



UiT The Arctic University of Norway

Faculty of Health Sciences

Activity-Based Protein Profiling (ABPP) and functional validation of uncharacterized enzymes in bacterial pathogens *Staphylococcus aureus* and *Klebsiella pneumoniae*

Md Jalal Uddin

A dissertation for the degree of Philosophiae Doctor - May 2024

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Activity-Based Protein Profiling (ABPP) and functional validation of uncharacterized enzymes in bacterial pathogens *Staphylococcus aureus* and *Klebsiella pneumoniae*

MD JALAL UDDIN



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Research Group for Host-Microbe Interactions

Department of Medical Biology

Faculty of Health Sciences

UiT- The Arctic University of Norway

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Abbreviations

ABP	Activity-based probe
ABPP	Activity-based protein profiling
FP	Fluorophosphonate
TAMRA	Tetramethylrhodamine
Cy3	Cyanine 3
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
SHs	Serine hydrolases
GHs	Glycoside hydrolases
TMT	Tandem mass-tag
SILAC	Stable isotope labeling by amino acids in cell culture
LC	Liquid chromatography
MS	Mass spectrometry
CRISPRi	Clustered regularly interspersed short palindromic repeats interference
EPS	Extracellular polymeric substance
ECM	Extracellular matrix
eDNA	Extracellular DNA
CPS	Capsular polysaccharide
LPS	Lipopolysaccharides
TLR	Toll-like receptor
IL	Interleukin
AMR	Antimicrobial resistance
AMP	Antimicrobial peptide
5 FU	5-Fluorouracil
SDS	Sodium dodecyl sulfate

List of papers

Paper I

Md Jalal Uddin, Hermen S. Overkleeft and Christian S. Lentz. **Activity-Based Protein Profiling in Methicillin-Resistant *Staphylococcus aureus* Reveals the Broad Reactivity of a Carmofur-Derived Probe.** *Chembiochem.* 2023 Nov 2;24(21):e202300473. doi: 10.1002/cbic.202300473. Epub 2023 Aug 29.

Paper II

Md Jalal Uddin, Hermen S. Overkleeft, Mona Johannessen and Christian S. Lentz. **Activity-Based Protein Profiling Identifies an Alpha-amylase Family Protein Contributing to the Virulence of Methicillin-Resistant *Staphylococcus aureus*.**

(Manuscript)

Paper III

Md Jalal Uddin, George Randall, Jiyun Zhu, Tulsi Upadhyay, Laura van Eijk, Paul B. Stege, Frerich M. Masson, Marco C. Viveen, Matthew Bogyo, Matthias Fellner, Marcel R. de Zoete, Mona Johannessen and Christian S. Lentz. **Activity-Based Protein Profiling Identifies *Klebsiella pneumoniae* Serine Hydrolases with Potential Roles in Host-Pathogen Interactions.**

(Manuscript)

Summary

S. aureus and *K. pneumoniae* often reside in the human body as harmless commensal bacteria but can cause various infections once they breach the host's immune defenses. These pathogens are increasingly difficult to treat due to the emergence of antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA) and carbapenem-resistant *K. pneumoniae*. Activity-based protein profiling (ABPP) is a powerful chemoproteomic method to uncover potential new druggable targets and to identify off-target effects of drugs. It uses functionalized covalent enzyme inhibitors called activity-based probes (ABPs) for directly capturing enzyme activity changes within their native biological systems. To search for new putative targets of antimicrobial drugs, this study aimed to identify active enzymes in these pathogens using ABPP and to validate them in the context of bacterial physiology and host-pathogen interactions.

In Paper I, multiple classes of enzymes were identified in MRSA using an ABP derived from carmofur, which is registered as an anti-neoplastic agent and also possesses antimicrobial and anti-biofilm properties. The broad reactivity and somewhat promiscuous nature of the carmofur probe suggest potential side effects and therapeutic implications in clinical settings due to the inactivation of multiple enzymes in both humans and microbes.

In Paper II, two previously uncharacterized glycosidases were identified in MRSA. Functional studies using CRISPRi-induced gene silencing showed that a protein from the α -amylase family, trehalase C (TreC), plays a role in bacterial virulence during infections of wax moth larvae *Galleria mellonella*.

In Paper III, seven previously uncharacterized serine hydrolases were identified in *K. pneumoniae*, most of which show little or no homology with the proteomes of gut commensal bacteria and humans. Functional validation using transposon mutants deficient in either of the putative lysophospholipase PldB, esterase YjfP, and patatin-like phospholipase YchK revealed pronounced growth defects in human-derived colonic organoid co-culture models and reduced virulence in *G. mellonella* infection models.

Overall, this study provides substantial insights into the molecular mechanisms underlying the virulence and cellular physiology of *S. aureus* and *K. pneumoniae*, offering promising targets such as TreC, PldB, YjfP, and YchK for developing new antimicrobial strategies. Inhibiting these enzymes could synergize with existing antibiotics and augment human immune defenses

1. Introduction

The rapid spread and emergence of antibiotic resistance among pathogenic bacteria to conventional chemotherapy is a significant global health concern due to the scarcity of novel antibacterial drug developments. Understanding the molecular mechanisms behind bacterial pathogenesis and virulence is crucial for both academic and clinical purposes, especially in light of the increasing resistance to existing antibiotics and antibacterial agents. Activity-based protein profiling (ABPP), a chemoproteomic technique, has recently become an attractive approach for dissecting the basis of these dynamic processes. It uses small molecules functionalized covalent enzyme inhibitors called activity-based probes to identify and characterize (unknown) protein functions, study key regulators and visualization of enzymatic activity in various disease states and discover and evaluate potential new enzyme inhibitors.

This thesis focuses on screening and identifying novel functional enzymes using ABPP within the bacterial pathogens *Staphylococcus aureus* and *Klebsiella pneumoniae*. Additionally, validates the role of some previously uncharacterized enzymes within bacterial physiology and the dynamics of host-pathogen interactions. This study will increase the understanding of the importance of using small molecule probes to search for new antibacterial targets that might work in combination with existing antibiotics and human immune defenses to combat bacterial infections.

1.1 Bacterial pathogens

Infectious diseases represent a considerable threat to the humankind, contributing to the global burden of diseases and ranking among the top three causes of death worldwide, alongside cancer and cardiovascular diseases¹⁻⁴. The rapid spread of these diseases is exacerbated by globalization, overpopulation, and the interconnectedness of communities, posing serious public health concerns, especially in low-resource settings with inadequate infrastructure and poor healthcare access^{4,5}.

Bacterial pathogens, mainly those resistant to multiple drugs, play a significant role in this threat, accounting for about 54% of all emerging infectious diseases⁶. Among these, the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are especially notorious for their drug resistance and the challenges they pose to healthcare systems⁷. *Staphylococcus aureus* and *Klebsiella pneumoniae*, two of the most formidable among these,

exemplify the difficulties in combating bacterial infections due to their ability to develop resistance to multiple antibiotics, leading to severe and often fatal infections^{8,9}.

The misuse of antibiotics, together with inadequate public health infrastructure and disease surveillance, especially in resource-limited countries, hampers the effective response and management of infectious diseases^{10,11}. This situation is further complicated by global trade and intensive livestock systems that facilitate disease transmission between species, and the movement of people and goods across borders, making disease containment increasingly challenging¹² (reviewed in ¹³). Therefore, there is an urgent need for improved diagnostics, treatments, and management strategies to combat infectious diseases.

1.1.1 *Staphylococcus aureus*

Staphylococcus aureus was first reported by Scottish physician Sir Alexander Ogston in 1880 from surgical abscess fluid¹⁴. He observed unique micrococci clustering together like grapes; thereby, he coined the organism *Staphylococcus*, a term originated from the Greek words ‘Staphyle’ and ‘kokkos,’ denoting ‘bunch of grapes’ and ‘berries,’ respectively^{15,16}. A few years later, in 1884, Rosenbach successfully identified and cultivated staphylococci from individuals and was given the name *Staphylococcus aureus* due to its unique yellowish pigmentation of colonies derived from the production of staphyloxanthin^{17,18}. *S. aureus* is a versatile Gram-positive bacterium with the unique ability to live as harmless commensals but has the potential to cause infections in both humans and animals. These bacteria are typically 0.5-1.0 μm in diameter, aerobic and/or facultatively anaerobic, catalase positive, oxidase negative, coagulase-positive, and non-spore-forming, with some strains even capable of forming capsules¹⁹⁻²¹. The cell wall of *S. aureus* is primarily composed of peptidoglycan (PG) and wall teichoic acid (WTA), which create a rigid, insoluble, heterogeneous matrix^{22,23}. This bacterium can grow in high-salt conditions with up to 10% NaCl concentration²⁴. Additionally, it can produce a range of toxins, including α , β , γ , δ , exfoliative, and enterotoxins, leading to hemolytic and cytolytic activities^{25,26}. Nearly 140 years since its discovery, *S. aureus* remains a major cause of human disease and is extensively studied staphylococcal species alongside *Staphylococcus epidermidis*²⁷.

1.1.3 Colonization and/or infection of *S. aureus*

S. aureus is a versatile and opportunistic bacterium that resides in different parts of the human body as a harmless commensal, yet it also has the potential to become a formidable pathogen,

capable of causing a wide range of infections²⁷. Humans are frequently colonized by *S. aureus* either for short or long periods at various stages throughout life²⁸. The primary site of *S. aureus* colonization in humans is the anterior nares (nostrils). Around 20-30% of the healthy adult population asymptotically carries *S. aureus* in their nostrils as a part of their normal flora^{29,30}. However, it can also temporarily colonize other parts of the body, including the skin, throat, perineum, vagina, and gastrointestinal tract³¹⁻³³.

