Host-microbe protein interactions during bacterial infection

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Summary

Interspecies protein-protein interactions are essential mediators of infection. While bacterial proteins required for host cell invasion and infection can be identified through bacterial mutant library screens, information about host target proteins and interspecies complex structures has been more difficult to acquire. Using an unbiased chemical cross-linking/mass spectrometry approach, we identified interspecies protein-protein interactions in human lung epithelial cells infected with *Acinetobacter baumannii*. These efforts resulted in identification of 3076 total cross-linked peptide pairs and 46 interspecies protein-protein interactions. Most notably, the key *A. baumannii* virulence factor, OmpA, was identified cross-linked to host proteins involved in desmosomes, specialized structures that mediate host cell-to-cell adhesion. Co-immunoprecipitation and transposon mutant experiments were used to verify these interactions and demonstrate relevance for host cell invasion and acute murine lung infection. These results shed new light on *A. baumannii*-host protein interactions and their structural features and the presented approach is generally applicable to other systems.

Introduction

Interspecies protein interactions and the underlying structural interfaces are essential for bacterial infection

The molecular-level arms race between hosts and pathogens is carried out on multiple fronts, but predominantly takes place through evolutionary adaptation of protein structural landscapes[1-5]. Bacteria commandeer host resources through evolutionarily optimized bacterial protein structures that bind with high specificity to host protein cognates. Pathogen proteins target diverse host proteins involved in metabolite acquisition [4], molecular trafficking to the cell membrane [2], cytoskeletal rearrangement [6], and cell adherence complexes [7]. As an example, iron is necessary for biochemical processes in both bacteria and hosts and can be sequestered by the vertebrate membrane protein transferrin to defend

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Further description of Experimental Procedures can be found in the Supplemental Experimental Procedures.

against bacterial infection[4, 8]. In response, bacteria such as *Neisseria gonorrhoeae* and *Haemophilus influenzae* have evolved transferrin-binding proteins (TbpA) capable of binding and scavenging iron directly from transferrin to overcome sequestration[8]. Barber and Elde[4] showed that single point mutations in transferrin alter TbpA affinity at the interface of the two proteins and are responsible for establishing the host range of the bacteria and modulating host nutritional immunity. Therefore knowledge of not only the proteins involved in host-pathogen protein interactions but also the manner of their interaction, i.e. structural insight into interfacial regions, can profoundly advance understanding of bacterial infection and provide insight for developing new antimicrobial therapies[4].

Technologies have evolved to allow large-scale protein interaction identification, but relevant information on host-pathogen interspecies interactions and structures is still limited

Two-hybrid[9], affinity purification mass spectrometry[10], and protein compliment[11] methods have made the large-scale study of protein-protein interactions (PPIs) possible. Although recent efforts with these techniques have demonstrated the ability to identify PPIs relevant to host-pathogen interactions, including the virus-human protein interactions of HIV[12] and H1N1[13], host pathogen PPIs remain a general challenge to identify. Furthermore, structural details pertaining to host-pathogen protein interactions are exceedingly sparse. Many aspects of host-pathogen interactions are mediated by membrane proteins, as exemplified by the transferrin case above. With roles in quorum sensing, secretion, adhesion and invasion, membrane proteins play pivotal roles in bacterial pathogenesis, yet they often require significant dedicated efforts for interaction studies, are less suitable for many large-scale methods, and are equally challenging for conventional structural characterization[14].

Alternative technologies have the potential to shed light on interspecies PPIs and their structural interfaces

Chemical crosslinking mass spectrometry (XL-MS) approaches are beginning to have a greater impact on protein interaction studies[15-20]. Because of the finite crosslinker length, covalent linkage of two amino acid sidechains indicates their proximity during the crosslinking reaction period. Identification of crosslinked peptide pairs provides useful distance constraints for development and assessment of structural models, as illustrated for the interactions of protein in purified complexes from the protein phosphatase 2A network[16]. Chemical crosslinking can be carried out with mixtures of proteins in cell lysates[19, 21, 22] or on living cells[23-25], whereby interaction identification and structural details on complexes can be performed in an unbiased manner[26-29]. This approach holds great potential for the determination of transient or long-lived interactions that have been chemically stabilized[22], particularly for the identification of protein interactors and structural details of membrane proteins[27]. For example, the outer membrane protein OmpA in *Escherichia coli* is important for adhesion to host cells, catheters and implants among its other roles[30]. OmpA has been among the most heavily studied bacterial membrane proteins over the past 30 or more years. However, the protein was only recently shown to exist as a multimer through *in vivo* crosslinked sites within its C-terminal

domain[31]. This finding was recently verified *in vitro* by site-directed mutation based on our reported crosslinked sites and native mass spectrometry measurements[32]. Our recent efforts have further shown that *in vivo* crosslinking can yield large scale interactions and structural details on complexes in pathogenic bacterial cells, such as *Pseudomonas aeruginosa*[27]. These results demonstrated that OprF, an OmpA-homolog in *P aeruginosa*, exists as a multimer *in vivo*. Additionally, these data showed the potential to gain information from living cells on the interactions and structures of membrane and soluble protein complexes in systems where molecular biology-based strategies are less developed or even unsuitable.

Given the unbiased capabilities for membrane protein interactions and structural characterization, chemical crosslinking technologies offer the potential for identification of interspecies interactions that can help increase our understanding of bacterial infection

The results presented here illustrate the initial crosslinking application of Protein Interaction Reporter technologies to the study of interspecies PPIs in human lung epithelial cells infected with the nosocomial pathogen, *Acinetobacter baumannii*. These efforts produced the first large-scale interspecies crosslink dataset, including 46 host-pathogen PPIs, several of which involve the key *A. baumannii* virulence factor OmpA.

Results

Determination of interspecies protein interactions

In vivo proteomic XL-MS analysis of proteins from infected lung epithelial cells generated 16,758 crosslinked peptide-peptide relationships (Figure 1A). Of these we identified 3,076 non-redundant peptide-peptide relationships across three biological replicates at a relationship false discovery rate of 0.24% (Table S1). Crosslinked peptide-peptide relationships were mapped to a network of 715 PPIs attributed to 488 human proteins and 113 bacterial proteins (Figure 1B, Figure 1C, Figure S1). Identified human proteins covered an abundance range of greater than five orders of magnitude (Figure S1C)[33]. Relationships between two peptides from the same protein constituted the majority of identifications (intraprotein PPIs). Two residues within a single protein have a high likelihood of being in close physical contact within a cell, are therefore more frequently cross-linked, and make up a higher proportion of interactions in crosslinked datasets[22, 34]. Importantly, intraprotein interactions define proximal residues within a protein, and thereby provide valuable structural coordinates for identified proteins even when no known structure exists for this protein (Figure 2). Alternatively, interprotein PPIs were derived from crosslinked relationships between peptides from two different proteins. Interprotein PPIs were used to generate interaction networks (Figure 1B, Figure 3) and yielded structural data pertaining to the interaction interfaces of protein complexes. Within the interprotein relationships, we determined that 3.7% of the total peptide-peptide crosslinks, and 6.4% of all PPIs, were interspecies interactions Figure 1B, Figure 1C). No bacterial proteins or interspecies interactions were detected in uninfected, crosslinked H292 cells (Table S2). Based on these cross-linked relationships we constructed a protein interaction network that included: host-host, pathogen-pathogen, and host-pathogen PPIs (Figure 1B).

