Mixed communities of mucoid and non-mucoid Pseudomonas aeruginosa exhibit enhanced resistance to host antimicrobials.

Sankalp Malhotra, Dominique H. Limoli, Anthony English, Erin Mulroney, Daniel J. Wozniak.

Abstract

Pseudomonas aeruginosa (*P.a.*) is a bacterial pathogen that causes chronic lung infections in cystic fibrosis (CF) patients. The CF lung is hyperinflammatory due to an abundance of neutrophil-derived antimicrobials, including reactive oxygen species [e.g. hydrogen peroxide (H2O2)] and antimicrobial peptides (e.g. LL-37). *P.a.* colonizes the CF lung by acquiring adaptive mutations. Mutation of *mucA* results in a mucoid phenotype due to the overproduction of the polysaccharide alginate. Mucoid isolates often revert to a nonmucoid phenotype through second mutations. Mucoid and nonmucoid strains are often isolated from the same patient sample, suggesting a selective advantage for the co-existence of these variants in the CF lung. We hypothesized that mucoid/non-mucoid P.a. may be differentially susceptible to neutrophil products, exhibiting heightened resistance to host factors in consortia. While clinical mucoid *P.a.* isolate (FRD1) was 10-fold more resistant to LL-37 compared to non-mucoid variants, FRD1 was also more sensitive to H2O2 compared to a non-mucoid, *algT* revertant. Resistance to H2O2 was dependent on catalase (katA) expression, which was likely regulated by two transcription factors, AlgT and AlgR. Extracellular release of KatA was found to be dependent on the expression of *lys*, which encodes an endolysin in *P.a.* already implicated in eDNA release. Co-cultures of mucoid/non-mucoid strains (mimicking CF lung co-isolates) exhibited greater tolerance to both H2O2 and LL-37 than mono-culture. These data provide an important rationale to study the interaction of mucoid/non-mucoid P.a. variants as contributors to CF pathology.

Introduction

Cystic fibrosis (CF) is a lethal genetic disease that manifests in early childhood and progresses to multi-organ dysfunction, lung failure, and premature death[1]. There are currently 30,000 patients living with CF in the United States (70,000 globally), with nearly 1000 new diagnoses each year[2]. Despite progress in diagnosis and management strategies, morbidity and mortality in CF continue to be closely associated with chronic respiratory infections complicated by antimicrobial resistance[3]. From infancy, CF patients suffer from accumulation of thick, dried mucus within their airways, which leads to entrapment of opportunistic bacteria[4]. During the chronic stages of disease, the Gram-negative bacterium *Pseudomonas aeruginosa* (*P.a.*) predominates in the CF lung, exacerbating pathology and hastening patient mortality[5]. The progression of CF lung disease is driven by a combination of host and bacterial factors. Infection with *P.a.* promotes excessive influx of neutrophils into the lung, which drives tissue damage, fibrosis, and compromised function[6]. Importantly, neutrophils in CF overproduce antimicrobials like reactive oxygen species (ROS) [e.g. hydrogen peroxide (H_2O_2) and hypochlorite (HOCl)] that are damaging to both the host and bacteria[7]. Neutrophils also produce LL-37, an antimicrobial peptide that functions both as an innate immune effector as well as an immunomodulator [6].

P.a. successfully colonizes the hyper-inflammatory environment of the CF lung via acquisition of stable mutations[8]. One striking *P.a.* adaptation occurs via mutation of the gene *mucA*, resulting in over-production of the bacterial exopolysaccharide, alginate[9]. This phenotypic switch from an alginate non-producing (non-mucoid) to an alginate-overproducing (mucoid) phenotype has negative consequences for CF patients[10]. Compared to non-mucoid isolates, mucoid *P.a.* exhibits enhanced resistance to multiple antibiotics[11] and to innate immune

effectors[10], including antimicrobial peptides. As such, mucoid conversion is also correlated with a dramatic decline in patient lung function and marks a transition to the chronic and progressively debilitating stages of disease[12]. In addition to the alginate polysaccharide, *P.a.* expresses enzymes that also protect it from the host response, including catalases encoded by *katA* and *katB*[13] that combat H2O2-stress.

Despite the well-documented recalcitrance of the mucoid *P.a.* phenotype, both mucoid and non-mucoid *P.a.* are often isolated together in CF patient sputum[14]. Non-mucoid *P.a.* present in the CF lung in chronic disease are often non-mucoid "revertants" that have acquired a second site mutation (following mucoid conversion via *mucA*). These second-site mutations often occur both *in vitro* and *in vivo* in the gene, *algT*, which encodes the central sigma factor responsible for mucoid conversion[15]. Functional AlgT is required to up-regulate the alginate biosynthetic operon, which begins with the gene *algD*. *mucA* encodes an anti-sigma factor that normally sequesters AlgT at the inner membrane. Mutation of *mucA* liberates AlgT, which can then up-regulate alginate biosynthesis[10,16].

AlgT is known to regulate multiple genes outside of the alginate biosynthetic operon as well. The AlgT regulon is predicted to consist of 300 ORFs[17]. However, downstream of AlgT, there are three known transcriptional regulators, encoded by the genes *algB*, *algR*, and *amrZ*, that also positively regulate alginate biosynthesis[18].

Given that mucoid/non-mucoid revertants of *P.a.* are co-isolated from CF lungs, we hypothesized that there may be an advantage for these multi-variant communities in evasion of the host response. Here, we show that mucoid *P.a.* resistance to LL-37 is alginate-dependent, whereas susceptibility to H2O2 is *algT*-dependent. *algT* mutants (non-mucoid revertants) produce KatA, which is released into the extracellular space in a cell-lysis dependent process that

relies on a bacteriophage endolysin encoded within the *P.a.* chromosome. We provide evidence that *katA* is transcriptionally regulated by *algR*. Finally, we show that mucoid/non-mucoid *P.a.* variants in consortia are more resistant to LL-37 and H2O2 than in single-variant, mono-cultures. These findings provide important insights regarding muti-variant *P.a.* interactions that enable this bacteria to evade critical components of the host-immune response.

Materials and methods

Strains and growth conditions. All *P. a.* strains used were streaked for isolation from frozen stocks at -80 degrees C onto *Pseudomonas* Isolation Agar (PIA), followed by growth in Luria Broth No Salt (LBNS) medium. *E. coli* (Hb101) strains were streaked for isolation from frozen stocks onto Luria Agar (LA), followed by growth in Luria Broth (LB). All gene mutations were made as previously described by overlap extension PCR[19]. When necessary to maintain a plasmid, *E. coli* strains were grown in LB with 100ug/mL ampicillin. *P.a.* strains with plasmid were grown in LBNS with 300ug/mL carbenicillin.

Alginate purification and quantitation. *P.a.* alginate was purified as previously described[20]. Briefly, clinical mucoid isolate, FRD1, was streaked on PIA and grown at 37 degrees C for 16-18 hours. Colonies were scraped directly from the solid medium and homogenized in 2mL 0.85% saline. Each 1mL of saline and bacteria collected from the plate was split into two equal, 500uL fractions and vortexed well. Bacterial cells were pelleted by centrifugation at 14,000 rpm for 30 minutes at room temperature. 500 uL 2% cetyl pyridinium chloride was added to the supernatant to precipitate the alginate (appeared as stringy, white substance). Tubes were

inverted to mix, then centrifuged at 14,000 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet (alginate) was dissolved in 500 uL of cold (-20 degrees C) 100% isopropanol. Here, the alginate partially dissolved, and appeared cloudy, white. The mixture was centrifuge at 14,000 rpm for 10 minutes at room temperature, followed by discarding the supernatant and draining the tube well. The alginate pellet was resuspended in 500 uL of saline and incubated 12-24 hours at 4 degrees C to become hydrated.

P.a. alginate was quantitated as described previously[20]. Brown algae-derived (seaweed) alginate was dissolved in saline and diluted two fold (2mg/mL to 62.5mg/mL) to generate standards. 50uL of alginate standards or sample were placed in duplicate into a 96-well plate. 200 uL of 25 mM sodium tetraborate in sulfuric acid to each well, followed by mixing the plate on a nutator. The plate was heated on a standard heatblock at 100 degrees C for 15 minutes, and allowed to cool to room temperature for 15 minutes. 50uL 0.1% carbazole was then added into each well, followed by heating the plate at 100 degrees C for 15 minutes. After cooling the plate to room temperature, absorbance was read on a Spectramax plate reader at wavelength of 550nm.