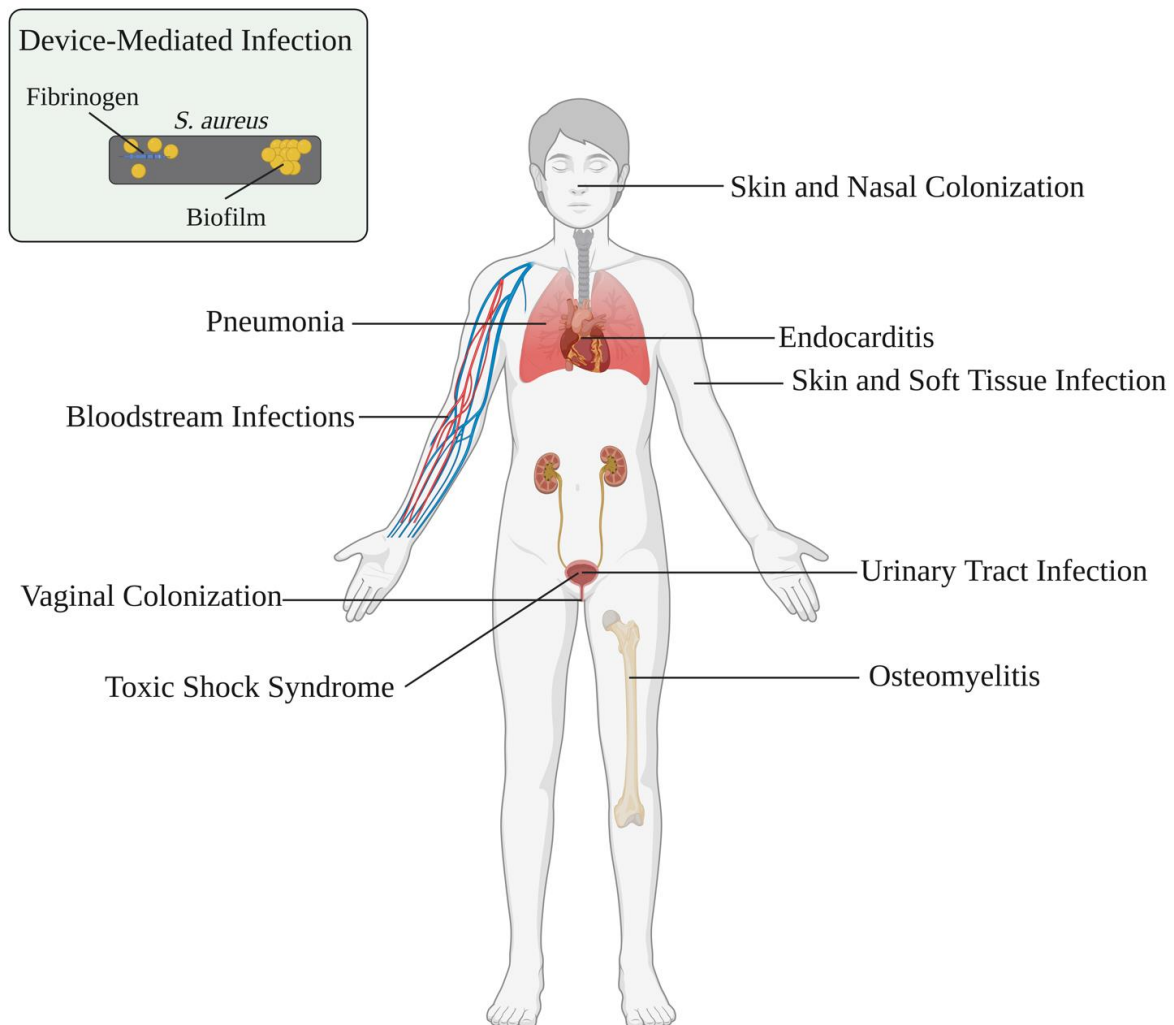


Figure 1. *S. aureus* colonizes asymptotically but can cause various infections. *S. aureus* has the capability to attach to and invade various human body tissues. The colonization of the skin and nasal passages increases the likelihood of developing pneumonia, sepsis, endocarditis, infections of the urinary tract, osteomyelitis, and infections of the skin and soft tissues. Infections related to medical devices can happen when these devices are covered with host substances like fibrinogen, creating a perfect environment for *S. aureus* to attach, colonize, and form biofilms. The figure was created with biorender.com. and adapted from ³⁴.

Colonization is recognized as a critical risk factor for the onset of *S. aureus* infections, notably in individuals who have compromised skin integrity or weakened immune systems³⁵. The likelihood of colonization and the progression to infection is influenced by many factors, including the various bacterial adhesion and immune-modulating proteins, host factors, and the presence of local microbiota. At the interface between host and microbe, there is a constant battle between the host's immune defenses and the pathogenic capabilities of the bacteria. Humans are defended against bacterial infection through innate and adaptive immune responses, and physical barriers like skin, epithelial layers, and mucous membranes. The skin barrier, protected by the secretion of antimicrobial peptides such as defensins and cathelicidins, usually prevents *S. aureus* from penetrating^{29,36}. However, any breach of these barriers significantly increases the risk of infection, which can vary from minor skin and soft-tissue infections (SSTI) to life-threatening conditions, including endocarditis, meningitis, osteomyelitis, hemolytic pneumonia, bacteremia, toxic shock syndrome, and device-related infections^{27,37}.

1.1.4 Virulence factors of *S. aureus*

The term “virulence” originates from the Latin “virulentus” which means “full of poison”. It describes a pathogen's capacity to cause disease or infection, effectively quantifying its pathogenicity^{38,39}. Bacteria possess various virulence factors that enhance their ability to cause disease^{40,41}. These factors are important for successful colonization and proliferation, leading to tissue damage and infection, and they play an essential role in evading host defense mechanisms (reviewed in ^{42,43})⁴⁴. These virulence factors are encoded by genes located both on the bacterial chromosome and extrachromosomal elements, often present on mobile genetic elements (MGEs) such as plasmids, pathogenic islands, or bacteriophages^{45,46}.

S. aureus can produce various virulence factors, which are crucial for its ability to adhere, invade, and cause disease within the host. These include over 24 cell wall-anchored (CWA) proteins, a plethora of toxins, and extracellular enzymes that facilitate host colonization and infection (**Table 1**) (reviewed in^{34,47-49}). Among the cell wall-anchored proteins, key components are the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), including fibronectin-binding proteins, clumping factors, and Protein A, which are vital for initial adhesion to host tissues. Additionally, *S. aureus* produces multiple toxins contributing to tissue damage and the immune system disruption. The secreted proteins encompass various extracellular enzymes, such as proteases, lipases, and hyaluronidases,

crucial for penetrating host tissues, evading the immune response, and promoting bacterial proliferation.

Table 1. Examples of *S. aureus* virulence factors involved in pathogenesis. The table is based on ^{34,47,48}.

Process involved with virulence	Protein/protein group	Associated molecule(s)/Bacterial determinant(s)	Abbreviation	Role in pathogenesis (examples)	References
Adhesion	CWA-MSCRAMM	Fibronectin binding protein A, B	FnBPA, FnBPB	Aids cell adhesion and host cell internalization	^{50,51} & reviewed in ⁴⁷
	CWA-MSCRAMM	Clumping factor A, B	ClfA, ClfB	Facilitates the nasal colonization and immune evasion	^{52,53} & reviewed in ⁴⁷
	CWA-MSCRAMM	Serine-aspartate repeat proteins C, D, E	SdrC, SdrD, SdrE	Binding to desquamated nasal epithelial cells and/or keratinocytes <i>in vitro</i>	^{54,55} & reviewed in ^{34,47}
	CWA-MSCRAMM	Bone sialoprotein-binding protein	Bbp	Adhesion to ECM	⁵⁶ & reviewed in ⁴⁷
	CWA-MSCRAMM	Collagen	Cna	Adhesion to collagen-rich tissue	^{57,58}
	CWA-NEAT motif Family	Iron-regulated surface determinant A, B, H	IsdA, IsdB, IsdH	Iron acquisition and immune evasion	⁵⁹⁻⁶²
	CWA- G5-E repeat Family	<i>S. aureus</i> surface protein G	SasG	Binding to desquamated nasal epithelial cells <i>in vitro</i>	^{63,64}
	CWA structurally uncharacterized	<i>S. aureus</i> surface protein X	SasX	Biofilm formation, cell aggregation, and squamous cell adhesion	⁶⁵ & reviewed in ⁴⁷
	Tandemly repeated three-helical bundles family	Protein A	SpA	Inhibition of opsonophagocytosis and inflammation	^{66,67} & reviewed in ^{34,47}
	SERAM	Extracellular adhesive protein	Eap	Impaired function in neutrophil and block complement activation	^{68,69}
	SERAM	Coagulases	Coa, vWbp	Ability to evade the host's immune response and protect the bacterium during abscess development and sepsis	⁷⁰⁻⁷²
	Cell-wall component	Wall teichoic acid	WTA	Facilitates the nasal colonization	Reviewed in ⁷³ & ⁷⁴
	Noncovalent attached cell proteins / major murein hydrolase	Autolysin	Atl	Aids host cell internalization and biofilm formation	^{75,76}
	Cytotoxin	α -hemolysin/ α -Toxin	Hla	Enhanced host survival, inflammation, and tissue injuries	⁷⁷⁻⁷⁹
	Exotoxins	Enterotoxins	SEs	Causes food poisoning and tissue injuries	^{80,81} & reviewed in ⁸²
	Exotoxin	Toxic shock syndrome toxin-1	TSST-1	Inhibit host immune responses	⁸³⁻⁸⁵
	Secreted enzyme	Hyaluronidase	HysA	Enhanced intracellular survival and inhibition of proinflammatory cytokine expression	⁸⁶⁻⁸⁸

Ability to produce toxins and enzymes	Exoenzyme	Staphylokinase	Sak	Activates host plasminogen and promotes bacterial dissemination	^{89,90} & reviewed in ⁴⁸
	Serine protease	V8 protease or GluV8	SspA	Contributes to <i>S. aureus</i> immune evasion and dissemination by breaking down self and host proteins	^{91,92} & reviewed in ⁴⁸
	Metalloprotease	Aureolysin	Aur	Contributes to <i>S. aureus</i> immune evasion and prevent complement activation	⁹³⁻⁹⁵
	Cysteine proteases	Staphopain A, B	ScpA, SspB	Prevents <i>S. aureus</i> from phagocytosis and thrives intracellularly in macrophages and dendritic cells	⁹⁶⁻⁹⁸
	Serine proteases	Serine protease-like proteins A to F	SplA to SplF	Role in shaping the <i>S. aureus</i> proteome	^{99,100}
	Phospholipases	β -toxin and phospholipase C	PI-PLC	Promote survival of <i>S. aureus</i> in human blood and neutrophils	¹⁰¹⁻¹⁰³
	Glycerol ester hydrolases	lipases 1 and 2	SAL1 and SAL2	Promote bacterial survival in biofilms and abscesses	^{104,105}
	Acetyl esterase/lipase	Fluorophosphonate-binding hydrolases B	FphB	Reducing the colonization efficiency of <i>S. aureus</i> in a mouse model	¹⁰⁶

CWA: Cell wall anchored protein, ECM: Extracellular Matrix, MSCRAMM: microbial surface component recognizing adhesive matrix molecule, NEAT: near iron transporter family, SERAM: secreted expanded repertoire adhesive molecules.

The expression of these virulence factors is highly adaptive and depends on the pathogen's requirements at various stages of infection; for example, adhesion proteins are predominantly expressed during the initial colonization stages, whereas the production of toxins becomes more pronounced during later stages of infection. Moreover, these factors support the bacteria's long-term persistence within the host by enabling it to evade detection or killing by the immune systems. The regulation of these virulence factors is precise and coordinated, changing with growth factors and conditions¹⁰⁷. Altogether, the extensive array of virulence factors produced by *S. aureus* underscores its versatility as a pathogen and its capacity to cause diverse human infections.