To verify that PIR technology could detect physiological protein complexes and interactions, we quantified the number of human PPIs that were (1) intraprotein interactions or (2) were previously annotated in a PPI databases (i.e. PrePPI[35] and IntAct[36]). Out of 566 unique human PPIs 70% were attributed to either intraprotein interactions or identified in a PPI database (Figure 1C). Subnetworks of PPIs from known complexes included: host cytoskeleton, heterogeneous ribonucleoproteins, integrins, histones, cohesin, and ATP synthase (Figure S2) [37]. Identified *A. baumannii* PPIs included interactions with virulence factors, membrane integrity proteins, metabolic complexes, transcription and translation machinery, and many Genes of UNKnown function (GUNKs) (Table S1). The last group represents a large, and potentially important, subset of the *A. baumannii* proteome. Though they make up approximately 30% of the all *A. baumannii* proteins (Figure S1D), functional characterization of GUNK proteins is a challenge [38]. Owing to structural and network information derived from identified crosslinked peptide relationships this work provides insight on functional roles for several GUNK proteins.

A. baumannii *interactions with host proteins*

Bacterial adherence and internalization require host-microbe protein interactions involving bacterial virulence factors binding host signaling complexes, host membrane adherence proteins, and host cytoskeletal proteins [7, 39, 40]. Bacterial membrane proteins and proteins secreted via OMVs were previously suggested to be a major mechanism by which *A. baumannii* adhere to host cells[41] and deliver effector molecules [42]. Interestingly, the bacterial proteins observed in interspecies PPIs were enriched for the presence of predicted signal peptides (p-value = 0.019)[43] and proteins observed in outer membrane vesicles (OMVs) (p-value = 0.026) [42] (Figure S1E). Furthermore, we observed crosslinked relationships involving bacterial proteins previously determined to be *A. baumannii* virulence factors, including: OmpA, Lon protease, Oxa-23, hemolysin, TolB, TonB, and several lipoproteins (Figure 3, Table S1) [44]. We found crosslinked relationships between several of these virulence factors (lipoproteins, OmpA and Lon protease) and human proteins from the cytoskeleton (keratin-7, keratin-8, keratin-18, actin B, plectin, Arp 2/3-1B) and junctional adherence proteins (desmoplakin [DSP], plakoglobin, plectin) (Figure 3B) [40, 41, 45].

Verification of OmpA as an essential virulence factor in Ab5075

Interactions identified between OmpA and host or bacterial proteins were of special interest as OmpA orthologs in other bacteria and *A. baumannii* strains are necessary for bacterial invasion [30, 41]. Verification of this critical role for OmpA in Ab5075 was necessary to better understand the significance of the *in vivo* cross-linking results and establish the pathogenic characteristics of this clinical isolate [46]. To investigate the role of OmpA in the *A. baumannii* strain Ab5075, we first tested bacterial invasion of a gene inactivation mutant of OmpA (transposon insertion, *tn-ompa*) in the Ab5075 genetic background. Invasion of host cells was severely attenuated for the *tn-ompa* mutant compared to wild type Ab5075 (WT, Figure 4A, Figure S3A). Second, pre-treatment with α-OmpA serum prior to infection significantly attenuated Ab5075's ability to invade host cells (Figure 4A). Third, we quantified bacterial invasion after pre-treatment of bacterial cells with novel, purified, and targeted OmpA antibodies (Figure 4A, Figure S3B). Three antibodies, targeting two

extracellular loops and a C-terminal portion of OmpA, were generated and pooled (Figure S3B). We observed that these targeted antibodies were able to attenuate bacterial invasion to the same degree as α-OmpA serum (Figure 4A). Finally in an acute murine lung infection model, all 5 mice treated with *tn-ompa* survived bacterial challenge, while 3 of the 4 mice treated with WT Ab5075 succumbed to infection within 36 hours (Figure 4B). Taken together, these findings establish the significance of OmpA in Ab5075 virulence and highlight new tools for studying Ab5075 virulence (i.e. *tn-ompa* strain and targeted antibodies)

Identification of interactions between a host desmosome and A. baumannii *proteins*

Bacterial outer membrane proteins have been proposed to be involved in host cell adherence and evasion of host immunity[47] and the outer membrane protein OmpA appears critical in several bacterial species[30, 41]. Therefore, identification of OmpA cross-linked to several host proteins in infected cells, including an obligate component of desmosomes and hemi-desmosomes (Figure 3B) offers new insight on OmpA function during cell invasion. Peptide sequences from DSP and OmpA were unique to the human and *A. baumannii* proteomes, respectively (Table S1).

Bacterial infiltration of host epithelial layers has been demonstrated to involve close proximal relationships between desmosomes and bacterial cells[48]. Desmosomes are cell adhesion complexes that mediate cell-to-cell contact in host epithelia [45, 49, 50], and – along with tight junctions, gap junctions, and adherens junctions – create a physical barrier to prevent bacterial intrusion through the epithelium[51]. DSP-OmpA crosslinked peptides that we identified correspond to sites within the C-termini of both proteins. Disruption of the DSP C-terminus has been reported to interfere with epithelial integrity [50], and therefore virulence factor binding the DSP C-terminal could serve to destabilize host cell-to-cell interactions to bypass the barrier function of host epithelia[52]. Sites of crosslinking within both proteins were mapped to C-terminal crystal structures of OmpA and DSP (Protein Databank: 4G4Y [OmpA] and 1LM5 [DSP] [53, 54]) (Figure 5A) [55]. Consistent with our crosslinking results, when we modeled the protein docking of the C-termini of DSP and OmpA, the linked lysine residues between the two proteins in the predicted model were within the crosslinker distance constraint for the BDP-NHP crosslinker (Figure S4B).

To validate this interaction, we immunoprecipitated DSP from Ab5075-infected H292 cells and uninfected H292 cells and blotted for the presence of OmpA. OmpA precipitated with DSP from infected H292 cells in the presence and absence of crosslinker (Figure 5B, Figure S4A), confirming the interaction observed through large-scale, *in vivo* crosslinking. Notably, two high-mass bands, both greater than 250,000 Da in size, were present only in the crosslinked sample (Figure S4A). These high molecular weight bands were presumed to be the crosslinked protein complex formed by DSP (>260 kDa) and OmpA (38 kDa). By confocal immunofluorescence, DSP and OmpA proteins colocalized during Ab5075 infection of H292 cells (Figure 6A). In addition, we observed an *A. baumannii* GUNK protein crosslinked with DSP. The GUNK identified in multiple peptide-peptide interactions with DSP was Ab57_2521 (later referred to as GUNK2), a signal-peptide containing protein that also physically interacts with OmpA in infected host cells. The random chance of

matching multiple interactors across species in this way was miniscule ($\rho = 2.35 \times 10^{-9}$, Figure 3B, Figure S5A).

