, pooled, centrifuged and protease inhibitors were added. Mucins were purified using size-exclusion chromatography on separate Sepharose CL-2B columns. Mucin fractions were then desalted, concentrated and lyophilized for storage at −80°C. Lyophilized mucins were reconstituted by shaking gently at 4°C overnight in the desired medium. Mass spectrometry is routinely used to monitor the composition of purified mucin extracts. This type of analysis has shown that mucin extracts purified from porcine stomach mucus, for example, are composed predominantly of MUC5AC, with small quantities of MUC2, MUC5B and MUC6, as well as histones, actin and albumin.

**Isolation of mucin oligosaccharides.** Here we applied non-reducing alkaline β-elimination amionolysis to dissociate non-reduced glycans from mucins. Purified mucins were dissolved in ammonium hydroxide saturated with ammonium carbonate and incubated at 60°C for 40 h to release oligosaccharide glycosylamines and partially deglycosylated mucins by centrifugal filtration through 3–5 kDa molecular weight cut-off membranes in accordance with the manufacturer’s instructions (Amicon Ultra). The resulting oligosaccharide glycosylamines were converted to reducing oligosaccharide hemiacetals by reductive amination as described for oligosaccharides. Neutral monosaccharides were obtained by hydrolysis in trifluoracetic acid for 3–5 h at 70°C. Lyophilized mucins were reconstituted by shaking gently at 4°C overnight in the desired medium.

**Capillary electrophoresis of oligosaccharides.** Reducing oligosaccharides released from mucins were labelled by reductive amination using the fluorescent tag 8-amino-1,3,6-trisulfonic acid, sodium cyanoborohydride and citric acid. Labelled oligosaccharides were analysed using polyvinylalcohol coated N-CHO capillaries in accordance with the manufacturer’s (SciEx/Beckman) protocol using a PA800 (Beckman) capillary electrophoresis instrument, detected with laser-induced fluorescence and analysed using the 32 Karat software. The relative sizes of separated oligosaccharides were determined by comparison with the migration times of glucose polymer standards. Chromatograms were constructed in GraphPad Prism (v.7.04).

**Capillary electrophoresis of monosaccharides.** The monosaccharide composition of released mucin glycans was determined using established methods. In brief, neutral monosaccharides were obtained by hydrolysis in trifluoracetic acid for 1 h at 80°C. The liberated monosaccharides were labelled with 8-amino-1,3,6-trisulfonic acid using reductive amination as described for oligosaccharides. Monosaccharides were analysed with capillary electrophoresis on a bare silica capillary with small quantities of MUC2, MUC5B and MUC6, as well as histones, actin and albumin.

**RNA preparation.** Total RNA was extracted using the MasterPure RNA Purification kit (Lucigen) and residual DNA was removed using the Turbo DNA-free kit (Ambion). The integrity of the total RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). 16S, 23S and 5S rRNA were removed using the Ribo-Zero Magnetic Kit (Bacteria; Epicentre).

**RNA-seq.** Gene expression analysis was performed using Illumina RNA-seq. RNA-seq was conducted for three biological replicates. The libraries were produced using the KAPA RNA HyperPrep kit (Kapa Biosystems). The libraries were sequenced using the Illumina HiSeq platform with a single-end protocol and read lengths of 40 or 50 nucleotides.

**Analysis of sequencing data.** Sequencing reads were mapped onto the *P. aeruginosa* PA01 reference genome, which is available for download from the Pseudomonas Genome Database (http://www.pseudomonas.com) using the Galaxy server. Gene expression values were normalized on the basis of library size and differentially expressed genes were identified using a negative binomial test with an FDR-adjusted P < 0.05. Expression changes in signature virulence genes identified by RNA-seq were validated using qPCR, which revealed strong concordance between the two methods (Supplementary Fig. 4).

**Functional category (pathway) assignments were downloaded from the Pseudomonas Genome Database. Pathway enrichment analysis was performed in MapMan (version 3.2.0b) using the MetaCore database (GeneGo, Inc.) and R (v.3.4.0) using the DISeq2 workflow.** For all analyses of the sequencing data, P values were adjusted for multiple comparisons using Benjamini–Hochberg correction to obtain FDR-adjusted P values.