1.1.5 *S. aureus* biofilm

A biofilm is often described as a complex, heterogeneous microbial community where cells adhere to a surface or to other cells and are surrounded by a protective extracellular polymeric substance (EPS)¹⁰⁸⁻¹¹⁰. This substance, also known as the extracellular matrix (ECM), varies in composition based on environmental factors and bacterial strain. It generally comprises elements like host factors, polysaccharides, proteins, and extracellular DNA (eDNA)¹¹¹⁻¹¹³. The recent perspective on biofilm formation highlights its dynamic nature, noting that biofilms do

not always conform to the classic mushroom-shaped structure and exhibit diverse formation scenarios. Biofilms can appear as either single or multiple discrete aggregates, or as more continuous films. These formations consist of microbial aggregates that are either attached to or associated with a surface (outstanding reviewed in ¹¹⁴). Biofilms can form on various surfaces, including medical devices, industrial or potable water systems, and human tissues^{115,116}.

Biofilms act as formidable barriers against immune system attacks and antibiotic treatments during infections. The biofilm matrix can prevent the penetration of immune cells, leading to inefficient phagocytosis and increasing the bacteria's ability to resist antibiotics^{117,118}. Additionally, cells within a biofilm can survive exposure to antibiotics without developing inheritable genetic resistance, termed as antibiotic tolerance, which reverts to normal sensitivity when they return to the planktonic state¹¹⁹. This tolerance may result from limited antibiotic diffusion within the biofilm or the presence of metabolically dormant persister cells that are inherently resistant to antibiotics^{119,120}. The ability of *S. aureus* to develop biofilms is closely linked to its involvement in various chronic infections, such as osteomyelitis and endocarditis. Moreover, *S. aureus* has become particularly notorious for its ability to establish biofilms on indwelling medical devices, significantly complicating clinical outcomes by increasing morbidity and mortality rates associated with biofilm-related infections (reviewed in¹²¹). The challenge in treating *S. aureus* biofilm infections lies in their resilience; often, eradicating the infection requires not only the use of antibiotics but also the physical removal of the infected device or tissue¹²¹.

The development of a biofilm is traditionally characterized by three major events: initial attachment of planktonic cells to a surface; maturation of the biofilm as cells proliferates and produce an extracellular matrix; and eventual dispersal, where cells revert to a planktonic state, enabling the seed new sites for biofilm formation^{122,123}. Moormeier et al. have proposed the addition of two stages, multiplication, and exodus, to outline a five-stage process in *S. aureus* biofilm development: attachment, multiplication, exodus, maturation, and dispersal^{124,125}.

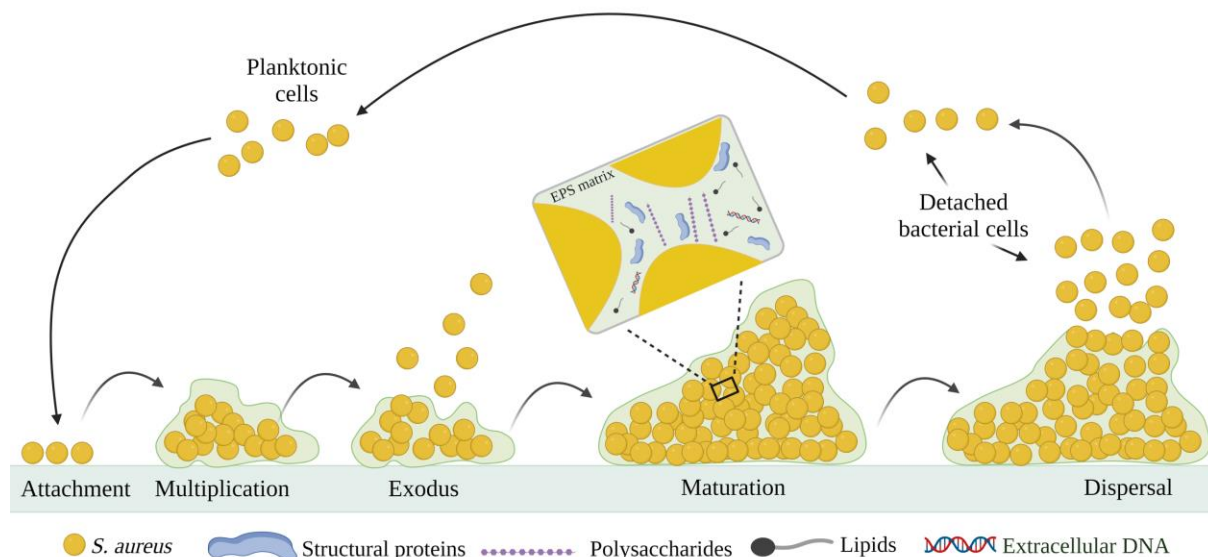


Figure 2. The development of *S. aureus* biofilms is characterized by five distinct stages. Initially, planktonic *S. aureus* adheres to either living (biotic) or non-living (abiotic) surfaces. Following attachment, the cells form a confluent ‘mat’, encapsulated within an extracellular matrix made of proteins and extracellular DNA (eDNA). A phase of exodus occurs next, during which a subpopulation of cells is detached from the main biofilm body. In the maturation stage, cells become interconnected through adhesive forces, leading to the substantial formation of the extracellular matrix and three-dimensional microcolonies. Finally, regulatory mechanisms trigger cell dispersal through the activation of proteases and/or phenol soluble modulins (PSM), facilitating the spread of cells to new locations and potentially initiating new biofilm formations. The figure was created with biorender.com. and modified based on¹²⁴.

Attachment involves the adhesion of *S. aureus* cells to both biotic and abiotic surfaces. On biotic surfaces (such as host tissues), this process is facilitated by cell-wall anchored proteins that recognize specific host matrix molecules. For abiotic surfaces, like medical devices, attachment is influenced by the surface properties of the bacteria and the physiochemical characteristics of the device¹²⁶. Studies have shown that the net charge of teichoic acids, along with specific surface proteins such as the major autolysin of *S. aureus*, known as Atl, which is essential for daughter cells separation during cell division, plays a role in the initial phase of biofilm formation^{75,127,128}. Among the key proteins involved in attachment are MSCRAMMs, which bind to various host matrix components, including fibronectin, fibrinogen, and collagen, playing a vital role in the binding of cells to host tissues (outstanding reviewed in¹²⁹). These proteins, alongside others such as fibronectin-binding proteins (FnBPA and FnBPB)¹³⁰, clumping factors (ClfA and ClfB)¹³¹, and serine-aspartate repeat family proteins (SdrC, SdrD, and SdrE)⁵⁴ initiate cell adherence and biofilm development.

Following the initial attachment, *S. aureus* cells enter a multiplication phase in the presence of sufficient nutrients, during which the adherent cells divide and aggregate, aided by factors that promote cell-to-cell interactions¹²⁴. Some proteins, including the MSCRAMMs, FnBPs, and ClfB, serve dual purposes in both attachment and multiplication phases^{124,132}, whereas other CWA proteins, such as Protein A¹³³, SasC¹³⁴, and Bap¹³⁵, are shown to play roles in biofilm multiplication. The exodus phase represents an early dispersal event characterized by the release of cell subpopulations and the restructuring of the biofilm¹²⁴. This is facilitated by the regulated, nuclease-dependent degradation of eDNA. This phase highlights the transition in biofilm integrity from protein-dependent biofilm structure to both proteins and eDNA^{124,125}.

An essential event in the maturation process of biofilms across bacterial species involves the development of microcolony structures. These structures enhance the surface area available for nutrient uptake and waste removal and facilitate the spread of biofilm cells to distant sites^{136,137}. Most of the ECM is produced during this phase, forming a three-dimensional structure around the biofilm cells^{125,138-140}. Studies have shown that the production of the exopolysaccharide poly-N-acetylglucosamine (PNAG), also known as polysaccharide intercellular adhesin (PIA), encoded by the *icaADBC* genes, plays an important role in biofilm maturation^{110,141}. The *ica* regulator (*icaR*) negatively regulates these genes expression, with most clinical *S. aureus* isolates containing the *ica* operon^{142,143}. However, *ica*-independent mechanisms also contribute to biofilm formation, involving components like teichoic acids and MSCRAMMs, as well as reliance on proteins such as Atl and fibronectin-binding proteins (FnBPs)¹⁴⁴.

Dispersal, the final phase of biofilm formation, involves the partial degradation of the ECM by nucleases and proteases, allowing cells to detach and potentially establish infections elsewhere or cause acute conditions such as sepsis^{122,145,146}. Several studies have shown that the dispersal of *S. aureus* biofilms is predominantly regulated by the *agr* quorum sensing system¹⁴⁷⁻¹⁴⁹.

1.1.6 *Klebsiella pneumoniae*

Carl Friedlander first reported *K. pneumoniae* in 1882 as a bacterium from the lungs of patients who had died from pneumonia¹⁵⁰. *K. pneumoniae* is a member of the Gram-negative *Enterobacteriaceae* family, along with other well-known pathogens, such as *Escherichia coli*, *Salmonella*, and *Shigella*¹⁵¹. It is a rod-shaped, facultative anaerobic, encapsulated, and non-motile bacteria, measuring between 0.3 to 2.0 μm in width and 0.6 to 6.0 μm in length, and often shows a slimy, mucoid appearance when grown on agar plates. Biochemically, it is

distinguished by its ability to ferment lactose, catalase-positive, and cytochrome oxidase-negative¹⁵² (reviewed in¹⁵³).

K. pneumoniae is capable of inhabiting various animal hosts as well as plants. It can also be found in different environments, such as soil, water, and drains. Moreover, *K. pneumoniae* can colonize different body parts including the respiratory tract, gut, nasopharynx, oropharynx, and skin^{154,155}. A key feature of *K. pneumoniae* is its encapsulated morphology. It produces a thick extracellular layer of polysaccharides known as a capsule¹⁵⁶. The capsule promotes immune evasion by hindering host clearance through phagocytosis, enhancing bacterial resistance to intracellular killing, and mimicking host glycans¹⁵⁷⁻¹⁵⁹. The capsule production is controlled by the capsule polysaccharide (cps) locus, which harbors several genes. *K. pneumoniae* is known to produce at least 79 types of capsules, which differ from one another by the structure and components of the repeating polysaccharide unit in the capsular polysaccharide^{156,160,161}. Some strains of *K. pneumoniae* are capable of producing hypercapsules through specific virulence genes, such as *c-rmpA*, *c-rmpA2*, *p-rmpA*, *p-rmpA2*, and *wzy-K1*. This phenotype is commonly found in K1 and K2 serotypes of the capsule and is associated with hypervirulence^{162,163}.