One hour bacterial killing. Bacterial strains were sub-cultured from overnight growth in LB or LBNS into fresh medium and grown to exponential phase (approximately OD_{600}= ~0.5 or 2x10^8 CFUs/mL). For H2O2 killing assays, bacteria were then mixed 1:1 with H2O2 diluted in LBNS (or LBNS alone, i.e. no treatment) and incubated for 1 hour at 37 degrees C, followed by plating on PIA to quantitate CFUs/mL. Data were expressed as log-fold killing relative to the no-treatment condition. For the LL-37 killing assays, bacteria at exponential phase were pelleted at 14,000 RPM for 3 minutes and resuspended in sodium phosphate buffer (SPB) at pH 6.4. Bacteria were then mixed 1:1 with LL-37 diluted in SPB (or in SPB alone) and incubated 1 hour

at 37 degrees C, followed by plating on solid medium (PIA for *P.a.* and LA for *E. coli*) to quantitate CFUs/mL.

Mono- vs. co-culture bacterial killing. Mono- vs co-culture killing assays were performed identically to the one hour killing assay protocol as described above. In the co-culture conditions, mucoid/non-mucoid *P.a.* strains were mixed 1:1 prior to exposure to either LL-37 or H2O2. Additionally, the mucoid/non-mucoid strains used here were differentially drug-marked to track their survival in the co-culture condition. The mucoid, FRD1, strain used was a spontaneous streptomycin resistant isolate, plated on PIA containing 150ug/mL streptomycin to quantitate CFUs/mL. The isogenic non-mucoid strains (*algT* and *algD* mutants) used were spontaneous rifampicin resistant isolates, plated on PIA containing 100ug/mL rifampicin to quantitate CFUs/mL.

Biolog growth inhibition and supernatant protection assays. Bacterial strains grown overnight (14-16 hours) in LBNS were diluted in fresh medium to OD_{600} of 0.24. To generate a master mix for each bacterial strain, per manufacturer's recommendations: 150uL of bacterial culture at OD 0.24 was added to 850uL LBNS with 12uL of 10X Biolog Dye (A). 50uL/well of these bacterial master mixes were added in triplicate to the Biolog 96-well plate. 50uL/well of H2O2 (diluted in LBNS at desired concentration) or LBNS alone was then added to each well containing bacteria. Plates were placed in the OmniLog incubator at 37 degrees C for 24 hours. The Biolog Dye is tetrazolium-based, and detects extracellular metabolites produced during of growth, resulting in a colorimetrically detectable byproduct. The output of the system is bacterial growth curves, which can be plotted (as Biolog units versus time in hours) using Biolog's kinetic

software (OL_FM_12) package. Each growth curve can be converted to parameters including area under the curve (AUC), maximum growth, maximum slope, and lag time via Biolog's parametric (OL_PR_12) software. AUC was used here as the most representative parameter corresponding to bacterial growth. Percent survival was calculated by taking the AUC of bacterial growth in the presence of H2O2 as a percentage of the no-treatment condition for each strain tested.

To assess if bacterial supernatants from various strains are sufficient to protect FRD1 from H2O2-stress, bacterial strains grown overnight (14-16 hours) in LBNS were centrifuged at 14,000 RPM for 3 minutes to pellet bacterial cells. Supernatants were collected and filter-sterilized via 0.2um pore-size filters. FRD1 grown overnight (14-16 hours) in LBNS was diluted in fresh medium to OD_{600} of 0.24. 500uL of this FRD1 culture was pelleted and resuspended with 500uL of supernatant from desired strains. A master mix with Biolog Dye A was then made as specified above: 150uL of FRD1 (resuspended in supernatant) was added to 850uL LBNS with 12uL of 10X Biolog Dye A. 50uL/well of these bacterial master mixes were added in triplicate to the Biolog 96-well plate, with 50uL/well of H2O2 (diluted in LBNS at desired concentration) or LBNS alone. Plates were placed in the OmniLog incubator at 37 degrees C for 24 hours. Data were plotted as bacterial growth curves or as percent survival (AUC) as described above. For each type of Biolog assay, three independent experiments were performed in triplicate.

Reverse transcriptase-PCR. RT-PCR was performed to measure mRNA levels of desired genes in bacterial strains of interest as described previously[18]. Here, bacterial strains were grown to exponential phase (OD_{600}= ~0.5-0.8). Bacterial cells were pelleted by centrifugation, and

resuspended/lysed in TRIzol for 5 minutes. Chloroform was added and the aqueous phase containing RNA was collected and applied to the genomic DNA eliminator column provided in the Qiagen RNeasy Kit. The column was centrifuged at 8000 x g for 30 seconds at 4 degrees C. The column was then discarded and 1 volume of 70% ethanol was added to the eluent. This sample was then applied to the RNeasy mini column (pink column). Following centrifugation, the column was washed with Buffer RW1 twice and then with Buffer RPE twice (with spins as above between each wash). An empty column spin for 2 minutes was performed to dry the RNeasy silica-gel membrane. The column was then transferred to a new collection tube and RNA was eluted with 50uL RNase-free water.

The RNA preparations were then converted to cDNA using the Thermo SuperScript III First-Strand Synthesis System, per manufacturer's instructions. Real time PCR was performed using Bio-Rad CFX1000 thermal cycler, gene-specific primer pairs, and SYBR green supermix. All samples were normalized to housekeeping gene *rpoD* and mRNA transcript fold-change was calculated relative to FRD1. All experiments were performed in triplicate on three independent occasions.

Catalase activity assays. To measure catalase protein activity in the cell-free supernatants of *P.a.* strains, a commercially available kit (BioVision Catalase Activity Colorimetric/Fluorometric Assay) was used per manufacturer's instructions. Supernatants were diluted 1:4 in assay buffer and added to a 96 well plate to a final volume of 78uL. A H2O2 standard curve was generated to include 0, 2, 4, 6, 8, and 10 nmol/well, also to a final volume of 78uL/well. 10uL of stop solution was added into the wells containing the standards. The positive control was catalase enzyme (diluted 1:16 in assay buffer). One additional well included the

catalase enzyme with stop solution (this was treated as the "high control" or negative control). 12uL fresh 1mM H2O2 was added into each well (samples and positive control) to start the reaction and incubated at room temperature for 30 minutes. 10uL of stop solution was then added into each sample well. Finally, for each well, a 50uL developed mix containing 46uL assay buffer, 2uL OxiRed Probe, and 2uL HRP solution was prepared and added to samples, controls, and standards. After 10 minutes of incubation at room temperature, absorbance was measured at OD 570nm using a Spectramax plate reader. Catalase activity was calculated by first calculating signal change: ΔA= absorbance of the high control- absorbance of each sample. After plotting the standard curve, linear regression was used to convert ΔA to get B nmol of H2O2 decomposed by catalase in a 30 minute reaction. Catalase activity cold then be calculated as follows: Catalase activity= B/30 x V x Sample dilution factor= nmol/min/mL= mU/mL, where one unit of catalase decomposes 1umol of H2O2 per minute at pH 4.5 at 25 degrees C.

Results

Alginate is sufficient to protect bacteria from LL-37 killing. We had previously shown that mucoid isolates of *P.a.* were more resistant to LL-37 mediated killing (at lethal doses) compared to isogenic, non-mucoid variants[21]. Here, we sought to determine if the exopolysaccharide, alginate, which is produced in excess by mucoid *P.a.*, is sufficient to protect non-mucoid *P.a.* from LL-37 stress. We purified alginate from a clinical, mucoid isolate, FRD1, and added it exogenously to an isogenic, *algD* mutant (at multiple concentrations) prior to exposing these strains to LL-37 for 1 hour. Bacteria were then plated for CFUs/mL on PIA and log-fold killing was calculated relative to no-treatment for each condition. As shown in our previous publication,

the *algD* mutant alone was 1-log more sensitive to LL-37 compared to the mucoid isolate. However, *P.a.*-derived alginate was sufficient to protect the *algD* mutant, even at the lowest added concentration of 10ug/mL (Fig. 1A). Here, we used three concentrations of alginate: 250ug/mL, 100ug/mL, and 10ug/mL. Alginate quantitation in CF patient sputum has shown a range of 10ug/mL to 100ug/mL of alginate[22], and an FRD1 culture grown to stationary phase contains approximately 250ug/mL alginate, which is a concentration used in prior work as well[23]. Notably, commercially available alginate derived from brown algae, which differs from *P.a.* alginate only in its acetylation state (i.e. it is still a polymer of mannuronic and guluronic acid), was sufficient to protect *E. coli* from LL-37 killing (Fig. 1B). This suggests that bacterial resistance to LL-37 is alginate-dependent and that this phenomenon is independent of bacterial genera.