Guilt-by-association: Functional significance of genes of unknown function

Defining the function and biological significance of GUNK proteins remains a challenge [38]. The unbiased determination of interactions between GUNK proteins and annotated gene products from a bacterial genome offers clues for putative functional characterization of GUNKs[56]. In addition to cross-linking infected host cells, we harvested Ab5075 alone and crosslinked the bacterial cells to improve network and structural coverage of bacterial interprotein and intraprotein crosslinks within Ab5075 (Table S3). Of particular interest were GUNK proteins we observed linked to OmpA, since OmpA appears necessary for bacterial infection (Figure 4). In both infected host cells and *A. baumannii* cells alone we observed crosslinks between OmpA and several GUNK proteins (Table S1, Table S3). We quantified bacterial invasion in host cells using transposon insertion mutants of three unannotated GUNKs most frequently identified cross-linked to OmpA – hereafter GUNK1, GUNK2, and GUNK3 (Figure 5C, Table S1). Transposon insertion mutants of GUNK1, GUNK2, and GUNK3 in the AB-5075 background showed reduced host cell invasion as compared to WT Ab5075 (Figure 5C). Two independent transposon mutants (A and B) for each GUNK gene were tested and observed to have consistent phenotypes (Figure 5C). While the attenuation of mutant bacterial invasion was not as great for these GUNK proteins as for OmpA, the reduction was still statistically significant compared to WT Ab5075 (p-values $< 2 \times 10^{-4}$, $< 7 \times 10^{-5}$, and $< 9 \times 10^{-7}$ for GUNK1, 2 and 3, respectively) (Figure 5C).

The intersection of PPIs and bacterial genes essential for persistence in host lungs

We compared our dataset of interspecies crosslinked proteins to a recently established set of *A. baumannii* genes determined to be essential for bacterial persistence in the murine lung [57]. Of the bacterial proteins we identified in crosslinked interspecies interactions (n=31), we observed a significant enrichment of essential persistence genes (7/31, p-value = 0.000664) (Figure S5B). Discrepancies between these two datasets can, in part be explained by three factors. First, a different, less virulent strain of *A. baumannii* (Ab17978) was used to test persistence in the murine lung[46, 57]. Second, not all proteins essential for invasion may interact with host proteins (proteins identified in persistence study but not in the proteomic interactome). Third, not all protein interactions between host and pathogen proteins may be essential (proteins identified in proteomic interactome but not in persistence study). Nonetheless, these results from *in vivo* host-pathogen cross-linking provide the first identification of host partners for seven *A. baumannii* proteins previously found to be essential for bacterial persistence in the host lung.

Discussion

Interspecies protein interactions are critical determinants in bacterial infection and pathogenesis[2]. Improved knowledge on which interspecies PPIs exist and how the involved proteins interact can greatly advance understanding of molecular mechanisms involved in host invasion and provide new opportunities for antibacterial therapies. As the growing threat of antibiotic resistant pathogens appears likely to surpass all currently known

antibiotics [58, 59], new knowledge and strategies that can help treat multidrug resistant bacterial infections are of critical importance. Many strains of *Acinetobacter baumannii*, such as the clinical strain Ab5075 used in these studies, exhibit increased resistance to all antibiotics, including carbapenems [46, 58]. Using unbiased *in vivo* protein crosslinking we identified physical interspecies PPIs during microbial infection to enable the first visualization of the *A. baumannii*-host interaction network. In total, more than 3000 cross-linked peptide pairs were identified defining the existence and structural features of more than 700 PPIs and 46 interspecies PPIs representing one of the largest host-bacteria protein interaction datasets and the first large-scale PPI analysis of host-pathogen interactions for *A. baumannii* (based on HPIDB2.0, Figure 6B) [60].

Previous studies have shown the outer membrane protein OmpA to be critical to attachment and invasion in many pathogenic bacteria [30], including *A. baumannii* [41, 61]. OmpA was also identified as a required gene for microbial persistence in murine lung infection ($\rho=7.6\times10^{-79}$)[57]. Though our experimental design did not specifically target OmpA, the membrane porin was identified in cross-linked relationships to several host proteins in infected epithelial cells. During infection OmpA migrates to at least three subcellular locations within host cells: mitochondria, nuclei and cell-surfaces [62, 63]. Consistent with these findings, we identified OmpA interspecies interactions with a mitochondrial protein that affects reactive oxygen species generation (GFM-1) [64, 65], a nuclear protein involved in the DNA damage response[66] and circadian rhythm regulation[67] (SFPQ), and the obligate desmosomal adhesion protein DSP (Figure 3B).

Bacterial targeting of adhesion complexes is a well-established mechanism for microbial intrusion and invasion [68, 69]. Gram-negative bacteria, such as *Staphylococcus aureus*[70] and *Campylobacter jejuni*[71], have previously been observed to bind and disrupt desmosomes during infection. Van Schilfgaarde et al. showed that bacterial intrusion into human lung epithelial cells placed infiltrating bacteria in close contact with desmosomal plaques[48]. The *in vivo* cross-linking results presented here linking OmpA and DSP are consistent with these observations and is corroborated by the fact that OmpA co-localizes with DSP in infected human cells (Figure 6A). OmpA has previously been shown to be a component of secreted *A. baumannii* OMVs[61]. OmpA and DSP could therefore colocalize after bacterial internalization [72] (Figure 4A, Figure 6A) or through the local release of bacterial OMVs [61] and the subsequent integration of these vesicular membranes into the host membrane [73]. For the latter case, altering desmosomal integrity could aid infiltration through host epithelia. Finally, because desmosomes integrate signals between the host cell surface and cytoskeleton [74], and a functional host cytoskeleton is required for *A. baumannii* pathogenesis [75], binding and targeted disruption of the desmosome by bacterial proteins could be a means for *A. baumannii* to modulate the requisite host cytoskeleton and facilitate epithelial intrusion and host-cell invasion. This speculation requires further investigation of course, but knowledge of the OmpA-DSP interaction will enable these future studies.

With extensive evidence linking OmpA and orthologous proteins to pathogenicity, the discovery of GUNK proteins linked to OmpA established a preliminary association between these GUNKs and bacterial invasion. While two GUNK proteins were *A. baumannii*-specific

(GUNK2 and GUNK3), GUNK1 shares sequence identity with hypothetical proteins from *Bacillus* (52%) and *Pseudomonas* (43%) species and META-domain/HslJ proteins from *Bordetella* (38%) and *E. coli* (32%). The HslJ protein has previously been related to antibiotic resistance to novobiocin [76], and overexpression of META proteins in *Leishmania* resulted in increased virulence[77]; META-domain proteins, often observed in hypothetical bacterial proteins [78], have also been implicated in bacterial motility. The determination of multiple crosslinked relationships with OmpA provided interaction-based evidence ('guilt-by-association' [56]) leading to further characterization of these GUNK proteins. We believe that sequence similarity potentiating the involvement of GUNK1 in motility/resistance and the attenuated host invasion by all three GUNK transposon insertion mutants (Figure 5C) further support these GUNK proteins as putative *A. baumannii* virulence factors. These findings established the utility of an unbiased approach to identify novel interactions and disseminate functional characterization from PPIs between known virulence factors and GUNK proteins.