**RT–qPCR analysis.** A list of the primers used in this study is provided in Supplementary Table 9. qPCR with reverse transcription (RT–qPCR) was performed using a two-step method. First-strand cDNA was synthesized from total RNA using the ProtoScript II First Strand CDNA Synthesis kit (NEB). The CDNA was used as a template for RT–qPCR using a SYBR PowerUp Master Mix kit (Applied Biosystems by Life Technologies) on a Roche LightCycler 480 real-time PCR system. Primers for RT–qPCR were designed on the basis of previously published literature or using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The genes *rpoD* and *proC* were used as endogenous controls. The elimination of contaminating DNA was confirmed using qPCR amplification of *rpoD* on control samples that did not have reverse transcriptase added during cDNA synthesis. Melting-curve analyses were used to verify single-product amplification. Changes in gene expression were calculated on the basis of mean changes in qPCR cycle threshold (AC) using the ΔΔCt method (fold change = 2−ΔΔCt).

**Dispersal of static *P. aeruginosa* biofilms.** Under static conditions, *P. aeruginosa* biofilm dispersal was assayed as previously described, with slight modifications. In brief, an overnight culture of PA01-GFP or PA01-GFP Δ*aiD* was diluted in ABTGC medium to an initial OD600 of 0.01, added to a glass-bottom or plastic 96-well plate and incubated for 48 h at 37°C under static conditions. The supernatant containing non-adherent cells was removed from the plate and the biofilm remaining in each well was washed at least three times with 0.9% NaCl. The biofilms were exposed to whole mucus, the solubilization buffer, ABTGC medium alone or ABTGC medium containing either 0.01% mucin glycans or monosaccharides. The biofilms were statically incubated at 37°C for 3 h. The plates were washed three times with 0.9% NaCl and resuspended in ABTGC medium, then examined using microscopy to determine the remaining biofilm biomass. Viable dispersed cells were quantified using CFU counts on LB agar plates. Image acquisition was performed using a confocal laser scanning microscope (LSM 800, Zeiss) equipped with a ×63/1.4 NA oil-immersion or a ×100/1.4 NA oil-immersion objective. The excitation wavelength for GFP was 488 nm. At least five stacks were recorded for each well and at least three independent wells. Biofilm quantification was performed using IMARIS (v.7.7.2).

**Measurement of protease activity.** Bacterial cultures were pelleted by centrifugation (13,200g for 3 min at room temperature), and the supernatants were filter-sterilized using 0.2 μm filters.

Protease IV activity. The activity of protease IV in cell-free culture supernatants was measured by breakdown of the chromogenic substrate Chromozym PL (Roche),
which reacts specifically with protease IV in *P. aeruginosa* culture supernatants. In brief, 10 µl pretreated sample and 3 µl Chromozym PL (7 nM) were combined in reaction buffer (20 mM Tris-HCl, pH 8.0) to a total volume of 100 µl in a microowell plate. The plates were assayed on a SpectraMax M Series Multi-Mode Microplate Reader (Molecular Devices) pre-equilibrated to 30°C by measuring the rate of increase in absorbance at 405 nm at 3 min intervals for 30 min. Protease IV activity was calculated using the following equation:

\[ F = E 	imes d 	imes A/\Delta A/\min \]

where \( F \) is the dilution factor, \( E \) is the extinction coefficient (which is 10.4 at 405 nm), \( d \) is the path length (at 100 µl volume in a microtiter plate, path length = 0.53 cm) in m, and \( A \) is the maximum change in absorbance at 405 nm. The protease activity was normalized to account for variation in bacterial cell growth on the basis of the OD_{600} of the culture at 5h. Relative changes were calculated on the basis of the protease activity in medium alone.