***algT* mutation confers resistance to H2O2.** Given that the alginate polysaccharide provided protection from LL-37, we proposed that it may be similarly protective against other neutrophil-produced antimicrobials such as ROS. Here, we used a high-throughput system called Biolog to investigate the capacity of isogenic mucoid *P.a.* isolate FRD1, and two non-mucoid strains (*algT* and *algD* mutants) to grow for 24 hours in the presence of two distinct ROS: HOCl and H2O2. The resulting bacterial growth curves (Supplemental Figure 1) were converted into to the parameter of area under the curve (AUC), which taken as a percentage of the no-treatment conditions provided an output of percent survival. We found that growth of all three strains was similar in the presence of 11.25mM of HOCl. Surprisingly, however, the *algT* strain was significantly more resistant to growth in the presence of 25mM H2O2 compared to both the mucoid and *algD* strains. To validate Biolog as an effective means to investigate bacterial

susceptibility to ROS, we exposed all three strains to a lethal dose of H2O2 (50mM) for 1 hour, plated surviving bacteria for CFUs/mL, and computed log-fold killing relative to no-treatment for each condition. Here too, we found that the *algT* strain was least susceptible to H2O2-killing compared to the mucoid and *algD* mutants. These findings suggest that H2O2 susceptibility in *P.a.* is *algT*-dependent and independent of mucoid status.

Various clinical mucoid *P.a.* isolates demonstrate susceptibility to H2O2, similar to FRD1. Given that mucoidy appeared to be non-protective versus H2O2 killing, we wanted to investigate whether this phenotype was specific to the clinical mucoid isolate, FRD1, or generalizable across mucoid *P.a.* strains. Here, we tested the H2O2 susceptibility of a panel of 10 clinical mucoid isolates (including FRD1) by Biolog. We found that 5/10 strains tested were statistically non-significant in their H2O2-susceptibility compared to FRD1, suggesting that a clear subset of mucoid *P.a.* isolates may be susceptible to H2O2 (Fig. 3). Within this H2O2-sensitive subset, each strain was significantly more sensitive to H2O2 compared to the *algT* mutant of FRD1.

Cell-free supernatants derived from the *algT* mutant protect FRD1 from H2O2 stress via KatA. We hypothesized that the *algT* mutant may be resistant to H2O2 due to the secretion of a soluble antioxidant. To test this experimentally, we filter-sterilized supernatants from stationary phase cultures of FRD1, *algT*, and *algD* strains. These supernatants were used to resuspend FRD1 prior to its growth in the presence of H2O2 for 24 hours. Only supernatants from the *algT* strains protected FRD1 from H2O2-killing (Fig. 4A). Furthermore, heat-inactivation of the *algT* supernatants (by heating at 80 degrees C for 30 minutes) abrogated protection of FRD1 from H2O2, suggesting that the soluble factor is a heat-labile protein (Fig 4A). The catalases, KatA

and KatB, have been shown to be two vital antioxidants produced by *P.a.* for defense against H2O2[24]. KatA is a constitutively expressed catalase that is found in both the *P.a.* cytoplasmic and periplasmic compartments, whereas KatB is an induced catalase that is found only in the *P.a.* cytoplasm[13]. *katB* expression is regulated by *oxyR* and is dependent on H2O2-exposure[25]. To determine whether either KatA or KatB may be the protective factor against H2O2 within *algT* supernatants, we generated *algT* Δ*katA* and *algT* Δ*katB* mutants. Supernatants derived from an *algT* Δ*katA* mutant showed abrogated capacity to protect FRD1 against H2O2, whereas deletion of *katB* had no effect (Fig 4B). This phenotype was complemented by expression of *katA* in-trans via a pHERD20T vector (under the control of an arabinose-inducible promoter) (Fig. 4C). This suggested that supernatants derived from the *algT* mutant protect mucoid *P.a.* via the presence of KatA.

***algT* indirectly represses *katA* transcription via *algR*.** Given that mutation of *algT* resulted in greater protection of *P.a.* from H2O2 via KatA, we proposed that *algT* may act as a repressor of *katA* transcription. Though sigma factors do not commonly function to suppress gene transcription directly, they can negatively regulate gene expression via secondary or downstream transcription factors. In alginate biosynthesis, three main transcription factors lie downstream of AlgT: AlgB, AlgR, and AmrZ (Fig 5A). If these transcriptions factors directly repress katA transcription, we reasoned that mutation of the genes that encode for these factors should result in increased resistance to H2O2 (i.e. the same effect as an *algT* mutation). We observed that *algR* mutation specifically results in enhanced resistance to H2O2 (Fig 5B). Additionally, by RT-PCR, we found that *katA* transcript is up-regulated in both the *algT* and *algR* mutant backgrounds relative to the isogenic mucoid, FRD1 isolate (Fig 5C). Finally, catalase protein activity was also

higher in the cell-free supernatants of the *algT* and *algR* mutants relative to *mucA* (Fig 5D), suggesting that *algT* is an indirect repressor of *katA* transcription via *algR.*

Extracellular release of KatA is dependent on *lys*-mediated explosive cell lysis. Previous work had shown that KatA appears to be released from *P.a.* during stationary phase of growth via cell lysis[25]. However, a clear mechanism for *P.a.* cell lysis as a driver of KatA secretion was not elucidated. Recently, a bacteriophage endolysin encoded by *lys* (PA0629) has been shown to mediate explosive cell lysis and eDNA release in *P.a.* (Figure 6A) Here, we sought to investigate whether *lys* has a role in KatA release in *algT* mutants as well. To this end, we generated both *algT Δlys* and Δ*lys*/p*lys* strains. We first wanted to validate the *Δlys* mutant by confirming that it has a deficiency in eDNA release. As such, we purified cell-free supernatants from FRD1, *algT*, *algT Δlys* and Δ*lys*/p*lys* strains. These supernatants were combined with 6X DNA running buffer and subjected to agarose gel electrophoresis. A high molecular weight band was observed for each of the strains (Fig 6B), which was suggestive of extracellular chromosomal DNA (eDNA). The intensity of this band (densitometry) was quantitated by ImageJ software (Fig 6C). We observed that the *algT* mutant appears to undergo more cell lysis (i.e. greater eDNA release) than the mucoid strain. Furthermore, deletion of *lys* in the *algT* background reduces cell lysis and this can be complemented by expression of *lys* in-trans under the control of an arabinose-inducible promoter.

We also saw that deletion of *lys* resulted in increased susceptibility to H2O2 (Fig 6D), and this was likely because of reduced catalase protein activity in the cell-free supernatants of this mutant (Fig 6E). To our knowledge, this finding for the first time links a specific mechanism for cell lysis and extracellular release of *P.a.* catalase (KatA). Finally, we wanted to know

whether *lys* gene transcription is negatively regulated by *algT/algR* in the same way as *katA*. RT-PCR showed that *lys* transcript was unchanged in the *algT* and *algR* mutant backgrounds, relative to the mucoid strain. This observation suggests two non-mutually exclusive explanations: 1) *lys* may be post-transcriptionally regulated or 2) another gene in the *lys*-mediated cell lysis pathway (e.g. *hol*, PA0614) may be transcriptionally up-regulated in the *algT/algR* mutant backgrounds. It has been previously seen that *hol* over-expression is sufficient to induce cell lysis[26].

Co-cultures of mucoid/non-mucoid isolates, which mimic multi-variant *P.a.* populations within the CF lung, exhibit enhanced resistance to host antimicrobials. Mucoid and non-mucoid *P.a.* morphotypes are commonly co-isolated from CF patients, despite the heightened resistance of mucoid *P.a.* to multiple antimicrobials. As such, we hypothesized that there may be a selective advantage to co-localization of mucoid/non-mucoid *P.a.* within patient lungs to evade the host response. To test this, we exposed mono- or co-cultures of mucoid and non-mucoid variants to either H2O2 or LL-37 for 1 hour, followed by plating for CFUs/mL. In these experiments, the mucoid (streptomycin-resistant) and non-mucoid (rifampicin-resistant) strains were differentially drug-marked to easily and independently track their survival in co-culture.

In mono-culture, the *algT* strain is clearly more resistant to H2O2 than the mucoid strains; however, in co-culture, the susceptibility of the mucoid strain to H2O2 is almost identical to that of the *algT* strain. This is because the *algT* strain is producing KatA, which is likely to be acting as a common good to protect both the mucoid/non-mucoid *P.a.* within the mixed community.

Similarly, in mono-culture, the mucoid strain is significantly more resistant to LL-37 than the non-mucoid, *algD* strain. However, in co-culture, the susceptibility of the mucoid and non-mucoid strain to LL-37 is similar, wherein the *algD* strain is likely rescued by the presence of the exopolysaccharide, alginate, from the mucoid isolate. As such, based on these *in vitro* experiments, there appears to be an advantage for multi-variant, mucoid/non-mucoid communities of *P.a.* for the evasion of two critical innate immune effectors.