The techniques underlying our study of *in vivo* protein crosslinking in bacterially infected human cells provide a unique opportunity to view networks of intercellular PPIs. Recent studies have suggested that interactomes, even incomplete ones, can enable discovery of molecular commonalities between phenotypically-related disease pathologies that may not share primary disease genes [79]. When extrapolated to the study of bacterial pathogenesis, overlapping interspecies interactomes could reveal common paradigms of infection exploited by microbes. *In vivo* cross-linking of host-pathogen systems is a generally applicable technology and future applications with other pathogens will help to explore the possibility of common strategies used among multiple pathogens.

Significance

Protein interactions are essential mediators of bacterial pathogenesis. While methods to elucidate bacterial proteins required for infection have been employed extensively, information regarding the host proteins targeted by bacteria and interspecies complex structures has been more difficult to determine. In this proof-of-principle study, we used chemical cross-linking of proteins in combination with large-scale mass spectrometry to identify interspecies interactions between proteins in cultured human cells and Gram-negative bacterial proteins during bacterial infection. These efforts resulted in identification of interspecies protein-protein interactions between human proteins and known bacterial virulence factors. Most notably we identified bacterial virulence factors interacting with host structural proteins that mediate host cell-to-cell adhesion. Our study shows the potential of chemical cross-linking of proteins in combination with large-scale mass spectrometry to shed new light on host-pathogen protein interactions and their structural features. We demonstrate the ability of said methods to interrogate protein interactions underlying complex pathogenic systems and the presented methodologies are generally applicable to other pathogen systems. The broad application of these methods could aid the rapid expansion of understanding of how diverse bacterial species target and manipulate host proteins during pathogenesis.

Experimental Procedures

Cell culture

Ab5075 cells were grown to stationary phase in nutrient broth (BD). H292 cells were grown in RPMI-1640 (Thermo) with 10% fetal bovine serum and 1% penicillin/streptomycin (37°C, 5% CO_2). Transposon mutants in Ab5075 were attained from Dr. Colin Manoil [80].

Crosslinker synthesis, infection, Stage-1 database creation, sample preparation

Biotin-Aspartate Proline-PIR *n*-hydroxyphthalimide (BDP-NHP) was synthesized as previously described [28, 81]. Confluent H292 cells were washed, released and pelleted before resuspended in crosslinking (XL)-buffer (0.17M potassium phosphate) containing Ab5075 cells at an MOI of 500 for 2 hours. Five experiments were run: three biological replicates of Ab5075-infected H292 cells (Figure S6), and two biological replicates of uninfected H292 cells (Table S2). BDP-NHP (8mM) was added to the cell suspension for 1 hour. **H**292 cells infected with **A**. *baumannii* (i.e. HA-1, HA-2, HA-3) and uninfected cells were washed, pelleted, and frozen at −80°C.

Infected H292 cell pellets were resuspended in lysis buffer: 8M urea, 100mM Tris-Cl pH 8.0, 150mM NaCl, and protease inhibitor tablets (Roche); then lysed by cryo-grinding and sonication. Proteins were reduced and alkylated. Protein lysates were diluted with 100mM Tris-Cl, pH 8.0. 1mg of protein was removed to enrich full length crosslinked proteins with monomeric avidin beads. Enriched, crosslinked proteins were digested with sequencing grade trypsin (Promega), and injected on a C-8 column eluting into an LTQ-XL or LTQ-Velos-FT-ICR mass spectrometer to create a search database of proteins that had been crosslinked (Stage-1 database)[28]. Digested Stage-1 peptide samples were shot in quadruplicate on a 4 hour reverse phase data dependent Top5 method. Spectra were searched using SEQUEST. 30880 peptide-spectral matches (10304 unique peptides) were identified at a false discovery rate (FDR) of less than 1%, based on a concatenated, target-decoy database of all human and *A. baumannii* (AB0057) proteins [28]. The remaining protein lysates were digested with sequencing grade trypsin. Digested peptides were desalted and fractionated by strong cation exchange (SCX). Eluted peptides were incubated with monomeric avidin beads to enrich crosslinked peptides.

LC-MS/MS/MS, ReACT, database searching, and data analysis

Crosslinked peptides were resuspended in 5% ACN/2% formic acid and injected onto an in-house pulled C-8 column (Magic, 200A, 5um) run on 4 hour gradients as with the Stage-1 mass spectral analysis; eluted peptides were analyzed on an LTQ-Velos-FT-ICR. Crosslinked peptides were fragmented in a data-dependent ReACT mode (Real-time Analysis for Crosslinked peptide Technology)[28]. Briefly, high-charge state precursor ions (MS^1, $z > 4+$) were isolated and fragmented at low energy ($Q = 0.20$) to release crosslinked peptides and a reporter ion (m/z = 752.41). Data-dependent selection of fragmented MS^2-ions that sum to the precursor mass minus the reporter ion were further fragmented for MS^3 spectra and peptide sequencing.

All parameters and filtering was done as previously described to identify unique PPIs and site-site interactions[28]. The final relationship FDR was calculated to be 0.24% [(2 * 20 decoy relationships with at least 1 reverse hit)/16758 total relationships]. The final PPI FDR was 1% [(2 * 4 unique decoy PPIs)/719 total unique PPI's]. All interaction data, including pep.xml files, can be found at http://brucelab.gs.washington.edu/xlinkdb/. Protein interaction networks were created using Cytoscape 3.0. Protein structures for OmpA and desmoplakin were downloaded from the Protein Databank (4G4Y and 1LM5, respectively). Protein structure interactions were modeled using PatchDock[55]. KEGG pathway enrichment p-values were determined using STRING v9.1 [37].

Gentamicin protection assays

H292 cells were plated in 24-well plates and allowed to attach for 16 hours. H292's were incubated with Ab5075 or Ab5075-*tn-ompa* for 3 hrs in serum-free, antibiotic-free RPMI-1640 media (RPMI). The supernatant was saved to normalize AB5075 growth during infection. H292 cells were incubated for 1 hour with 200μg/ml gentamicin in RPMI at 37°C. Lysis buffer (PBS with 0.1% Trition-X100) was added to each well, and the plate was shaken at 200rpm for 10 min. Lysates (50ul) were cultured on NB-agar plates (16 hours, 37°C). Colonies were counted and normalized to WT AB5075 growth. Values are the average of technical duplicates from at least three experiments performed on three different days. For antibody blocking of OmpA-based invasion, Ab5075 cells were pre-incubated with α-OmpA or PBS-control serum (Dr. Michael McConnell) or purified Genscript synthesized antibodies. Pre-treated Ab5075 cells were then incubated with H292 cells as above.