As a Gram-negative bacterium, *K. pneumoniae* has also lipopolysaccharides (LPS) in its outer membrane, also known as endotoxin. The LPS is made up of lipid A, a core oligosaccharide, and O-antigens, which act as a protective layer against complement-mediated killing. *K. pneumoniae* has at least 8 O-antigen serotypes, of which O1 antigen is the most prevalent among clinical strains¹⁶⁴. Additionally, two types of fimbriae are widely distributed in *K. pneumoniae*: Type 1 and 3 fimbriae, which are encoded by the *fim* and *mrkABCD* operons, respectively. These fimbriae play a role in bacterial adhesion, invasion of host cells, and biofilm formation¹⁶⁵.

1.1.7 Colonization and dissemination and/or infection of *K. pneumoniae*

The relationship between *K. pneumoniae* and hosts is complex and variable, where *K. pneumoniae* can exist as either a harmless commensal, an opportunistic pathogen, or a direct pathogen. *K. pneumoniae* is commonly found colonizing the gut as a commensal organism, though the frequency of *K. pneumoniae* carriage differs based on variables like age, geographic location, and recent interactions with healthcare settings (reviewed in¹⁶⁶).

Studies from the United States and Australia report a community prevalence of gut colonization by *K. pneumoniae* at approximately 4–6%^{167,168}. However, this prevalence rises to around 25% among individuals who have recently been in contact with healthcare services in these countries

and England¹⁶⁹. Notably, countries like Korea, Japan, Singapore, Taiwan, and Malaysia report much higher rates of healthy adult carriage, ranging from 18% to 87%^{170,171}. The duration of colonization in the gut is poorly understood but can last over a year^{172,173}. The significance of *K. pneumoniae* extends beyond mere colonization in the gut; it has been linked to chronic gastrointestinal diseases, including inflammatory bowel disease and colorectal cancer¹⁷⁴.

Globally, the majority of *K. pneumoniae* infections are opportunistic, linked to healthcare settings, and can lead to extraintestinal infections like pneumonia, urinary tract infections, sepsis, and wound infections, which may progress to bloodstream infections¹⁵⁵ (**Figure 3**). The most vulnerable groups include neonates, the elderly, immunocompromised, and hospitalized patients. Intestinal carriage of *K. pneumoniae* significantly increases the risk of healthcare-associated infections, especially among intensive care and oncology patients, with a fourfold increased risk of infection^{167,175}.

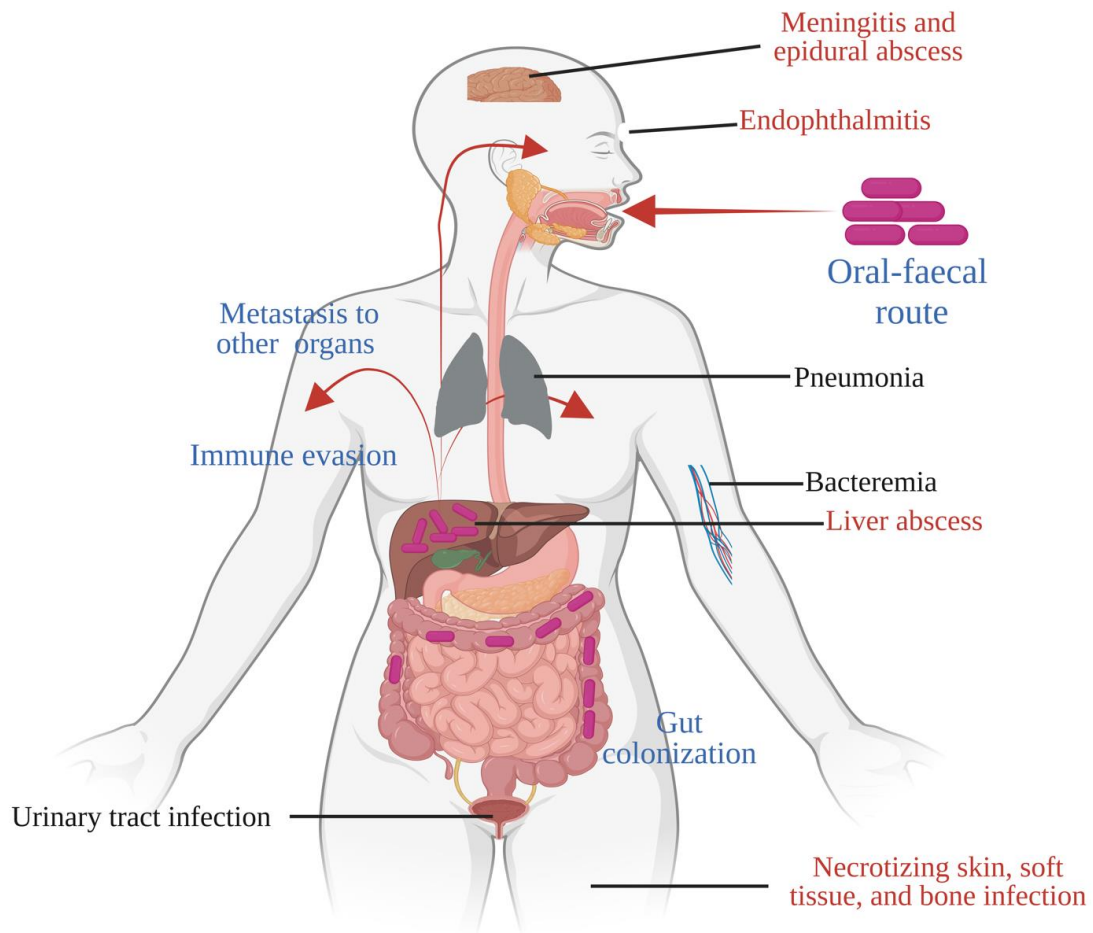


Figure 3. Common sites of *K. pneumoniae* colonization and the associated diseases. *K. pneumoniae* primarily enters the body through the oral- faecal route and can reside mainly in the gastrointestinal (GI) tract. The colonization of the gut increases the likelihood of dissemination to other parts of the body, evading the immune system and causing various extraintestinal diseases. Infections caused by hypervirulent *K. pneumoniae* (indicated in red) typically occur outside of hospital settings and can affect the central nervous system, liver, eyes, and soft tissue. Classical *K. pneumoniae* infections, which are known for rapidly developing resistance to multiple antibiotics, are more likely to arise within hospitals. Both variants of *K. pneumoniae* (highlighted in black) can lead to bloodstream infections, pneumonia, and urinary tract infections. The figure was created with biorender.com. and adapted from ^{166,176,177}.

The treatment of healthcare-associated *K. pneumoniae* infections faces significant challenges due to the emergence of multidrug-resistant (MDR) and carbapenem-resistant *K. pneumoniae* strains. A meta-analysis has shown the mortality rate of carbapenem-resistant strains-associated healthcare infections to be twice as high as that for infections caused by carbapenem-susceptible strains¹⁷⁸. This increasing resistance crisis has led to a renewed focus on limited treatment options, including the use of colistin and β -lactam/ β -lactamase inhibitor combinations, despite

increasing resistance, and an emphasis on developing vaccines and other preventive measures against *K. pneumoniae*^{179,180}.

Outside healthcare environments, *K. pneumoniae* can be a true pathogen causing severe community-acquired infections in healthy individuals without typical healthcare-associated risk factors¹⁸¹. These community-acquired infections include a range of conditions such as endophthalmitis, pneumonia, necrotizing fasciitis, non-hepatic abscesses, meningitis, and pyogenic liver abscesses, even without underlying biliary disease¹⁸². Such infections, often involving hypervirulent *K. pneumoniae* strains, are known for their potential to infect unusual or multiple sites, which can lead to bacteremia and metastatic spread¹⁸² (**Figure 3**). Specific host risk factors, such as alcoholism (for pneumonia)¹⁸³ and diabetes (for pyogenic liver abscess)¹⁸⁴, have been identified alongside numerous pathogen-related risk factors.

Despite hypervirulent *K. pneumoniae* strains rarely being multidrug-resistant, there are increasing reports of these strains carrying AMR plasmids, highlighting the convergence of AMR and virulence^{185,186}. This convergence poses a significant challenge, leading to invasive and difficult-to-treat infections, with at least one fatal outbreak reported in China, where carbapenemase-producing hypervirulent strains are becoming increasingly common¹⁸⁷⁻¹⁹⁰. The global healthcare community faces a dual challenge: managing the spread of *K. pneumoniae* in healthcare settings, where it acts as an opportunistic pathogen, and addressing the threat of hypervirulent strains that cause severe community-acquired infections. This situation underscores the need for continued vigilance, research into new treatments and preventive measures, and a comprehensive approach to managing both colonization and infection by *K. pneumoniae*.

1.1.8 Virulence of *K. pneumoniae*

Multiple virulence factors are described in *K. pneumoniae* that enhance the severity of infections and/or the propensity to cause disease, including capsular polysaccharides (CPSs), lipopolysaccharides (LPSs), fimbriae, pili, outer membrane proteins (OMPs), and siderophores¹⁹¹, with more recently identified factors like other iron uptake systems, efflux pumps, and a type VI secretion system (**Figure 4**).

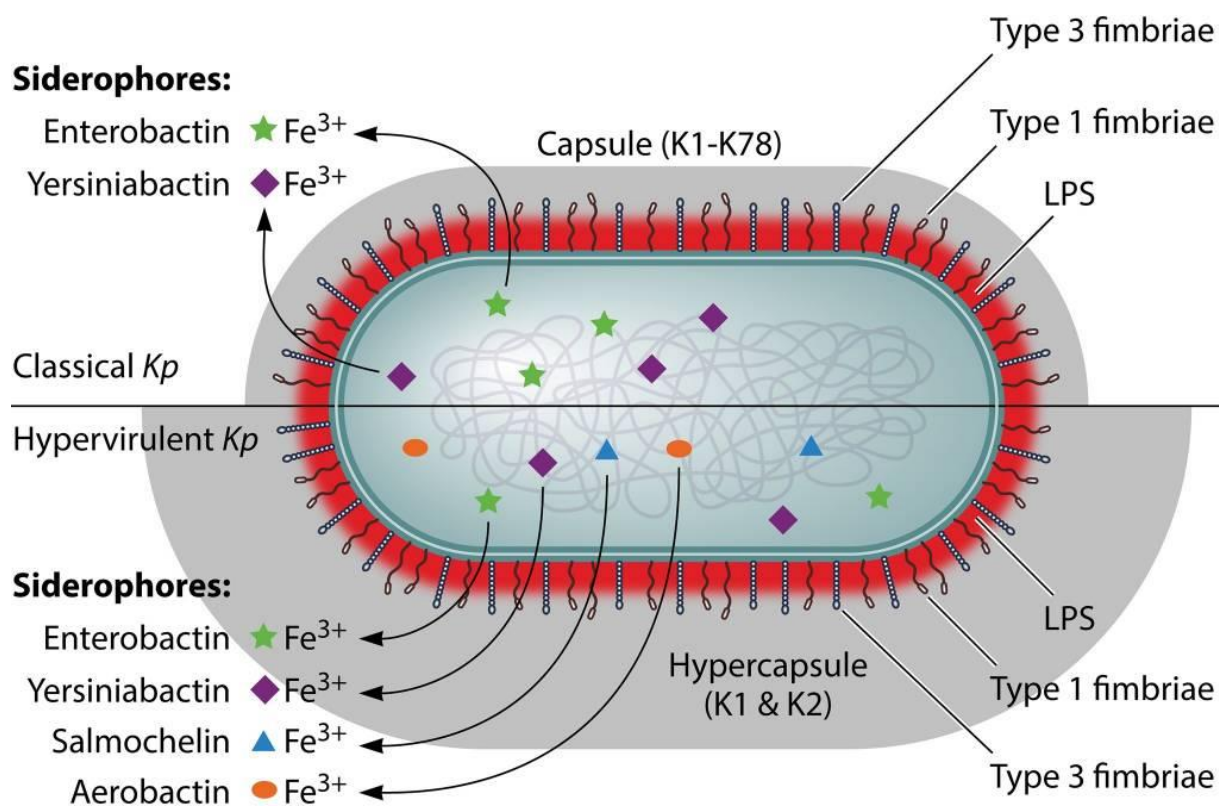


Figure 4. Key virulence factors in both classical and hypervirulent strains of *K. pneumoniae* play a crucial role in initiating infections and ensuring survival within the host. The figure was reprinted with permission from *Microbiology and molecular biology reviews (MMBR)*: Paczosa et al. (2016)¹⁹¹.