Figure legends

Figure 1. Alginate is sufficient to protect both *P.a.* and *E. coli* from LL-37-mediated killing. (A) One hour treatment with lethal dose of LL-37 (25ug/mL) of clinical mucoid isolate, FRD1 (mucA), isogenic *algD* strain, or *algD* strain plus exogenously added alginate at three different concentrations (250ug/mL, 100ug/mL, and 10ug/mL), followed by enumeration of surviving bacteria by plating on Pseudomonas Isolation Agar (PIA) for CFUs. Data are represented as log-fold killing compared to no-treatment for each strain/condition. To isolate alginate, FRD1 colonies growing on PIA were scraped, homogenized, and alginate precipitated via cetyl pyridinium chloride. Alginate was quantitated by the carbazole assay. (B) Log-fold killing of *E. coli* strain, Hb101, by three different concentrations of LL-37 with or without the presence of alginate. Here, commercially available brown-algae derived alginate was added to Hb101 at a concentration of 250ug/mL. Experiments were performed in duplicate. The mean +/- SEM is indicated.

Figure 2. The non-mucoid *algT* revertant is significantly more resistant to hydrogen peroxide than the clinical mucoid isolate, FRD1.

(A) Biolog growth curve data for strains grown for 24 hours in the presence of 11.25mM HOCl (Supplemental Fig 1C) plotted as percent area under the curve (AUC), relative to the no-treatment condition (B) Biolog data for strains grown in the presence of 25mM H_2O_2 (Supplemental Fig 1B) as percent area under the curve (AUC). (C) 1 hour treatment of *P.a.* strains with 50mM H2O2 plotted as log-fold killing. Experiments were performed in triplicate on three independent occasions. The mean +/- SEM is indicated. Statistical significance was measured using an unpaired two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001

Figure 3. Multiple clinical mucoid isolates exhibit sensitivity to H2O2 similar to FRD1.

Biolog growth curve data for strains grown for 24 hours in the presence of 25mM H2O2 plotted as percent area under the curve (AUC), relative to the no-treatment condition. Dotted line represents H_2O_2 sensitivity of FRD1. Strains marked as "ns" were were as susceptible as FRD1 to H_2O_2 ("not significant" statistically compared to FRD1). Experiments were performed in triplicate on three independent occasions. The mean +/- SEM is indicated. Statistical significance was measured using an unpaired two-tailed Student's *t*-test. ***p<0.001

Figure 4. Supernatants derived from the *algT* revertant are sufficient to protect mucoid *P.a.* from H_2O_2-killing via KatA.

(A) Cell-free supernatants derived from the mucoid, *algT*, or *algD* strain were used to resuspend the mucoid isolate, FRD1, prior to 24-hour growth in the presence of 25mM H_2O_2 by Biolog. Supernatants were collected and filter-sterilized from strains after 14-16 hours growth (stationary

phase). Supernatants were heat-inactivated (H.I.) at 80 degrees C for 30 minutes. Growth curve data are plotted as percent area under the curve (AUC), relative to the no-treatment condition.(B) Supernatants derived from *algT* Δ*katA* and *algT* Δ*katB* mutants were used to resuspend FRD1 prior to 24-hour growth in presence of H_2O_2 by Biolog. (C) Supernatants derived from *algT* Δ*katA* and *algT* Δ*katA* p*katA* strains were used to resuspend FRD1 prior to 24-hour growth in presence of H_2O_2 by Biolog. The mean +/- SEM is indicated. Statistical significance was measured using an unpaired two-tailed Student's *t*-test.. *p<0.05, **p<0.01, ***p<0.001

Figure 5. *katA* transcription is negatively regulated by *algT*, via *algR*.

(A) Model for indirect regulation of *katA* transcription by *algT* through one of three downstream transcription factors, *algB, algR,* and *amrZ* (B) Biolog of *P.a.* strains in the presence of 25mM H_2O_2. Data are plotted as percent area under the curve (AUC), relative to the no-treatment condition. (C) *katA* and *algD* mRNA levels quantitated by RT-PCR, normalized to the mucoid strain, FRD1. *rpoD* was used as the housekeeping gene in these experiments. (D) Quantitation of catalase protein activity within cell-free supernatants using BioVision Catalase Activity Colorimetric Assay Kit. Experiments were performed in triplicate on three independent occasions. Statistical significance was measured using a two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001

Figure 6. Deletion of *lys* abrogates protection of mucoid *P.a.* by *algT* supernatants.

(A) *lys* (PA0629) endolysin gene cluster/putative operon (PA0628- PA0632), which mediates explosive cell lysis and eDNA release in *P.a.* (B) eDNA was visualized by subjecting cell-free supernatants from *P.a.* strains to 1% agarose gel (with ethidium bromide) electrophoresis. eDNA

was observed as high molecular weight (>3000bp) band. (C) eDNA band intensity (densitometry) was quantitated via ImageJ (D) Supernatants from *algT* and *algT* Δ*lys* mutants were used to resuspend FRD1 prior to 24-hour growth in presence of H_2O_2 (E) *lys* mRNA levels quantitated by RT-PCR, relative to the mucoid strain, FRD1 (F) Quantitation of catalase protein activity within cell-free supernatants using BioVision Catalase Activity Colorimetric Assay Kit. Experiments were performed in triplicate on three independent occasions. Statistical significance was measured using a two-tailed Student's *t*-test. *p<0.05, **p<0.01, ***p<0.001

Figure 7. Mucoid and non-mucoid *P.a.* variants in co-culture exhibit enhanced resistance to both H_2O_2 and LL-37. (A) Mono- and co-cultures of mucoid strain, FRD1, and *algT* revertant exposed to 25mM H_2O_2 for 1 hour. Mucoid strain used was a spontaneous streptomycin resistant isolate and the non-mucoid *algT* revertant was a spontaneous rifampicin resistant isolate. Surviving bacteria were enumerated by plating on respective (antibiotic-containing) medium to differentiate between mucoid and *algT* strains. Percent survival was calculated relative to the no-treatment condition for each strain. (B) Mono- and co-cultures of FRD1, and *algD* mutant exposed to 50ug/mL LL-37 for 1 hour. Mucoid strain used was a spontaneous streptomycin resistant isolate and the non-mucoid *algD* mutant was a spontaneous rifampicin resistant isolate. Data are represented as log-fold killing compared to no-treatment for each strain/condition. Experiments were performed in duplicate on three independent occasions. Statistical significance was measured using a two-tailed Student's *t*-test. *p<0.05, **p<0.01

Figure 8. Model for mucoid/non-mucoid revertant co-existence in the CF lung. Mucoid *P.a.* provides multi-variant community protection from LL-37 via alginate production. Non-mucoid members of the community provide protection from H2O2 via catalase production.

Supplemental Figure 1. The non-mucoid *algT* revertant is more resistant to growth in the presence of H2O2 compared to the isogenic mucoid isolate, FRD1.

(A) 24 hour Biolog growth curves of mucoid strain, FRD1 (mucA), isogenic *algT* and *algD* mutants grown in the presence of medium alone (B) 24 hour Biolog growth curves of *P.a.* strains-of-interest in the presence of 25mM H_2O_2 (C) Biolog growth curves of *P.a.* strains in the presence of 11.25mM HOCl. Experiments were performed in triplicate on three independent occasions. The mean growth reading per hour across three independent experiments is indicated.

References

1. Spoonhower, K. A. & Davis, P. B. Epidemiology of Cystic Fibrosis. *Clin. Chest Med.* **37,** 1–8 (2016).
2. Cystic Fibrosis Foundation. Patient Registry. Annual Data Report 2012. 28 (2012).
3. Langton Hewer SC. Smyth, A. Antibiotic strategies for eradicating Pseudomonas

aeruginosa in people with cystic fibrosis. [Review] [60 refs][Update of Cochrane Database Syst Rev. 2006;(1):CD004197; PMID: 16437476]. *Cochrane Database Syst. Rev.* (2009). doi:10.1002/14651858.CD004197.pub3.Copyright

4. Moreau-Marquis, S., Stanton, B. A. & O'Toole, G. A. Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway. *Pulm. Pharmacol. Ther.* **21,** 595–9 (2008).

5. Bendiak, G. N., Ratjen, F. & Ph, D. The Approach to Pseudomonas aeruginosa in Cystic Fibrosis. *Crit. Care* **1,** 587–595 (2009).

6. Brennan, S. Innate immune activation and cystic fibrosis. *Paediatr. Respir. Rev.* **9,** 271–280 (2008).

7. Galli, F. *et al.* Oxidative stress and antioxidant therapy in cystic fibrosis. *Biochim. Biophys. Acta* **1822,** 690–713 (2012).

8. Ciofu, O., Tolker-Nielsen, T., Jensen, P. ??strup, Wang, H. & H??iby, N. Antimicrobial resistance, respiratory tract infections and role of biofilms in lung infections in cystic fibrosis patients. *Adv. Drug Deliv. Rev.* (2014). doi:10.1016/j.addr.2014.11.017

9. Boucher, J. C., Yu, H., Mudd, M. H. & Deretic, V. Mucoid Pseudomonas aeruginosa in cystic fibrosis : characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection . Mucoid Pseudomonas aeruginosa in Cystic Fibrosis : Characterization of muc Muta. **65,** 3838–3846 (1997).