Confocal immunofluorescence and brightfield microscopy

Confluent H292 cells were infected with Ab5075 (MOI = 100) on 3.5cm plates with glass coverslips (No. 1.5, Mattek). For confocal immunofluorescence, cells were fixed (formalin), blocked (3% milk in PBST) and incubated overnight at 4°C with α-DSP antibody (rabbit, Abcam) and primary mouse α-OmpA serum (Dr. Michael McConnell) in blocking buffer. Microscopy was performed with a Nikon A1 confocal mounted on a Nikon TiE inverted microscope (Garvey Cell Imaging Lab) at 20× magnification, n=1 (air), NA=0.75. Depth of field measured based on λ = 595nm. For brightfield microscopy, H292 cells were grown to confluency on 24 well plates, incubated in crosslinking buffer +/− Ab5075 cells (MOI=100), washed with PBS and imaged at 20× magnification.

Murine acute lung infection

WT Ab5075 or *tn-ompA* were streaked on LB agar or LB agar with 5ug/ml tetracycline (LBtet) from frozen stocks. PBS (50ul) containing ~2×10^8 CFU/ml bacteria were administered intratracheally to anesthetized mice as described before[82]. Animals that became moribund, distressed or were unable to eat/drink were euthanized using a CO_2 chamber. Experiments were approved by the University of Washington Institutional Animal Care and Use Committee (protocol number 4113-01).

Co-immunoprecipitation (Co-IP) and western blotting

H292 cells were grown to confluency and washed prior to co-incubation with Ab5075 cells (MOI=500) for 2 hours at RT. Subsequently, either DMSO or BDP-NHP in DMSO was added to the samples followed by 1 hour incubation. Cells were pelleted and resuspended in IP buffer (10mM Tris-Cl pH 8.0, 100mM NaCl, 1% Triton-X100, 1mM EDTA, protease inhibitors, 100μg/ml lysozyme) and incubated on ice for 30min to lyse bacterial cells; samples were syringe pumped (27.5 gauge needle) and sonicated to further lyse cells and fragment DNA.

Lysates were pre-cleared with 50μl of Protein-G-agarose (Thermo) mixed at 4°C for 1 hour. α-Desmoplakin antibody was added for 16.5 hours at 4°C. For immunoprecipitation, 50μl of Protein-G-agarose was added for 3 hours at 4°C. Beads were pelleted and thoroughly washed. Co-IP proteins were eluted at 95°C for 10 min with a 3:1 ratio of 1% SDS, 15% glycerol, 50mM Tris-Cl pH 8.0, 150mM NaCl: XT Sample Buffer (Biorad). Finally, proteins were detected by SDS-PAGE and western blot (primary: α-OmpA serum; secondary: α-mouse antibodies [IRDye, Li-Cor]).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- 3076 cross-linked peptide pairs identified from infected cells.

- Forty-six interspecies protein-protein interactions were identified from cells.

- Interspecies interactions involving unannotated *A. baumannii* proteins were identified.
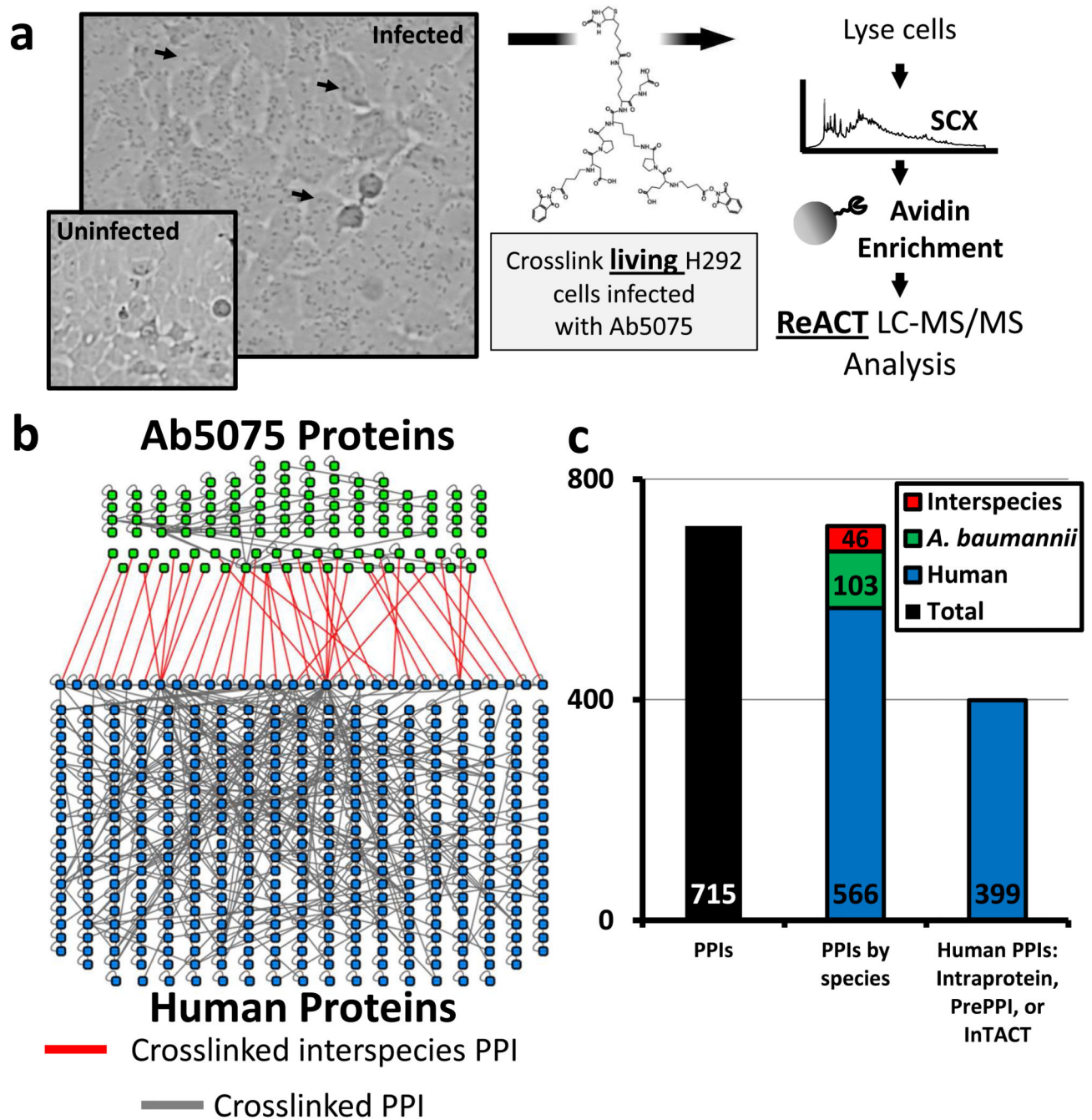
- Structural features of OmpA-desmoplakin interaction revealed.