**Alkaline protease activity.** Alkaline protease activity was tested using a modified method that was described previously. Samples containing 1 mg of Hidex powder azure (Sigma) dissolved in buffer (0.075 ml) consisting of 20mM Tris-HCl, pH 8.0 and 1 mM CaCl₂ were mixed with 0.025 ml of the culture supernatants. The reaction mixtures (0.1 ml) were incubated at 37°C for 3 h. Undissolved substrate was removed by centrifugation at 4,000g for 5 min. The absorbance of the reaction mixtures was determined at 595 nm using a SpectraMax M Series Multi-Mode Microplate Reader (Molecular Devices). Protease activity was expressed in terms of protease Um⁻¹, where one unit is equivalent to an increase of 1.0 OD₅₉₅h⁻¹ at 37°C. Protease activity was normalized to account for variation in bacterial cell growth on the basis of the OD_{600} of the culture at 5h. Relative changes were calculated on the basis of the protease activity in the medium alone.

**Measurement of siderophore fluorescence.** Pyoverdine and pyochelin levels were simultaneously quantified on the basis of characteristic fluorescence spectra, as previously described. In brief, fluorescence was measured on a SpectraMax M Series Multi-Mode Microplate Reader (Molecular Devices). Pyoverdine production was quantified using an excitation wavelength of 400 nm and an emission wavelength of 600 nm. Pyochelin fluorescence was quantified using an excitation wavelength of 350 nm and an emission wavelength of 410 nm. To account for the background fluorescence of pyoverdine, pyochelin production was calculated using the following equation:

\[ z = w - 36 - 7y^2 - 0.0413xy \]

where \( w \) is the actual value of pyochelin production, \( x \) is the fluorescence measured at an excitation/emission of 350 nm/410 nm and \( y \) is the pyoverdine fluorescence measured at an excitation/emission of 400 nm/600 nm.

**Quantification of bacterial growth and polyostrene-attached biomass.** Growth curves were measured in microtiter plates on a SpectraMax M Series Multi-Mode Microplate Reader (Molecular Devices) by measuring the absorbance at 600 nm (OD₆₀₀) and by CFU counts. Adherent biomass was quantified using crystal violet staining. Cells attached to the wells of the plate were washed three times with 0.9% NaCl and stained with 0.1% crystal violet for 15 min at room temperature. The wells were then washed three times with 0.9% NaCl and ethanol was then added to each well. After 15 min, crystal violet staining was quantified by measuring absorbance at 595 nm (OD₅₉₅) and then normalized to the density of the culture (OD₆₀₀) at 5h.

**Human cell culture.** Authenticated mycoplasma-free HT-29 cells (ATCC, HTB38), a human carcinoma cell line with epithelial morphology, were obtained directly from the American Type Culture Collection. Cell morphology was authenticated mycoplasma-free HT-29 cells (ATCC, HTB38), cell morphology was characterized by phase-contrast microscopy, and cells were periodically tested for contamination by PCR. The cells were cultured in DMEM with GlutaMAX (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C in 5% CO₂ and 95% air. Cells were split 1:12 and passaged as the culture reached confluence. To prepare for co-culture with bacteria, HT-29 cells were detached from the glass surface with 0.05% trypsin and 1 mM EDTA (Gibco), resuspended in DMEM and FBS, counted, diluted to the appropriate density in DMEM and FBS, and seeded in 96-well plates to confluence (approximately 5 × 10⁵ HT-29 cells).

**Examination of bacterial interactions with epithelial cells.** In all of the experiments, control and experimental HT-29 cells were treated identically except that the co-culture medium did not contain bacteria in the uninected controls. Bacteria were co-cultured with HT-29 cell monolayers at an initial multiplicity of infection of 20 (1 × 10⁸ CFU for 1–8 h. Subsequently, HT-29 cells were processed and assayed in the cell-function assays described below.

**Analysis of bacterial attachment.** After 1 h of co-culture, non-adherent bacteria in the supernatant were aspirated and quantified by serial dilution as CFUs. HT-29 cell monolayers were washed three times with phosphate-buffered saline (PBS, Gibco) and cells were lysed with 1% Triton X-100 (Sigma) and removed from the growth surface. The attached bacteria were serially diluted in PBS and quantified as CFUs. Epithelial cytotoxicity assay. The killing of HT-29 cells by *P. aeruginosa* was measured using the membrane-impermeable nuclear stain propidium iodide, which enables continuous quantitative measurement of cell viability over time owing to its optimal limit of DNA binding, without background from bacterial cell death (Supplementary Fig. 14). After co-culture for 1–10 h, propidium iodide fluorescence (excitation/emission 535 nm/617 nm) was measured using a SpectraMax M Series Multi-Mode Microplate Reader (Molecular Devices). Between measurements, plates were kept in an incubator at 37°C, 5% CO₂ and 95% air. At the end of the experiment, maximal fluorescence was measured following treatment of each well with 1% Triton X-100 to permeabilize all cells and label all nuclei with propidium iodide, which corresponds to 100% cell death. Background fluorescence of propidium iodide was measured in uninfected control cells (exposed to medium alone or the appropriate concentration of MUC5AC) at the beginning of the experiment. Percentage cytotoxicity was calculated as:

\[ (f_{0} - f_{s}) / (f_{M} - f_{s}) × 100 \]

where \( f_{s} \) is the initial fluorescence, \( M \) is the maximum fluorescence after addition of Triton X-100 and \( f_{s} \) is the fluorescence at any given time. Mucin dose response curves were assessed at 7.5h.

**Confocal imaging.** Image acquisition was performed using a confocal laser scanning microscope (LSM 800, Zeiss) equipped with a ×63/1.4 NA oil-immersion objective. Images were analysed using Zeiss ZEN v2.1 imaging software. The excitation wavelengths for GFP and propidium iodide were 488 nm and 555 nm, respectively. The 3D images of attached and unattached cells were created with IMARIS v.7.7.2. Plots were generated in GraphPad Prism. Quantification of bacterial aggregate volume was performed using IMARIS v.7.7.2.

**Examination of bacterial interactions with porcine burn wounds.** Interactions between *P. aeruginosa* and full-thickness burn wounds were assessed as previously described. In brief, female Yorkshire pigs (n = 4) weighing between 32 and 36 kg were anesthetized and the dorsal trunk was shaved and surgically prepared. Under aseptic conditions, a 6.08 × 5.08 cm full-thickness burn wounds were created on the back of the pig using an electrically heated burn device with controlled pressure delivery for 50 s. One day after the thermal injury, mid-log phase cultures of *P. aeruginosa* were topically inoculated onto the wound site at a concentration of 1 × 10⁸ CFU in 250 µl of 20% PF-127 (Sigma) prepared in PBS with 0%, 0.05% (0.5 µg ml⁻¹) or 0.5% (5.0 µg ml⁻¹) MUC5AC, or 0.01% (0.1 mg ml⁻¹) MUC5AC glycan or monosaccharides. The inoculated and treated wound was rubbed with a sterile spatula for 30 s. On days 1 and 4 after inoculation, treatment was reapplied to the wound. On days 1 and 7 after inoculation, full-thickness wound-tissue biopsies were collected for microbiological analysis using a 6 mm sterile disposable punch biopsy tool. Treatments for each wound site were randomized, and were not performed blinded. Viable bacterial counts were determined from three randomly selected 6 mm punch biopsies from each wound site. Biopsies were weighed and placed in separate sterile polypropylene culture test tubes containing 1 ml of PBS. All of the samples were homogenized using a Pro Scientific Bio-Gen Series Pro200 hand-held homogenizer for 45 s. The resulting solutions were serially diluted and plated on *Pseudomonas* isolation agar (Difco) with rifampicin (100 µg ml⁻¹) in at least triple incubated and inoculated at 37°C overnight. CFUs were calculated per gram of tissue.

**Calculation of sample size.** Statistical experts in the OSU Center for Biostatistics performed sample size calculations on the basis of power analyses. To determine the minimum sample sizes necessary to detect some of the effects under investigation, data from previous preliminary studies were used. Although power analysis (based on α = 0.05, two-tailed tests) indicated that six animals per group would be required to achieve statistical significance, 1–2 animals per group showed significant effects. As the data were not normally distributed, nonparametric Kruskal–Wallis one-way ANOVA was used.

All care of laboratory animals was in accordance with institutional guidelines, and approved by the Ohio State University Institutional Animal Care and Use Committee (IACUC) under protocol 2012A00000041-R1.