Among these, CPS is a key virulence factor for *K. pneumoniae* (**Figure 5**). It consists of an acid polysaccharide with three to six repeating sugar units. It is synthesized through a Wzy-dependent polymerization pathway under the regulation of the *cps* gene cluster^{192,193}. The process initiates with enzymes called glycosyltransferases assembling sugar units, followed by their transportation to the inner cell membrane, ultimately leading to their export to the cell surface¹⁹⁴. The thick capsule on the surface of *K. pneumoniae* hinders phagocytosis by blocking its interaction with immune cells. Notably, the K1-CPS variant, found in hypervirulent *K. pneumoniae* strains, significantly reduces macrophage interactions with bacteria compared to less virulent types¹⁹⁵. Additionally, CPS can diminish inflammatory responses by blocking IL-8 secretion from respiratory epithelial cells and interfering with Toll-like receptor signaling, thus preventing the immune system from responding effectively. CPS also protects the bacteria against antimicrobial peptides released by epithelial cells and inhibits the secretion of these antimicrobial substances by interfering with TLR-mediated responses. Moreover, it can prevent the maturation of dendritic cells and the activation of the complement system, crucial components of the host immune defense¹⁹⁶.

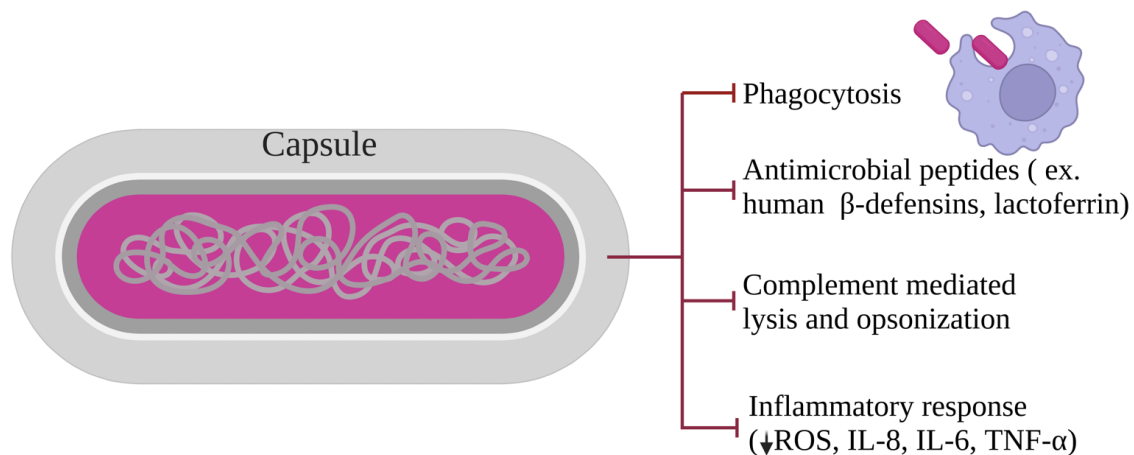


Figure 5. The capsule plays multiple roles in enhancing the virulence of *K. pneumoniae*. It serves several key functions. Firstly, it protects the bacteria from being engulfed and destroyed by immune cells by preventing phagocytosis and opsonophagocytosis. Secondly, the capsule acts as a barrier to the bactericidal effects of antimicrobial peptides, including human beta-defensins 1 to 3 and lactoferrin, by binding these molecules away from the bacterial outer membrane. Thirdly, it prevents complement components, such as C3, from attaching to the bacterial membrane, thereby inhibiting complement-mediated destruction and opsonization. Lastly, the capsule helps avoid the full activation of the immune system. This is evident from the reduced production of reactive oxygen species (ROS), IL-8, IL-6, and TNF- α , by facilitating the activation of a NOD-dependent pathway and preventing LPS from being recognized by immune cell receptors. The figure was created with biorender.com. and modified based on ¹⁹¹.

Another important virulence factor, LPS, especially its O-antigen, blocks the complement system from attacking the bacteria, thus protecting it from immune complement-mediated killing¹⁹⁷. It also influences the bacterium's resistance to antimicrobial peptides, including those used in treatments. On the other hand, lipid A and core polysaccharides of LPS confer resistance to host-derived antibacterial agents and prevent phagocytosis^{198,199}. Moreover, the lipid A part of LPS can trigger a strong immune reaction by activating TLR4, which in turn stimulates cytokine production and recruits immune cells.

Fimbriae (or pili), thin filamentous structures on the bacterial surface, promote attachment to host cells and biofilm formation, enabling infection spread within tissue¹⁹⁴. Type 1 fimbriae in *K. pneumoniae* are known for their roles in urinary tract infections²⁰⁰, whereas type 3 fimbriae mediate adhesion to epithelial cells in kidney and lung tissues²⁰¹. Conversely, type 3 and kpc fimbriae mainly contribute to biofilm formation^{202,203}. Additionally, the study showed that KPF-28 fimbriae facilitate the adherence of *K. pneumoniae* to human colon carcinoma cell lines, implying their participation in bacterial colonization of intestinal tissue²⁰⁴.

Siderophores are iron-chelating molecules secreted by bacteria for iron acquisition, essential for bacterial growth and virulence²⁰⁵. *K. pneumoniae* produces multiple siderophores, including enterobactin from its core genome and others like salmochelin, yersiniabactin, and aerobactin from its accessory genome^{191,206-208}. Each contributes differently to infection severity and immune evasion. Notably, yersiniabactin, is often associated with respiratory infections²⁰⁹, while aerobactin provides iron for *K. pneumoniae* replication and contributes to inducing inflammation and bacterial dissemination²¹⁰.

Outer membrane proteins (OMPs), like OmpA, can suppress inflammatory responses in airway epithelial cells, prevent phagocytosis, and resist host antimicrobial molecules^{199,211}. Additionally, other essential parts of OMPs, like porins, play an important role in the uptake of nutrients and expelling harmful substances, including antibiotics, from the bacteria²¹². Furthermore, efflux pumps, particularly AcrAB, are recognized for their role in antibiotic resistance and virulence by pumping out antibiotics, antimicrobial peptides, and other harmful substances to the outside of the bacteria^{213,214}.

The type VI secretion system (T6SS), a syringe-like structure for delivering effector molecules and toxins into cells, is found in various Gram-negative bacteria, including *K. pneumoniae*²¹⁵. Recent studies have started to characterize the effectors of the Type VI secretion system (T6SS) in *K. pneumoniae*. The necessity of phospholipase D family protein (PLD1) for virulence has been demonstrated in the hypervirulent strain Kp52.145 in a pneumonia model²¹⁶. Likewise, in the carbapenemase-producing strain HS11286, the phospholipase Tle1KP has been shown to facilitate both inter- and intra-species killing, and its secretion is increased by antibiotics²¹⁷. The T6SS, found in both the core and accessory genomes, enhances competitiveness against other bacteria in colonization sites and increases survival in infection sites²¹⁸.

In summary, these virulence factors of *K. pneumoniae* act as a complex arsenal that enhances the bacterium's pathogenicity, enabling the bacterium to invade host tissues, evade the immune response, and establish infections.

1.1.9 Antibiotic resistance

The emergence of antibiotic resistance is a major problem in treating bacterial infections. Numerous public health institutions have described the rapid increase of antibiotic-resistant bacteria as a “crisis” or a “nightmare scenario”, warning of “catastrophic outcomes”²¹⁹. In 2013, the Centers for Disease Control and Prevention (CDC) declared that humankind has entered a period known as the “post-antibiotic era,”²²⁰ and by 2014, the World Health Organization

(WHO) warned that the situation regarding antibiotic resistance is reaching critical levels²²¹(reviewed in²²²).

Since Alexander Fleming discovered penicillin in 1928, antibiotics have dramatically changed the era of modern medicine, offering hope against bacterial infections²²³. However, our reliance on antibiotics has come at a significant cost, a concern Fleming himself highlighted in his 1945 Nobel Prize acceptance speech²²⁴. He warned about the risks of antibiotic misuse and the ease with which bacteria could develop resistance if not treated with adequate doses. Despite his warnings, these concerns were overlooked, leading to the current crisis of antibiotic resistance (Figure 6) (reviewed in²²⁵).