Figure 1. Identification of protein interactions from infected lung epithelial cells
(a) H292 cells were infected with Ab5075, crosslinked using BDP-NHP. Digested peptides were enriched and analyzed by LC-MS/MS. (b) PPI map of human (blue) and bacterial (green) proteins. Interspecies crosslinks are highlighted in red. (c) Total number of PPIs within the dataset, their breakdown by species (Human-human, Ab5075-Ab5075, or Ab5075-Human), and the relative matched interactions from orthogonal data (i.e. previously observed in PrePPI[35]/IntAct[36] or intraprotein PPIs within a single protein).

Ab57_2983 *(A. baumannii)*
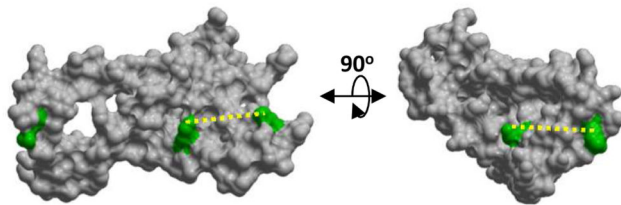
Oxa-23 *(A. baumannii)*
(PDB: 4JF4)
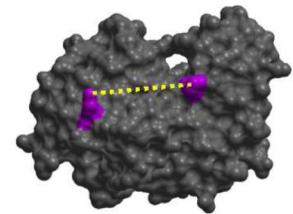
Plakoglobin (*Homo sapiens*)
(PDB: 3IFQ)
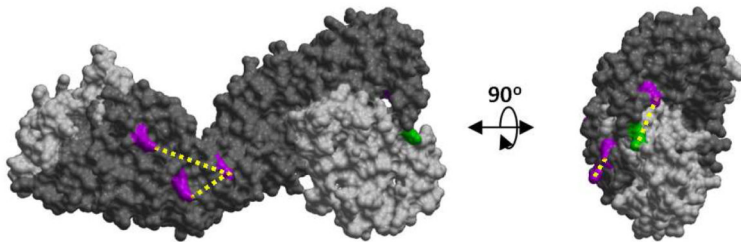
■ **Predicted structure**

■ **PDB structure**

■ **Lysine mapped to predicted structure**

■ **Lysine mapped to PDB structure**

Figure 2. Intraprotein interactions mapped to known and predicted protein structures
Crosslinked sites identified in the large-scale proteomic infection experiment were mapped
to Phyre2 predicted structures [83] (green lysine sites, light grey model) or known crystal
structures (magenta lysine sites, dark grey model) for the bacterial proteins Ab57_2983 and
Oxa23 and the human protein plakoglobin. For plakoglobin, while the central domain has
been crystalized (PDB: 3IFQ), the N- and C- terminal portions have not been crystalized, yet
a site within the C-terminus was identified in an intraprotein crosslinked relationship and
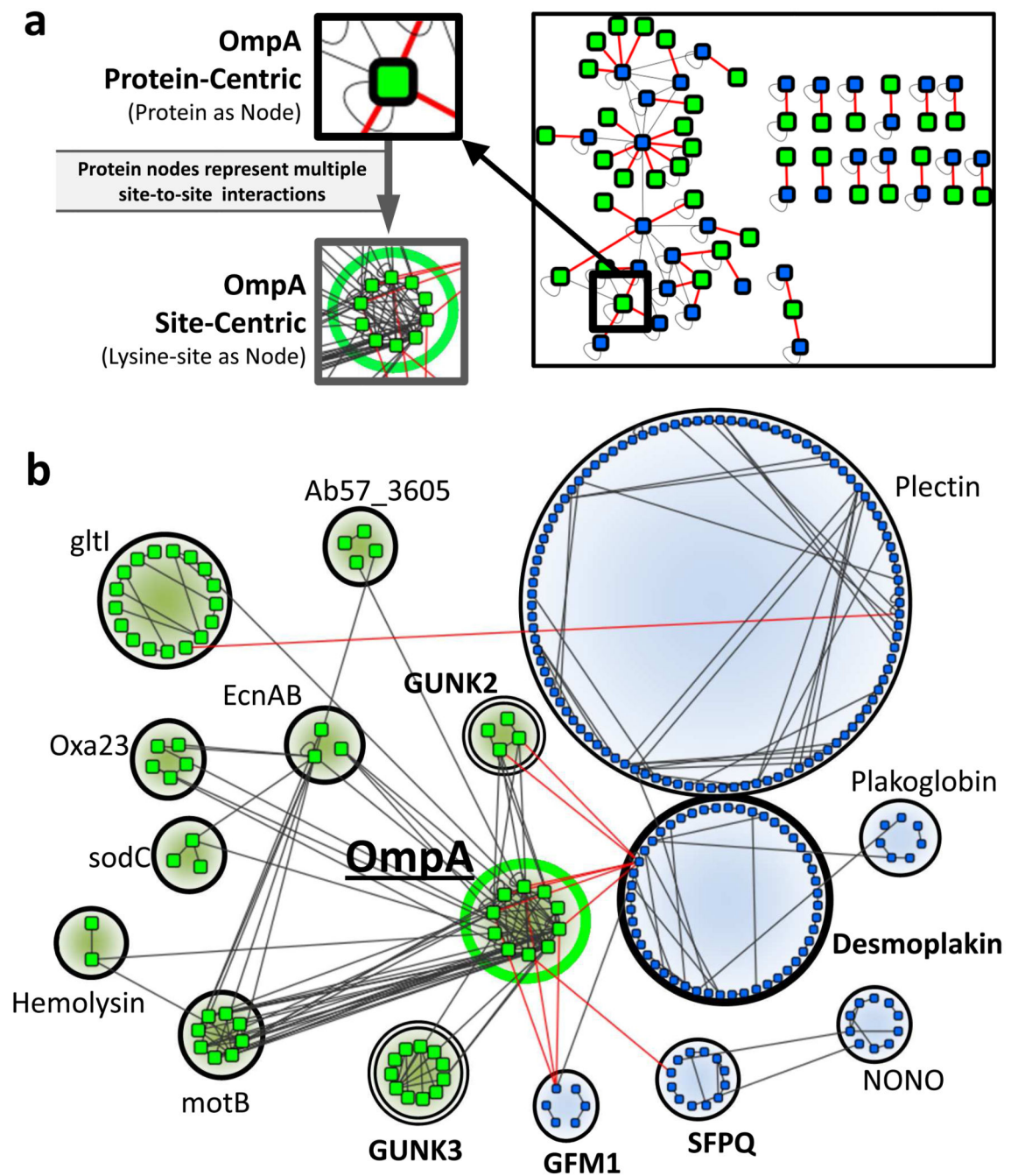shown within the predicted structural model of this region.