**Statistical analysis.** Unless noted otherwise, experiments were performed with at least three biological replicates consisting of at least three technical replicates, and results are presented as mean ± s.e.m. Statistical significance was assessed using ordinary one-way ANOVA followed by Dunnett’s multiple comparisons test, or one-sample Student’s t-tests for normalized data unless otherwise noted. Adjusted P values of <0.05 were considered to be significant.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** High-throughput sequencing data presented in Figs. 1 and 4 are deposited in the Gene Expression Omnibus (GEO) under accession number GSE136097. All other data that support the findings of this study are available from the corresponding author on reasonable request.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed |
|-----|-----------|
| ☑   | The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☑   | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑   | The statistical test(s) used AND whether they are one- or two-sided |
| ☑   | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| ☑   | A description of all covariates tested |
| ☑   | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☑   | A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☑   | For null hypothesis testing, the test statistic (e.g. \(F\), \(t\), \(r\)) with confidence intervals, effect sizes, degrees of freedom and \(P\) value noted |
| ☑   | Give \(P\) values as exact values whenever suitable. |
| ☑   | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☑   | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☑   | Estimates of effect sizes (e.g. Cohen’s \(d\), Pearson’s \(r\)), indicating how they were calculated |
| ☑   | Clearly defined error bars |
| ☑   | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection: Zeiss ZEN 2.1 imaging software (Thornwoods, NY, USA).

Data analysis: IMARIS 7.7.2 (Bitplane, Switzerland). PRISM (GraphPad Software). MATLAB R2016b. RStudio version 3.4.0 and the DESeq2 workflow (Love et al. 2014). Galaxy Servers (Afgan et al. 2016).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

High throughput sequencing data presented in Figures 1, 4, and S1 were deposited to the Gene Expression Omnibus (GEO), accession code is included in the text and data will be available upon publication.
Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- Life sciences
- Behavioural & social sciences
- Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
RNA-seq data were generated from 3 biological replicates. Supporting RNA-seq data presented in the supplement were generated using 2 biological replicates. All experiments performed in 96-well plates were performed in at least biological triplicate (exact number of replicates indicated by dots overlaying bars), and each replicate measure comprised of 3 technical replicates. In vivo experiments were performed on two pigs, 3 punch biopsies were collect from each and analyzed for bacterial load for a total of 6 replicates.

Data exclusions
No data were excluded from the analysis.

Replication
Experiments were performed at least in triplicate unless noted otherwise. Media, strain, and time points were varied for the RNA-seq experiments to confirm reproducibility under different experimental conditions.

Randomization
Treatment application sites were randomized in the porcine burn wound infection experiments.

Blinding
Blinding was not used in our study.

Reporting for specific materials, systems and methods

Materials & experimental systems
- n/a Involved in the study
- x Unique biological materials
- x Antibodies
- x Eukaryotic cell lines
- x Palaeontology
- x Animals and other organisms
- x Human research participants

Methods
- n/a Involved in the study
- x ChIP-seq
- x Flow cytometry
- x MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials
MUC5AC and MUC5B were purified by standard procedures described in the manuscript.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
ATCC

Authentication
HT-29 cells obtained from ATCC were certified authentic, authenticity was monitored based on morphology.

Mycoplasma contamination
HT-29 cells obtained from ATCC were certified mycoplasma free.

Commonly misidentified lines
No commonly misidentified lines were used.
### Animals and other organisms

Policy information about [studies involving animals](#): ARRIVE guidelines recommended for reporting animal research

| Category                  | Description                                      |
|---------------------------|--------------------------------------------------|
| Laboratory animals        | Yorkshire pigs                                   |
| Wild animals              | This study did not involve wild animals.          |
| Field-collected samples   | This study did not involve field-collected samples.|

### Human research participants

Policy information about [studies involving human research participants](#)

| Category                      | Description                                                                 |
|-------------------------------|-----------------------------------------------------------------------------|
| Population characteristics    | Whole saliva samples were collected from healthy volunteers. Volunteers were not screened for a particular age or gender. MUC5B was isolated and purified from pooled saliva samples. |
| Recruitment                   | Participants were recruited by word-of-mouth, which could lead to selection bias. Specifically, participants were largely adults in their 20s-30s. This is unlikely to affect salivary mucin composition or the outcomes of this work. |