10. Govan, J. R. & Deretic, V. Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiol. Rev.* **60,** 539–574 (1996).

11. Hengzhuang, W., Wu, H., Ciofu, O., Song, Z. & Høiby, N. Pharmacokinetics/pharmacodynamics of colistin and imipenem on mucoid and nonmucoid Pseudomonas aeruginosa biofilms. *Antimicrob. Agents Chemother.* **55,** 4469–4474 (2011).

12. Li, Z. *et al.* Infection and Lung Disease Progression in Children With Cystic Fibrosis. **293,** 581–588 (2005).

13. Brown, S. M., Howell, M. L., Vasil, M. L., Anderson, A. J. & Hassett, D. J. Cloning and characterization of the katB gene of Pseudomonas aeruginosa encoding a hydrogen peroxide-inducible catalase: purification of KatB, cellular localization, and demonstration that it is essential for optimal resistance to hydrogen peroxide. *J. Bacteriol.* **177,** 6536–44 (1995).

14. Ciofu, O. & Bjarnsholt, T. Pseudomonas aeruginosa biofilms in Cystic Fibrosis. *Future Microbiol.* **5,** 1663–1674 (2010).

15. Ciofu, O. *et al.* Investigation of the algT operon sequence in mucoid and non-mucoid Pseudomonas aeruginosa isolates from 115 Scandinavian patients with cystic fibrosis and in 88 in vitro non-mucoid revertants. *Microbiology* **154,** 103–113 (2008).

16. Martin, D. W. *et al.* Mechanism of conversion to mucoidy in Pseudomonas aeruginosa infecting cystic fibrosis patients. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8377–81 (1993).

17. Damkiaer, S., Yang, L., Molin, S. & Jelsbak, L. Evolutionary remodeling of global regulatory networks during long-term bacterial adaptation to human hosts. *Proc Natl Acad Sci U S A* **110,** 7766–7771 (2013).

18. Xu, B., Soukup, R. J., Jones, C. J., Fishel, R. & Wozniak, D. J. Pseudomonas aeruginosa AmrZ binds to four sites in the algD promoter, inducing DNA-AmrZ complex formation and transcriptional activation. *J. Bacteriol.* **198,** 2673–2681 (2016).

19. Heckman, K. L. & Pease, L. R. Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc* **2,** 924–932 (2007).

20. Jones, C. J., Ryder, C. R., Mann, E. E. & Wozniak, D. J. AmrZ modulates pseudomonas

aeruginosa biofilm architecture by directly repressing transcription of the psl operon. *J. Bacteriol.* **195,** 1637–1644 (2013).

21. Limoli, D. H. *et al.* Cationic Antimicrobial Peptides Promote Microbial Mutagenesis and Pathoadaptation in Chronic Infections. *PLoS Pathog.* **10,** (2014).

22. Pedersen, S. S., Kharazmi, A., Espersen, F. & Hoiby, N. Pseudomonas aeruginosa alginate in cystic fibrosis sputum and the inflammatory response. *Infect. Immun.* **58,** 3363–3368 (1990).

23. Leid, J. G. *et al.* The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-gamma-mediated macrophage killing. *J. Immunol.* **175,** 7512–7518 (2005).

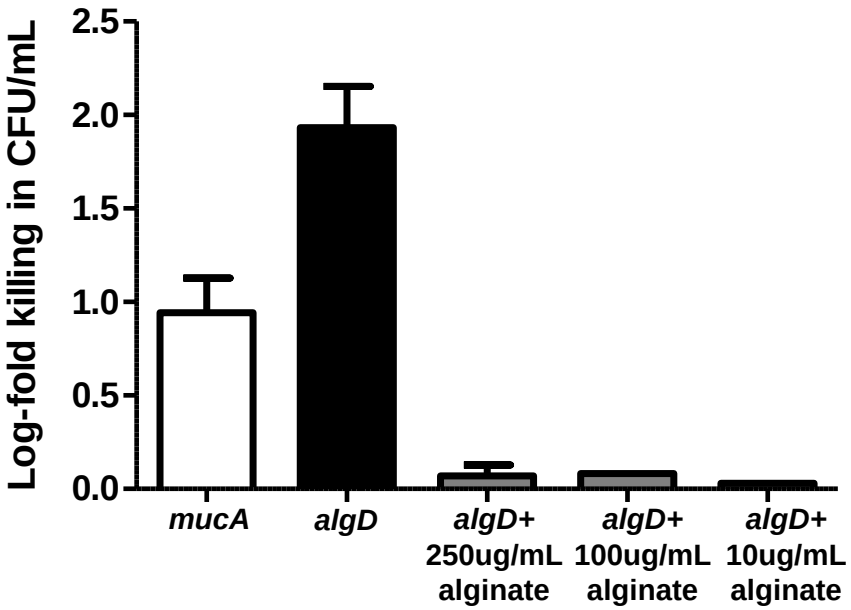
24. Britigan, B. E. *et al.* Antioxidant Enzyme Expression in Clinical Isolates of Pseudomonas aeruginosa : Identification of an Atypical Form of Manganese Superoxide Dismutase. **69,** 7396–7401 (2001).

25. Heo, Y.-J. *et al.* The major catalase gene (katA) of Pseudomonas aeruginosa PA14 is under both positive and negative control of the global transactivator OxyR in response to hydrogen peroxide. *J. Bacteriol.* **192,** 381–90 (2010).

26. Turnbull, L. *et al.* Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nat. Commun.* **7,** 11220 (2016).

Figure 1

A.


Log-fold killing in CFU/mL
2.5
2.0
1.5
1.0
0.5
0.0
mucA
algD
algD+ 250ug/mL alginate
algD+ 100ug/mL alginate
algD+ 10ug/mL alginate

B.

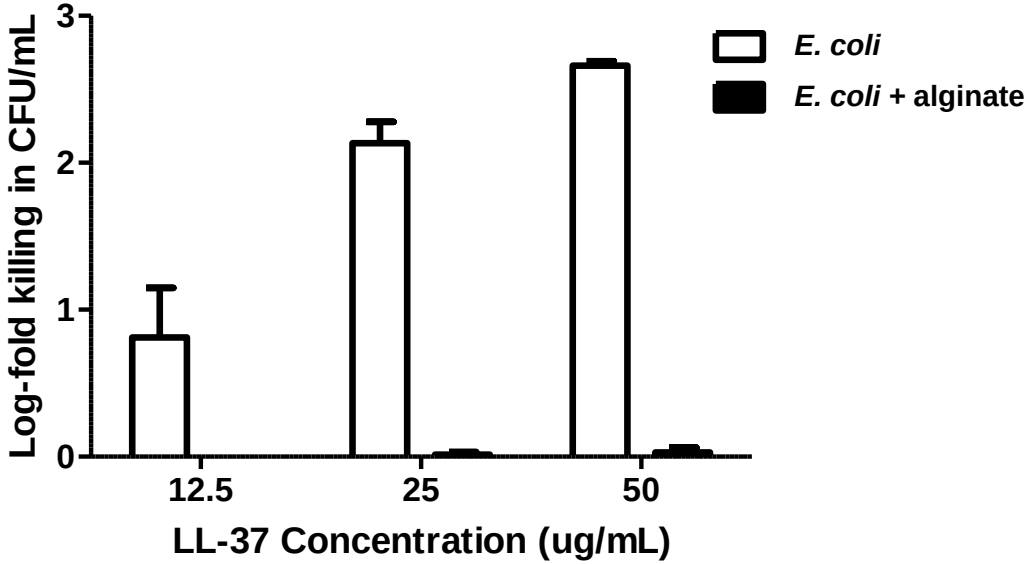

Log-fold killing in CFU/mL
3
2
1
0
12.5
25
50
LL-37 Concentration (ug/mL)
E. coli
E. coli + alginate