Figure 3. Depth of interspecies and OmpA specific intercellular interactions
(a) Force-directed network of the interspecies protein interactions identified between *A. baumannii* and human proteins. Insets depict how multiple site-to-site crosslink interactions underlie each PPI. Interspecies crosslinks are red. (b) Site-to-site interactions for all proteins (bacterial and human) interacting with OmpA in the cell infection model. Nodes are individual lysine sites identified in crosslinked relationships between human (blue nodes) and bacterial (green nodes) proteins. Interspecies links are red.
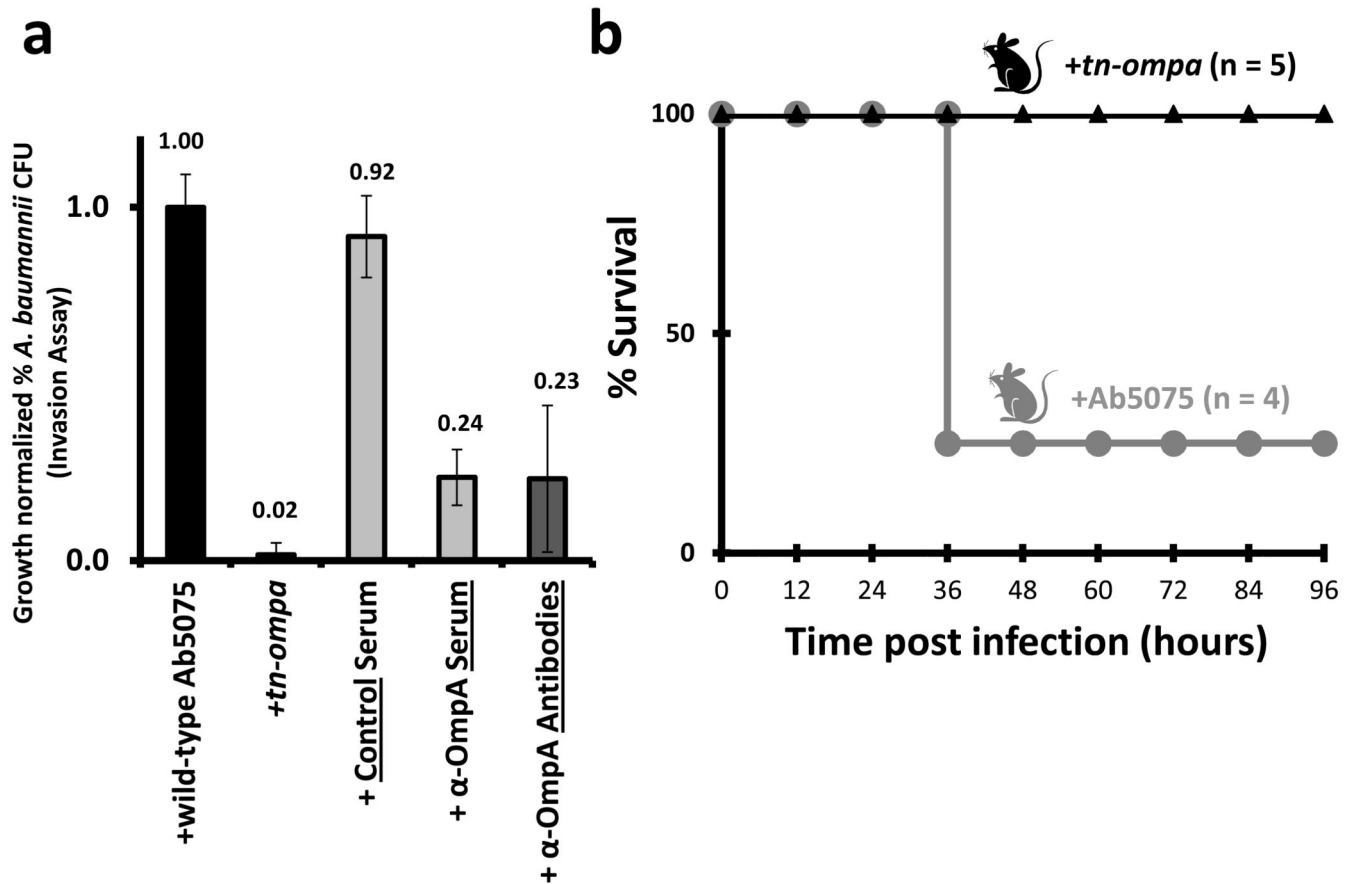
Figure 4. OmpA-related virulence in Ab5075

(a) Ab5075 invasion assays comparing WT Ab5075, Ab5075 with transposon disruption of OmpA (*tn-ompA*), and WT Ab5075 treated with control IgG serum, α-OmpA serum, or purified α-OmpA antibodies. Mean +/− SEM. (b) Murine intratracheal infection with WT Ab5075 (grey) or *tn-ompa* (black, log-rank p-value = 0.025).
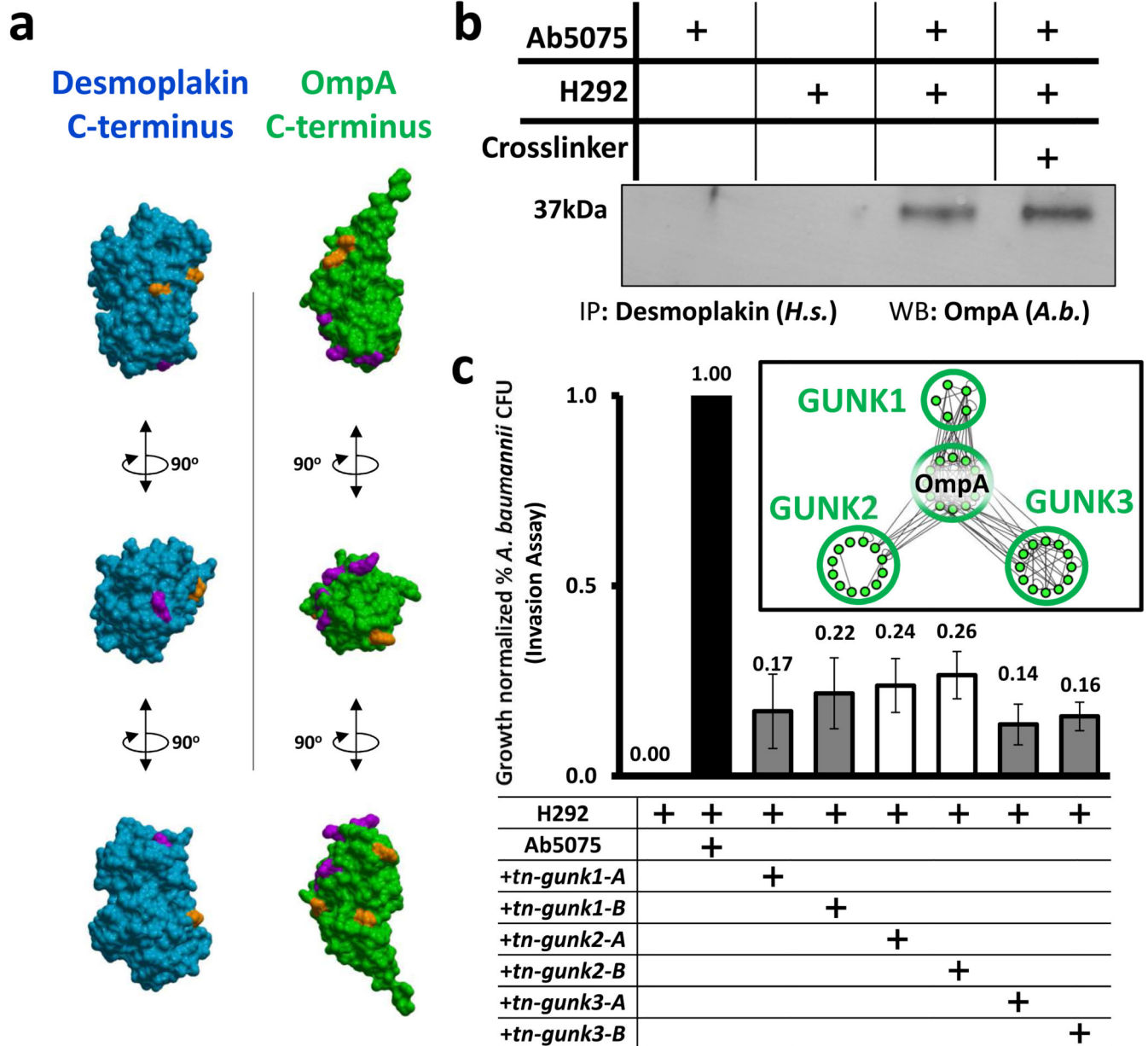
Figure 5. Functional significance of uncharacterized interspecies interactors and OmpA interactors