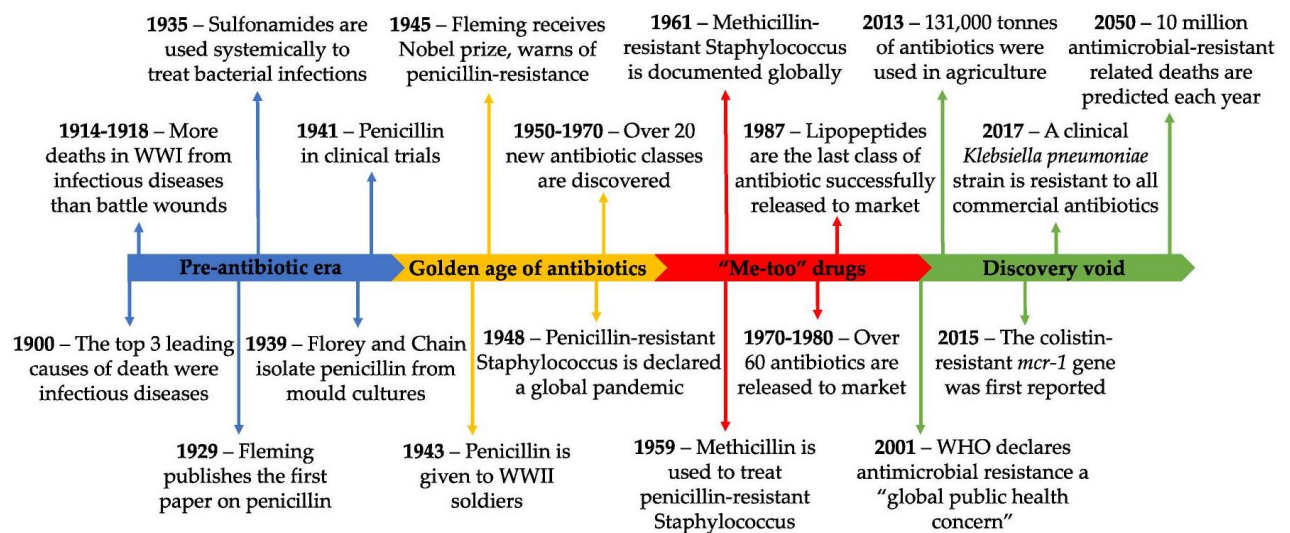


Figure 6. An overview of key milestones in the timeline of antibiotic resistance. WHO stands for the World Health Organization; WWI/II refers to World War I and World War II. *Browne et al. (2020)*²²⁵.

The primary concern lies in the increasing frequency of treatment failures. Infections that were once easily treatable with standard antibiotic therapy are now becoming difficult, if not impossible, to cure. This situation is further exacerbated by the slow pace of new antibiotic development. Pharmaceutical research and development have not kept pace with the rate of resistance development. The discovery and approval of new antibiotics are complex, time-consuming, and costly processes, resulting in fewer new drugs reaching the market²²⁶⁻²²⁸. The WHO now recognizes antimicrobial resistance as one of the top ten global public health challenges facing humanity²²⁹. It is predicted that by 2050, antibiotic-resistant infections could result in up to 10 million deaths annually and lead to a loss of productivity that could cost the global economy up to \$100 trillion^{230,231}.

Among the most concerning gram-positive pathogens is methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA strains emerged just two years after methicillin was introduced into clinical practice to fight *S. aureus* infections²³². MRSA now represents a significant threat to global health, leading to complications in both community and healthcare settings. The rise of MRSA has necessitated the use of second-line antibiotics, which are often associated with less favorable outcomes and an increased risk of severe, life-threatening infections²³³. MRSA claims more American lives annually than HIV/AIDS, Parkinson's disease, emphysema, and homicide combined^{234,235}. In 2019, MRSA was responsible for over 100,000 deaths worldwide²³⁶.

On the other hand, the Gram-negative carbapenemase-producing *K. pneumoniae* is becoming increasingly concerning due to its resistance to nearly all available antibiotic drugs, leading to scenarios reminiscent of the pre-antibiotic era²³⁷. The emergence of multidrug-resistant (MDR) and, more alarmingly, pandrug-resistant strains of *K. pneumoniae* has significantly affected medical practices in all fields²³⁸. As a result, in 2017, the WHO declared *K. pneumoniae* a critical pathogen that urgently requires new treatment options⁹. Furthermore, in 2019, the CDC identified carbapenem-resistant *K. pneumoniae* as an urgent threat to human health. In 2020, the European Antimicrobial Resistance Surveillance Network, along with the Central Asian and European Surveillance of Antimicrobial Resistance, reported that 38% of invasive *K. pneumoniae* were resistant to at least one category of antibiotic, with resistance to third generation cephalosporins being the most common²³⁹. In Europe, it is reported that infections caused by MDR *K. pneumoniae* exceed 90,000 cases annually, leading to more than 7,000 deaths, and accounting for 25% of the total disability-adjusted life years lost²⁴⁰.

Bacteria have a remarkable ability to rapidly develop resistance to new antibiotics through various mechanisms (reviewed in²⁴¹⁻²⁴³) (**Figure 7**). This includes spontaneous resistance that arises due to the mutations, as well as horizontal gene transfer. The key strategies bacteria use to resist antibiotics include the inactivation of drugs, modification of drug targets, the use of efflux pumps to remove drugs from the cell, decreased cell membrane permeability to prevent drug entry, and increased production of target molecules to counteract the drug's effect.

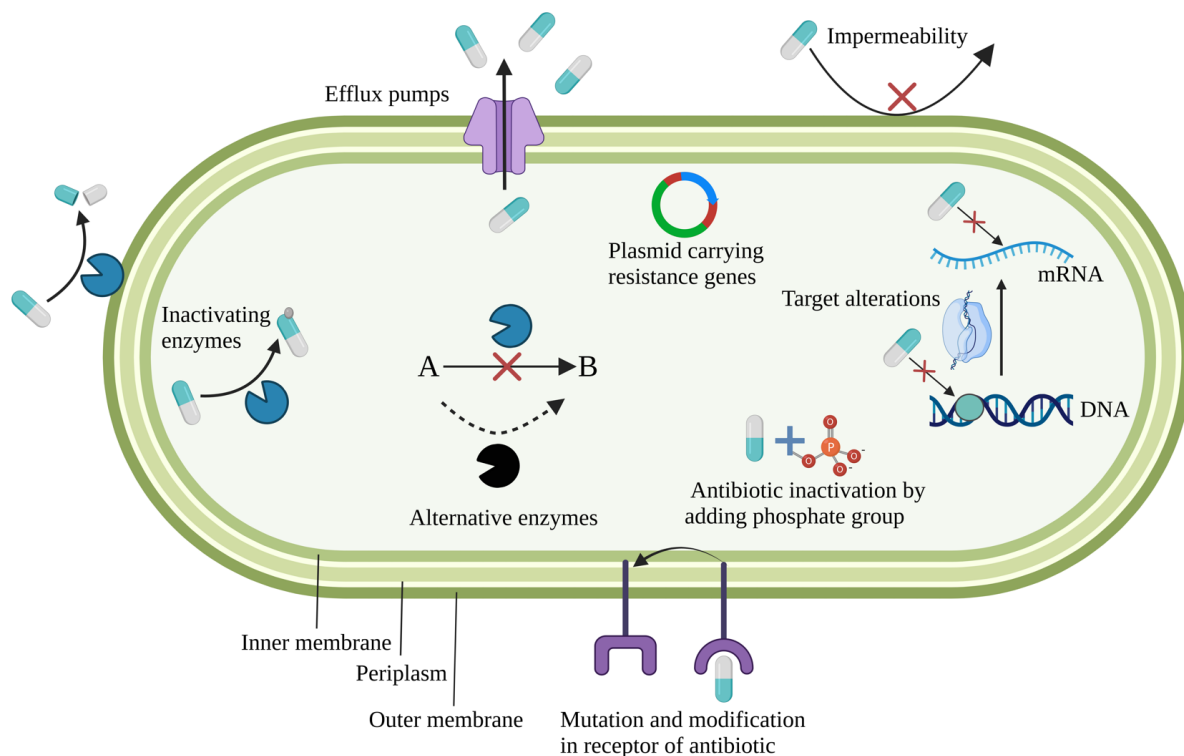


Figure 7. Strategies for different antibiotic resistance mechanisms are illustrated here, using gram-negative bacteria as an example. The figure was created with biorender.com. and adapted from ²⁴¹⁻²⁴³.

To address the growing antibiotic resistance crisis, novel therapeutic strategies are urgently needed. These should focus not only on the development of new drugs but also on ensuring no resistance development and helping immune systems to effectively combat pathogens.

1.1.10 Targeting bacterial virulence as a new strategy to combat antibiotic-resistant pathogens

Targeting bacterial virulence offers a novel approach to treating bacterial infection, particularly antibiotic-resistant pathogens, focusing on neutralizing the mechanisms bacteria use to infect and damage their hosts rather than killing them completely (reviewed in ²⁴⁴⁻²⁴⁸). This approach involves inhibiting virulence factors - molecules like toxins and enzymes that enable bacteria to invade host tissues and evade the immune system. By disarming bacteria, the strategy aims to reduce their infectivity, allowing the immune system to clear the infection more easily. Unlike conventional antibiotics that aim to eliminate bacteria, which often lead to resistance development, targeting bacterial virulence factors provides a less confrontational approach, potentially minimizing or reducing the likelihood of resistance development. In addition, this approach focuses on targeting not just bacterial viability but also functions essential for infection, such as those virulence factors necessary for causing host damage and disease. The

major advantages of this strategy are that it expands the range of bacterial targets, helps in preserving the host's natural microbiome, and exerts less selective pressure, which could result in decreased resistance. However, it poses challenges due to the complexity of bacterial virulence mechanisms and variability in human immune responses. Although it is currently in the early stages of research, this strategy holds promise as a complementary or alternative solution to traditional antibiotics in the fight against multidrug-resistant bacterial infections^{247,249}.

Since most virulence factors are secreted enzymes and proteins that remain functionally active during pathogenesis and play pivotal roles in a pathogen's ability to cause disease, robust tools for their precise identification and characterization are essential. Additionally, understanding the mechanisms of action of antivirulence agents and the ability to monitor the pathogen's response and resistance to these interventions is crucial.

2.1. Activity-Based Protein Profiling (ABPP)

Recent developments in 'omics'-based approaches have significantly advanced our understanding of the genetic, transcriptomic, and proteomic aspects of bacterial cells²⁵⁰. Despite this progress, these techniques are often inadequate in accurately identifying specific functional key regulators of a given cellular state at the protein level. This is particularly because of catalytic active enzymes are typically regulated by post-translational modifications and protein complex formation^{251,252}. This means that changes in their activity may not always correlate with their expression levels as determined by conventional genomic or proteomic methods. To overcome this challenge, a chemoproteomic approach called activity-based protein profiling (ABPP) has been developed. ABPP uses active-site directed small-molecule probes called activity-based probes (ABPs) for directly capturing enzyme activity changes within their native biological systems, thus offering a more precise insight into enzyme function and regulation than traditional 'omics' methods alone. ABPs rapidly and irreversibly bind with their target enzymes by covalently modifying the active site of catalytically active enzymes via a specific chemical reaction (reviewed in²⁵³⁻²⁵⁷).

ABPs typically consist of three components (**Figure 8A**): 1) a reactive group (also referred to as a 'warhead'), often an electrophilic group that covalently binds with a conserved active site nucleophile; 2) a linker region or binding group that can modulate the reactivity and specificity of the probe; 3) a reporter tag that enables the identification, enrichment, and/or visualization of labeled enzymes (reviewed in²⁵³⁻²⁵⁷). The reporter tags can be either a reporter group that is

directly connected to the ABPs in a one-step (direct) labeling process (**Figure 8B, C**) or can be attached in a later stage via bioorthogonal ligation chemistry in a two-step labeling approach (**Figure 8D**)²⁵⁸.