Figure 2

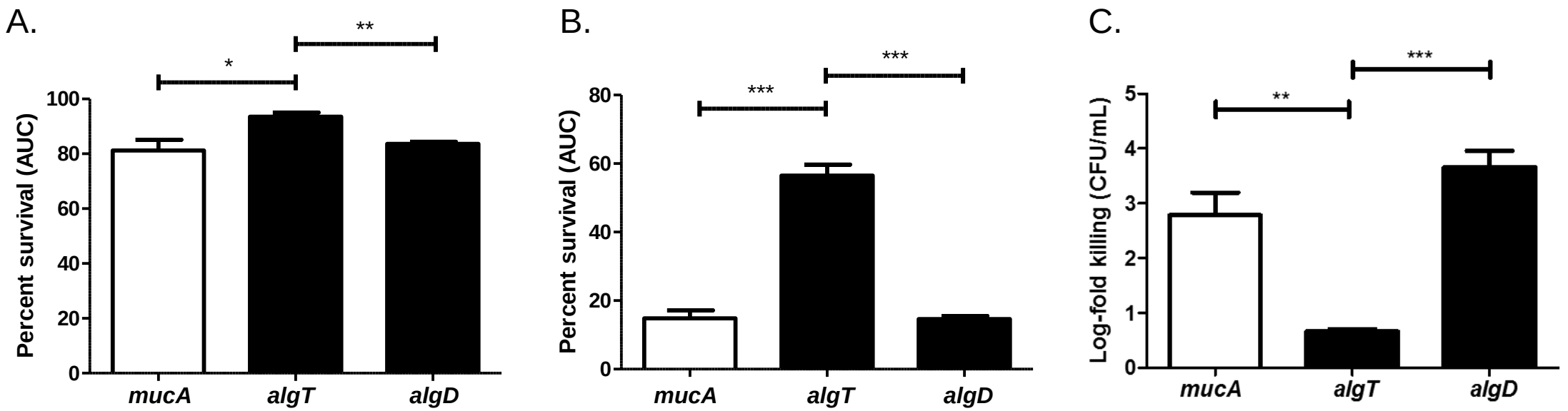

A.
B.
C.
Percent survival (AUC)
Log-fold killing (CFU/mL)
mucA
algT
algD
*
**
***

Figure 3

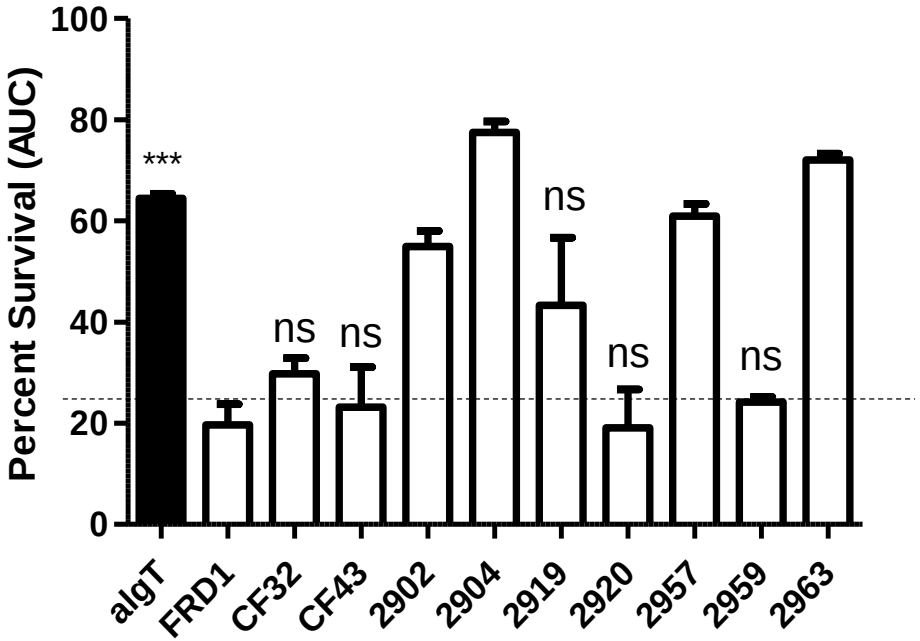

Percent Survival (AUC)
100
80
60
40
20
0
***
ns
ns
ns
ns
algT
FRD1
CF32
CF43
2902
2904
2919
2920
2957
2959
2963

Figure 4

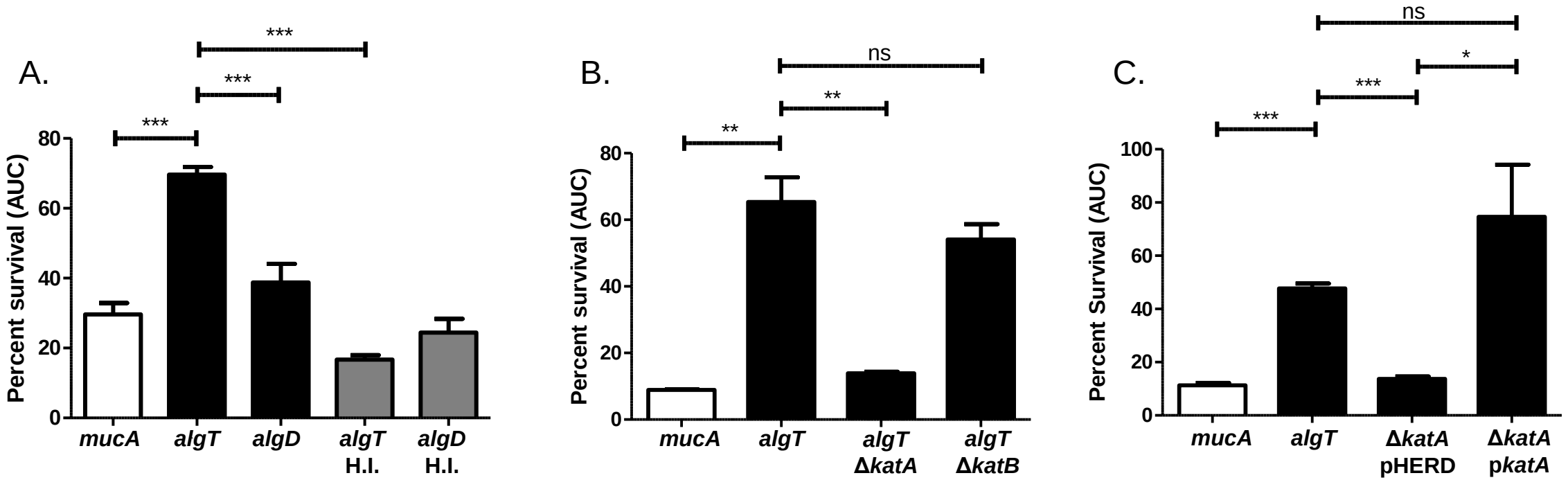
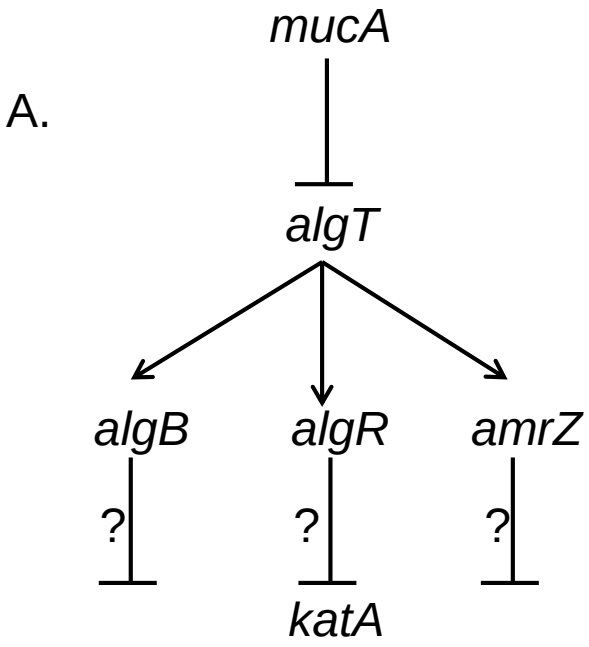
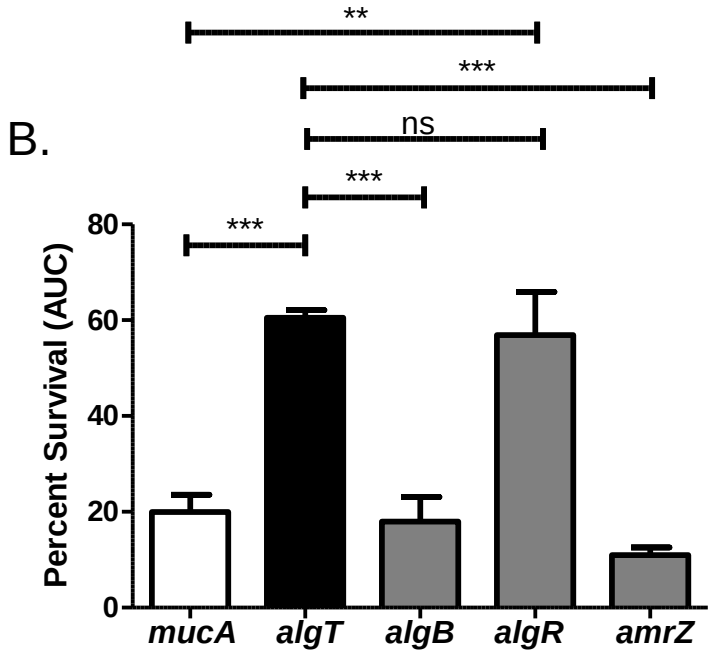
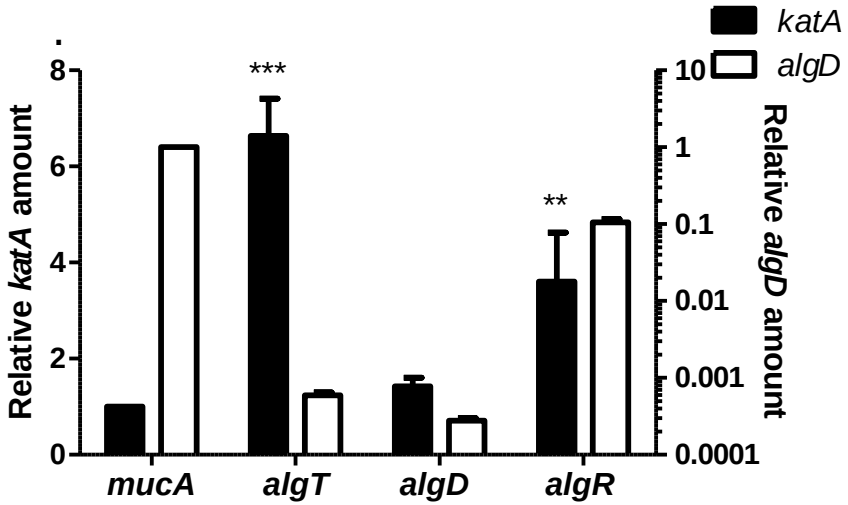