(a) Space filling models for crystal structures of OmpA (green, PDB: 4G4Y) and DSP (blue, PDB: 1LM5). Identified sites of crosslinking are highlighted in orange. Sites crosslinked between DSP and OmpA are highlighted in magenta. (b) Immunoprecipitation of DSP from Ab5075 cells, H292 cells alone or Ab5075-infected H292 cells (with and without crosslinker). (c) Ab5075 invasion assays comparing WT Ab5075 to Ab5075 with transposon disruption mutants of GUNK1, GUNK2, GUNK3 (Ab57_1108, Ab57_2521, and Ab57_2983). Mean +/− SEM. Inset: crosslinked site interactions between GUNK proteins and OmpA from Ab5075 crosslinked alone (nodes: non-redundant crosslinked lysines; edges: crosslinked relationships).
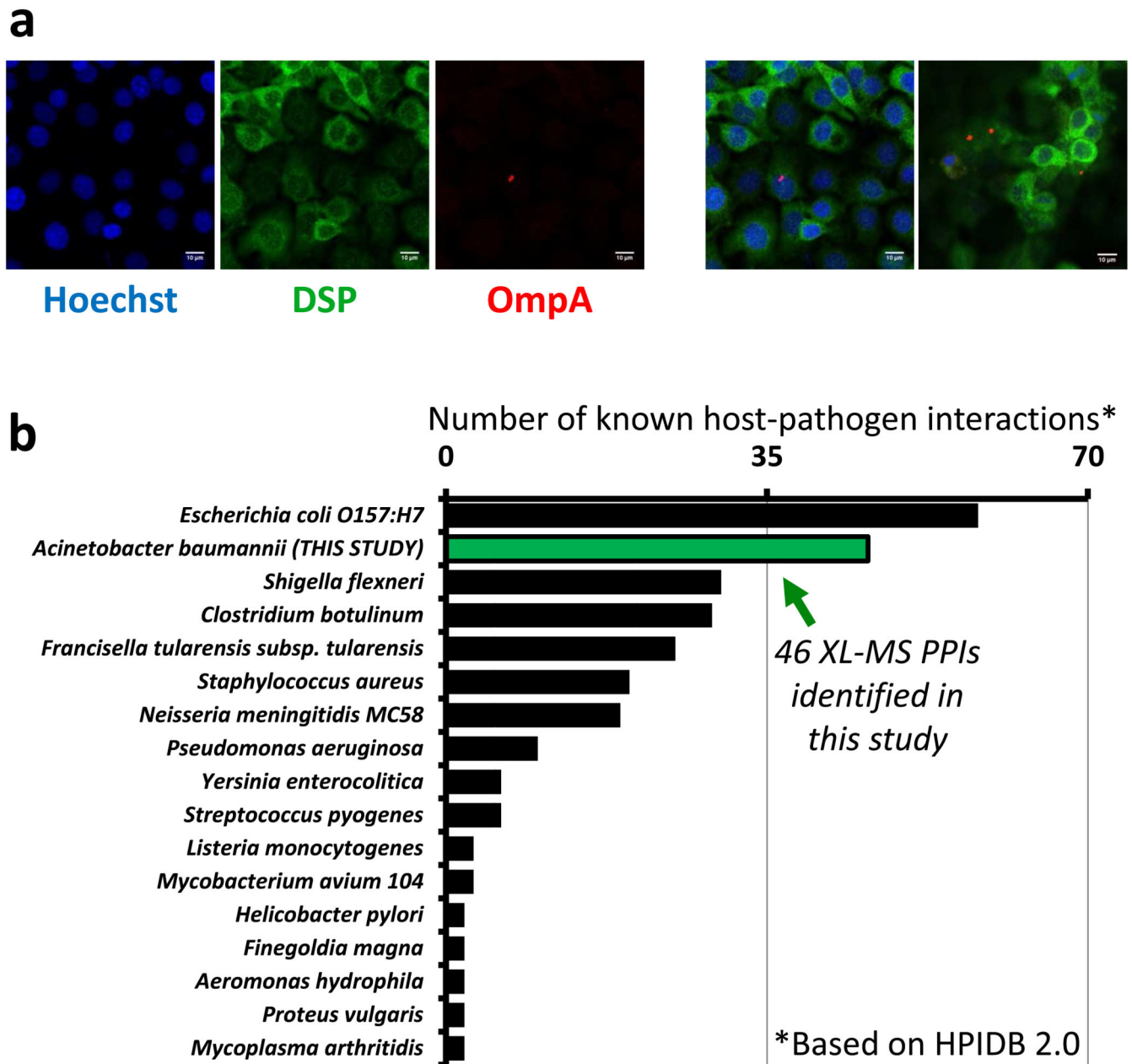
Figure 6. Ab5075 host-pathogen interactions
(a) Confocal micrographs of Ab5075-infected H292 cells stained with primary antibodies against desmoplakin (green) and OmpA (red). DNA was stained with Hoechst 33342 (blue). Confocal images and overlaid images from replicate analyses at a minimal thickness of 1.1μm, scale bars represent 10μm. (b) The 46 host-pathogen interactions identified in this study (green bar) compared to the number of interspecies interactions identified for other bacterial species (HPIDB 2.0 database) [60]. If substrains were present in the database, the strain with the highest number of interactions was shown. Due to scale, HPIDB 2.0 host

pathogen interactions for *Yersinia pestis* (n=4018), *Bacillus anthracis* (n=3061), and *Francisella tularensis subsp. tularensis* SCHU S4 (n=1346) were not shown.