ABP probe scaffold can be used for dual purposes-visualization and the pull-down and enrichment of targets, depending on the specific functional handle integrated into it. As a result, the applications of ABPs are diverse, ranging from identifying enzyme activities and drug targets in chemical proteomics to the non-invasive *in vivo* imaging of enzymatic functions^{257,259}.

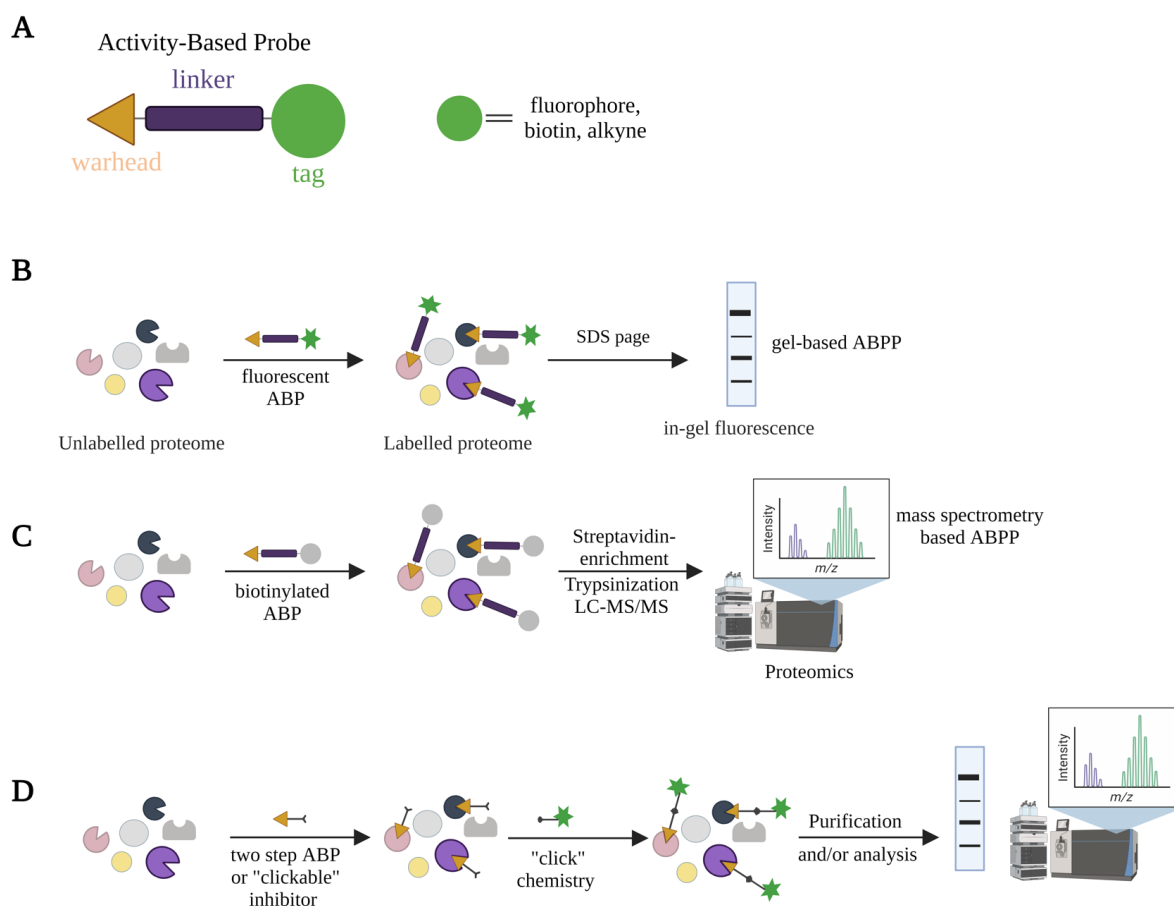


Figure 8. A general workflow for activity-based protein profiling (ABPP). **A)** Schematic structure of activity-based probes (ABPs) consists of an electrophile (often referred to as a “warhead”), a specific linker, and a reporter tag. ABPs selectively bind to active enzymes based on their activity levels. **(B, C)** Schematic representation of the direct ABPP method involves incubating a complex proteome with a probe containing a reporter tag. The enzymes that bind to the probe can then be visualized using a fluorescent tag through SDS-PAGE and in-gel fluorescence analysis (known as 'gel-based ABPP'). Alternatively, they can be enriched using a biotin tag for analysis via LC-MS/MS (termed 'mass spectrometry-based ABPP'). **D)** Schematic outline of the two-step ABPP method that requires bio-orthogonal chemistry (“click” chemistry) to introduce a reporter tag to a two-step process. Subsequently, labeled proteins can either be visualized or enriched. The figure was created with biorender.com.

ABPs can be used to label complex protein samples under different experimental conditions, including cell or tissue lysates (*in vitro*), within live cells (*in situ*), or even in living organisms (*in vivo*). The ABP-labeled proteins can be detected by using various analytical methods depending on the type of reporter tags used. For example, fluorescent reporter tags allow for quick analysis through a gel-based assay, where the labeled proteins are separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (**Figure 8B**). In-gel fluorescence scanning using fluorescent ABPs, while informative, comes with its limitations. It primarily detects the most abundant targets while their identity remains undefined. On the other hand, affinity tags such as biotin allow for the enrichment of target proteins using a streptavidin resin. Subsequent tryptic-digested peptides are analyzed by liquid chromatography-mass spectrometry (LC-MS) (**Figure 8C**). This mass spectrometry-based ABPP method, might be lower in throughput, but its higher sensitivity can identify low-abundance proteins and the depth of information makes it an extremely attractive technique^{260,261}.

2.1.1 Comparative and competitive ABPP

ABPP typically uses two common experimental setups: comparative ABPP and competitive ABPP (**Figure 9**). Comparative ABPP is highly useful for studying the activities of a particular (or enzyme family) across two or more proteomes, such as those from healthy versus diseased samples, or comparing pathogens versus commensal bacteria. This approach enables the identification of previously uncharacterized enzymatic activities involved in specific biological processes^{260,262} (**Figure 9A**). Insights into variations in enzyme activity enhance our understanding of biological pathways and can assist in identifying new therapeutic targets. A significant advantage of ABPP over gene expression analysis is its ability to measure the enzyme activity directly, including changes due to post-translational modifications.

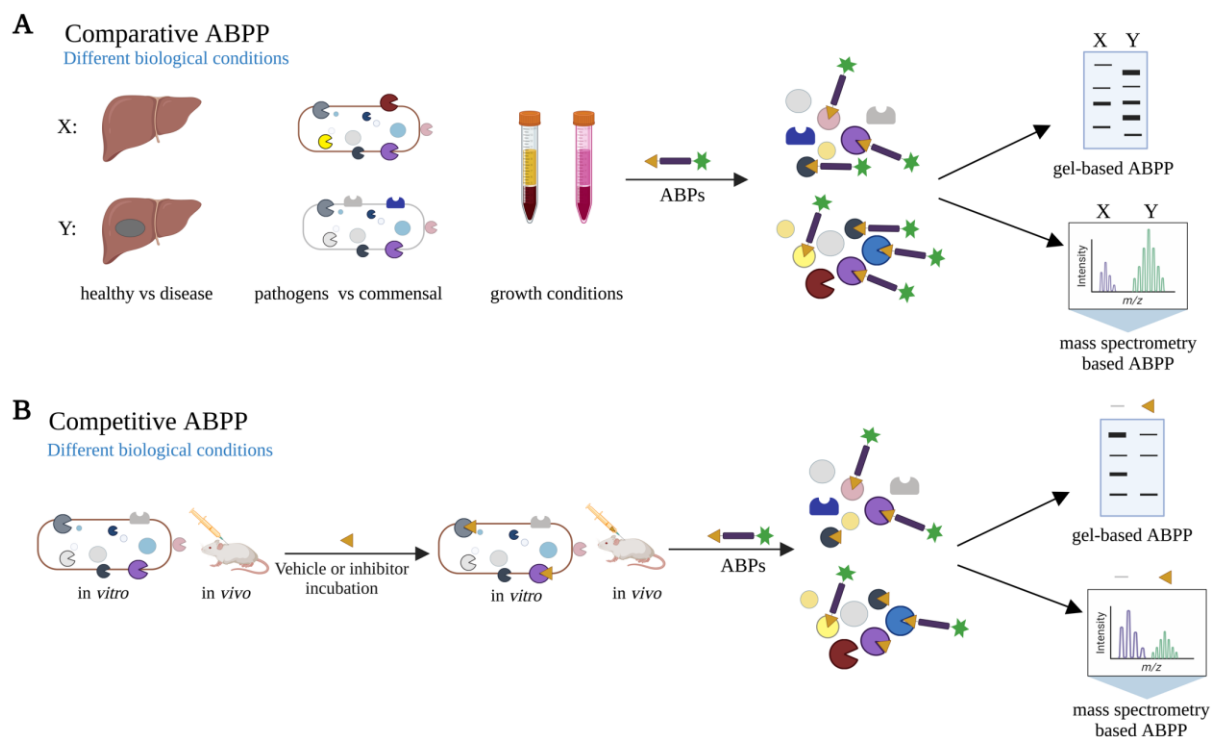


Figure 9. General workflow of comparative and competitive ABPP. **A)** Comparative ABPP involves analyzing proteomes from different biological samples, such as healthy versus diseased conditions, pathogens versus commensal bacteria, or varying growth conditions, by comparing them through gel- or mass spectrometry (MS)-based ABPP analysis of samples treated with activity-based probes (ABPs). **B)** Competitive ABPP is used for assessing target engagement and profiling off-target effects, followed by incubation with an ABP and subsequent analysis via gel or MS-based ABPP. The figure was created with biorender.com.

ABPP not only identifies relevant and druggable enzymes in a context of human disease, but it also enables the direct development of selective inhibitors for enzymes of interest. Competitive ABPP is a powerful technique that allows the testing of inhibitors against targets in their native environment without the need to express recombinant protein or even knowing the enzyme's native substrates²⁶³. Competitive ABPP also helps to guide lead identification, optimization, and pre-clinical trials in the drug development process. This technique involves pre-incubating a native proteome with a small molecule inhibitor, allowing for the identification of target enzymes by their reduced probe labeling post-incubation (**Figure 9B**)^{255,264}.

For example, in the profiling of ABPP in *S. aureus*, which is known for causing hospital-acquired infections and forming biofilms resistant to antibiotics²⁶⁵, Lentz et al. discovered 12 fluorophosphonate binding hydrolases (Fphs), most of which were previously uncharacterized²⁶⁶. By screening a library of serine-reactive electrophiles against these hydrolases, they found selective inhibitors, such as Chloroisocoumarin **12**. This compound effectively inhibited one of these enzymes, FphB, reducing the virulence of *S. aureus* in a

mouse model, indicating its potential role in the initial stages of tissue colonization²⁶⁶. In a subsequent study, competitive ABPP was applied to screen a library of 1,2,3-triazole ureas, which resulted in identifying additional specific inhibitors for the Fphs enzymes²⁶⁷. One such promising inhibitor was later developed into a fluorescent ABP **13**, enabling selective imaging of heterogeneity of FphE enzyme activity within a population of genetically identical bacteria and uncovering compensatory functions within the Fphs enzyme network²⁶⁷.