A.
***
***
***
Percent survival (AUC)
0
20
40
60
80
mucA
algT
algD
algT H.I.
algD H.I.
B.
ns
**
**
Percent survival (AUC)
0
20
40
60
80
mucA
algT
algT ΔkatA
algT ΔkatB
C.
ns
*
***
***
Percent Survival (AUC)
0
20
40
60
80
100
mucA
algT
ΔkatA pHERD
ΔkatA pkatA

Figure 5


A.
mucA
algT
algB
algR
amrZ
?
?
?
katA


B.
**
***
ns
***
***
Percent Survival (AUC)
0
20
40
60
80
mucA
algT
algB
algR
amrZ


C.
katA
algD
Relative katA amount
Relative algD amount
***
**
0
2
4
6
8
0.0001
0.001
0.01
0.1
1
10
mucA
algT
algD
algR

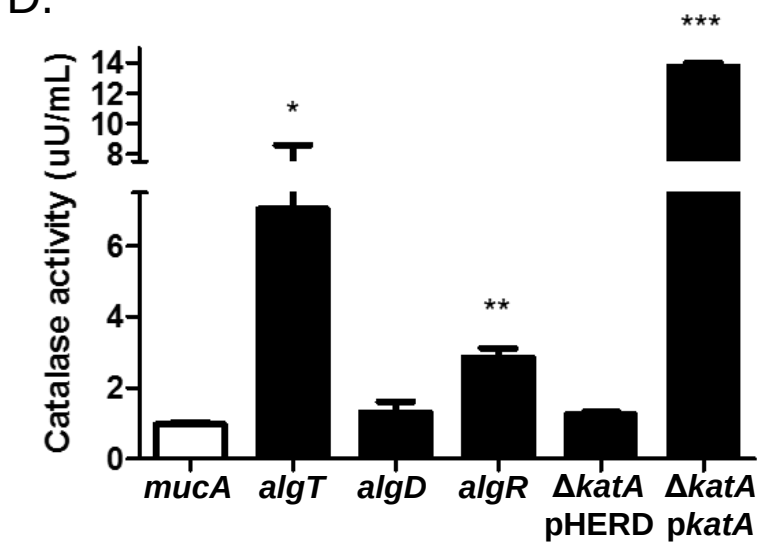
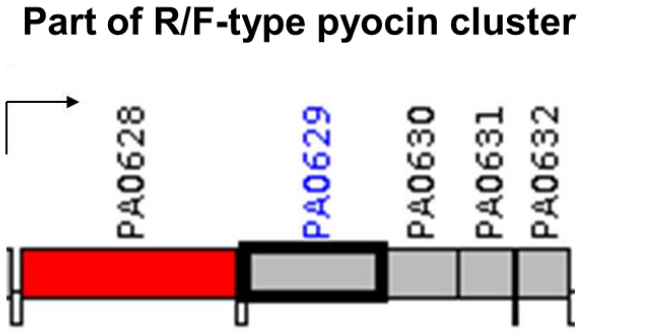

D.
Catalase activity (uU/mL)
*
**
***
0
2
4
6
8
10
12
14
mucA
algT
algD
algR
ΔkatA pHERD
ΔkatA pkatA

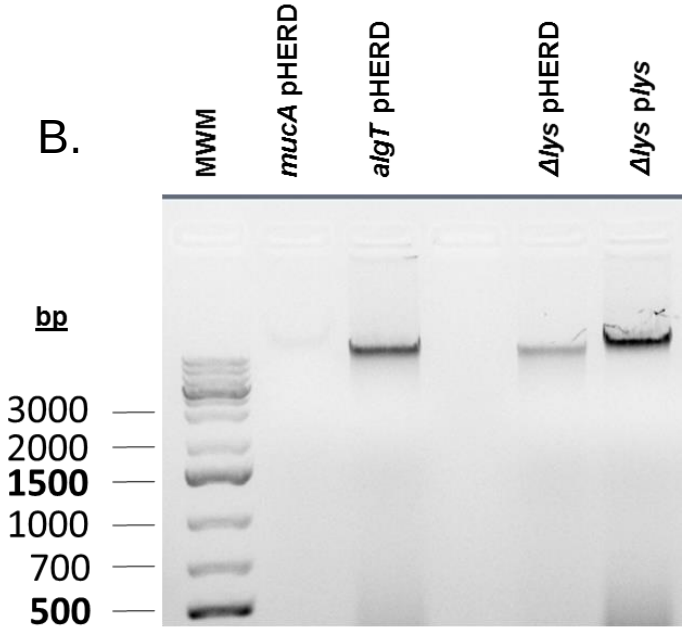
Figure 6

A.

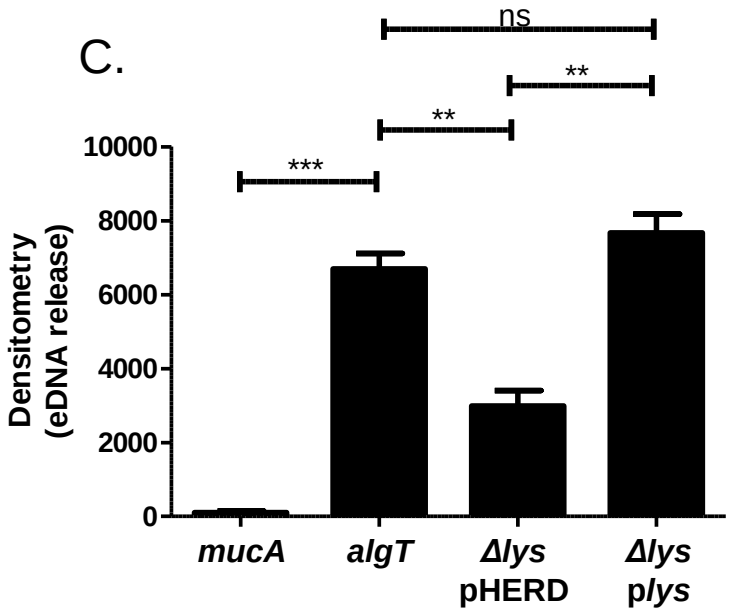


Nucleic Acids Res. (2016) doi: 10.1093/nar/gkv1227 (Pseudomonas genome database).

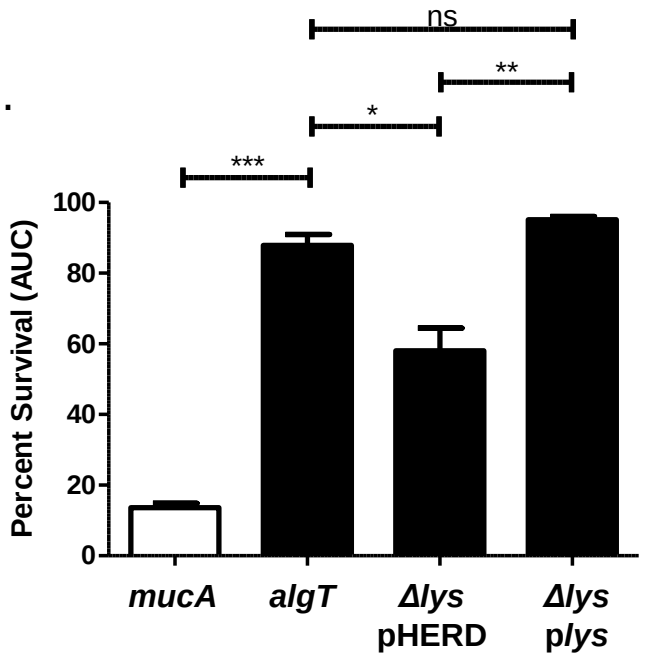
B.



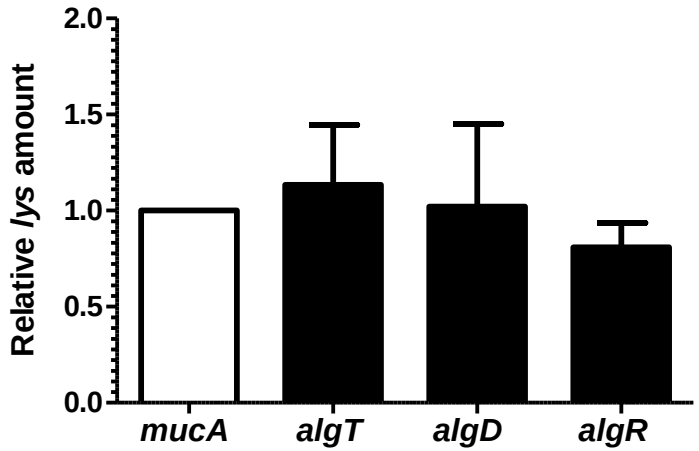
C.



D.



E.



F.

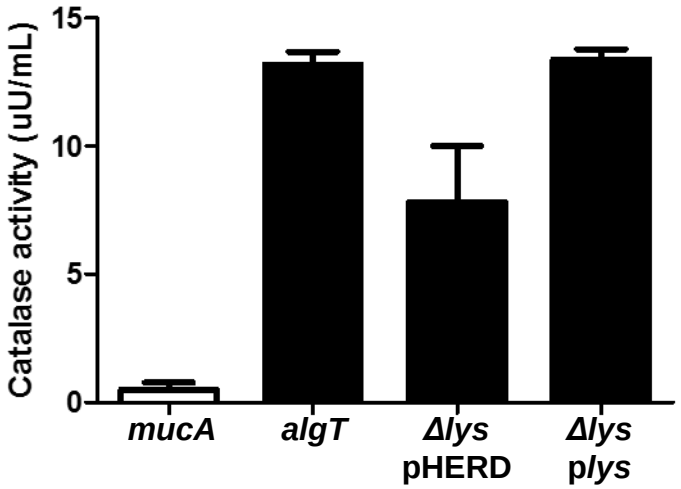


Figure 7

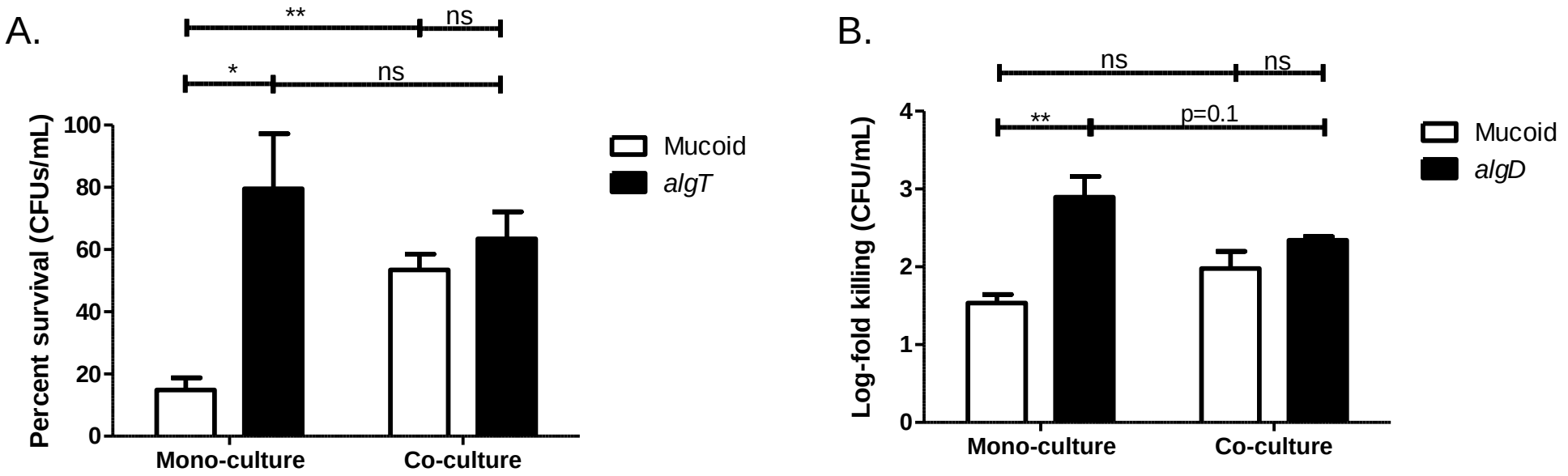
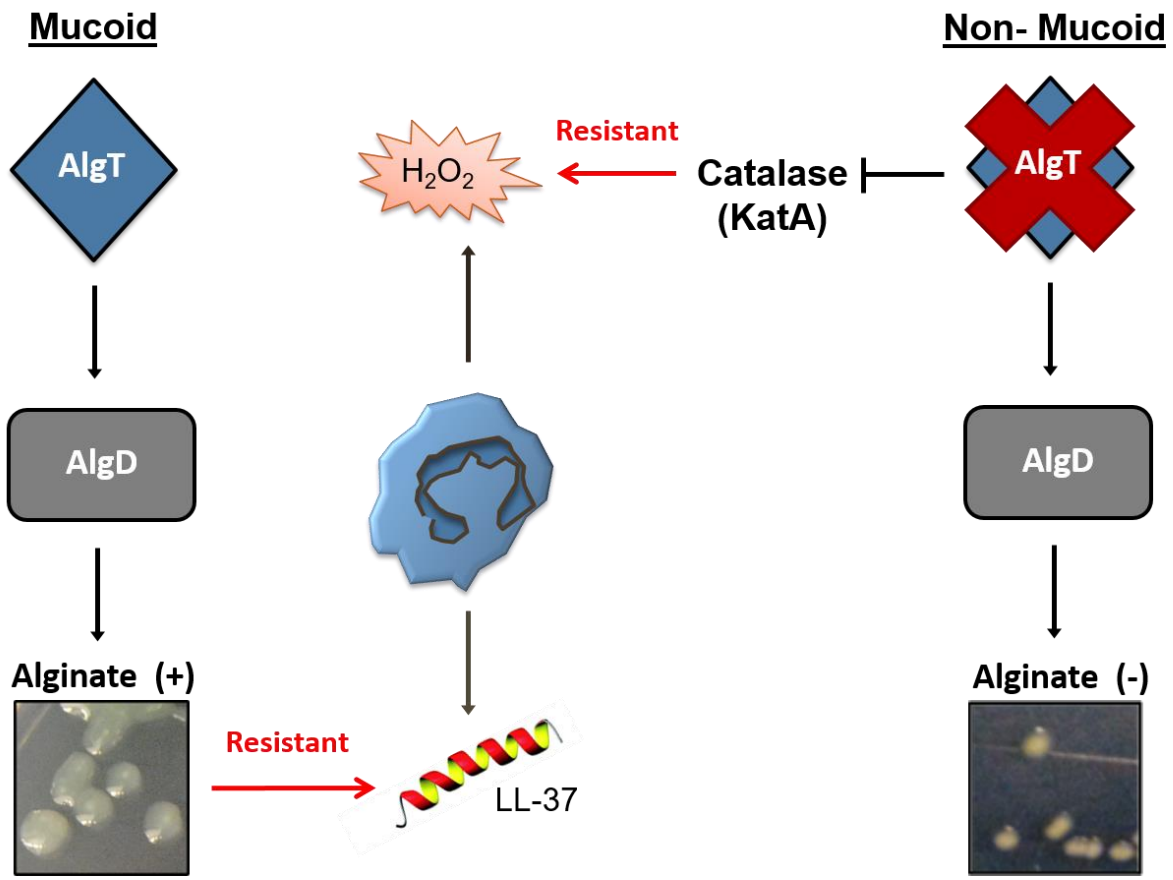

A.
**
ns
*
ns
Percent survival (CFUs/mL)
100
80
60
40
20
0
Mono-culture
Co-culture
Mucoid
algT
B.
ns
ns
**
p=0.1
Log-fold killing (CFU/mL)
4
3
2
1
0
Mono-culture
Co-culture
Mucoid
algD

Figure 8


Mucoid
Non- Mucoid
AlgT
H_2O_2
Resistant
Catalase
(KatA)
AlgT
AlgD
AlgD
Alginate (+)
Alginate (-)
Resistant
LL-37

Supplemental Figure 1