Another approach, inspired by natural products, involved the use of β -lactone-containing molecules by the Sieber lab and others to design a series of inhibitors^{268,269}. Among these, the compound **14** was notably effective in suppressing the growth of *M. tuberculosis* at low concentrations²⁷⁰. By using an analog of this compound with an alkyne handle, Lehmann and colleagues identified two target enzymes, antigen 85 and polyketide synthase 13, both involved in mycolic acid biosynthesis. This compound not only inhibited these targets in vitro but also showed synergistic effects with other antibiotics, highlighting the potential of mimicking critical bacterial metabolites for developing novel antibiotics²⁷⁰.

Recently, two research teams independently identified new chemical classes, 1,2,3-triazole urea and 7-urea chloroisocoumarin, which inhibit the growth of *M. tuberculosis* by interfering with cell wall biosynthesis^{271,272}. Using competitive ABPP, both teams identified certain serine hydrolases as potential targets. Li et al. used 1,2,3-triazole urea derivatives, employing active (AA692) and inactive (AA702) isomers from a series of hits to narrow their focus to five serine hydrolases²⁷¹. On the other hand, Babin et al. focused on 7-urea chloroisocoumarin chemical class, specifically JCP276, to identify nine hydrolase targets. Notably, inhibiting these hydrolases individually led to growth defects in *M. tuberculosis*, indicating that JCP276 may exhibit beneficial polypharmacological effects²⁷².

These instances underscore the versatility and effectiveness of ABPP in drug discovery, particularly in identifying and developing selective inhibitors for specific enzymes within complex biological systems.

In recent decades, a wide range of probes has been developed to target various enzyme families, including serine hydrolases^{273,274}, cysteine and threonine proteases^{275,276}, kinases²⁷⁷, cytochrome P450s²⁷⁸, and glycosidases²⁷⁹. The work in this thesis mainly focuses on activity-based protein profiling of the serine hydrolase and glycosidases.

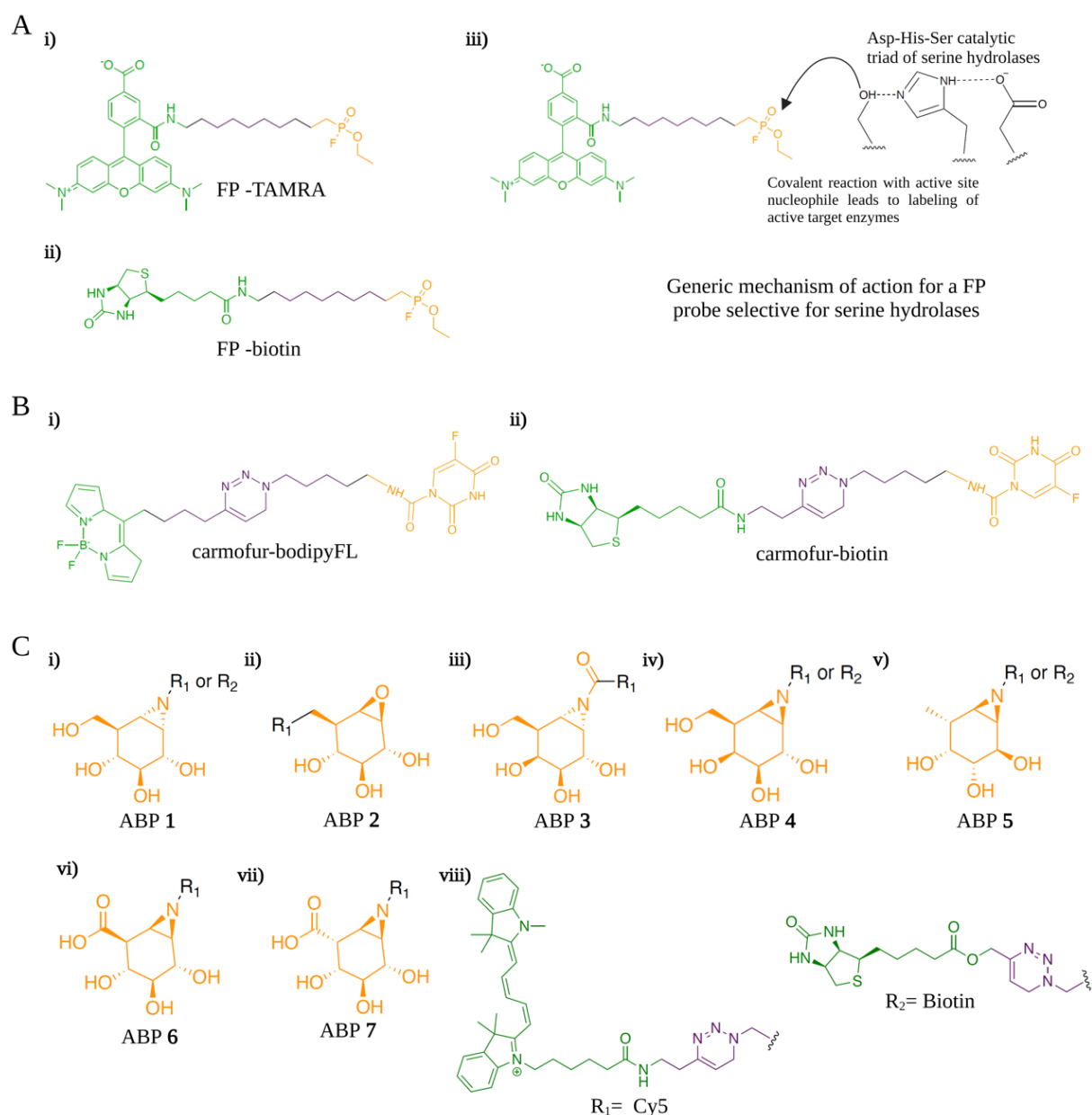


Figure 10. Activity-based probes (ABPs) used in this study. **A**) Structures of broad-spectrum serine hydrolase probes, including fluorophosphonate (FP)-TAMRA (i) and FP-biotin (ii), and the labeling mechanism of FP-TAMRA on the catalytic serine residue within the active sites of serine hydrolases (iii). **B**) Carmofer-derived probes, such as carmofer-bodipyFL (i) and carmofer-biotin (ii). **C**) Structures of glycosidases ABPs (**1-7**) equipped with Cy3 (R₁) or biotin (R₂) moieties.

2.1.2 Serine hydrolases and serine-reactive probes

Serine hydrolases (SHs) represent one of the largest and functionally diverse enzyme families found widely across all domains of life, including humans, plants, and microorganisms²⁸⁰. SHs are critical for various biological functions in mammals, playing central roles in numerous processes^{281,282}. They also play an essential role in pathogens like bacteria and viruses, where

they contribute to infection-related pathways, including virulence^{266,283} and antibiotic resistance^{284,285}. Because of their critical functions in both normal cellular processes and disease mechanisms, serine hydrolases are considered promising targets for therapeutic interventions against various diseases²⁸², including bacterial infections^{266,286}, cancer²⁸⁷, and neurological disorders²⁸⁸. Structurally, serine hydrolases are categorized into two main groups based on their functional roles. The first group consists of proteases, enzymes that specialize in breaking down peptide bonds in proteins. The second group comprises metabolic hydrolases, which include a diverse range of enzymes such as peptidases, amidases, lipases, esterases, and thioesterases. These enzymes are primarily involved in the processing of amide, ester, or thioester bonds found in small molecular metabolites, peptides, or in the post-translational modifications of proteins^{280,281}.

More than 60% of metabolic serine hydrolases (mSHs) are characterized by adopting an α , β -hydrolase structure and utilizing a catalytic trio of Ser-His-Asp^{289,290}. However, a variety of distinct, evolutionarily unrelated subclasses of mSHs exist that employ different structural folds and catalytic mechanisms. Among these are the amidase signature enzymes, which use a Ser-Ser-Lys trio^{291,292}, and the patatin domain-containing lipases, which use a Ser-Asp dyad²⁹³.

Lipases (lipid hydrolases) are the major catalytic members of the mSH family and the main driver of lipid catabolism^{294,295}. Lipid hydrolases are part of the broad family of carboxylic ester hydrolases (EC 3.1.1). This group includes several subclasses: carboxylesterases (EC 3.1.1.1), arylesterases (EC 3.1.1.2), triacylglycerol lipases (EC 3.1.1.3), phospholipases A2 (EC 3.1.1.4), lysophospholipases (EC 3.1.1.5), acetyl esterases (EC 3.1.1.6), acetylcholinesterases (EC 3.1.1.7), and cholinesterases (EC 3.1.1.8)²⁹⁶. Each subclass plays a distinct role in lipid metabolism, breaking down various lipid components into simpler molecules.

Bacterial phospholipases can play multiple roles in the infection process across various hosts. By hydrolyzing structural membrane lipids, some of these enzymes lead to the lysis of host cells. This action aids in bacterial colonization and/or dissemination and supplies essential nutrients for the pathogens survival and replication²⁹⁷⁻²⁹⁹. Phospholipases are also categorized based on their cleavage site into 1) carboxyl ester acyl hydrolases, 2) phospholipase Cs (PLCs), and 3) phospholipase Ds (PLDs)³⁰⁰. The group of carboxyl ester acyl hydrolases includes phospholipase As (PLAs), phospholipase Bs (PLBs), and lysophospholipase As (LPLAs). Based on where they cleave the ester bond, PLAs are classified into PLA₁ (EC 3.1.1.32), which hydrolyzes the fatty acid at the glycerols sn-1 position, and PLA₂ (EC 3.1.1.4), which

hydrolyzes the sn-2 position. PLBs (EC 3.1.1.5) can cleave acyl groups at both the sn-1 and sn-2 positions of glycerophospholipids and possess lysophospholipase activity. Phosphoric diester hydrolases, PLCs (EC 3.1.4.3) and PLDs (EC 3.1.4.4) cleave glycerol-oriented or alcohol-oriented phosphodiester bonds, respectively. PLCs produce a phosphorylated head group (such as inositol triphosphate [IP3]) and diacylglycerol (DAG), while PLDs release a head group (like choline) and phosphatidic acid (PA)³⁰⁰.

Since the common catalytic mechanism of serine hydrolases (SHs) involves the formation of an acyl-enzyme intermediate during substrate processing, it has enabled the design and development of ABPs that target the active serine as a reactive nucleophile (as reviewed in ³⁰¹). Over the last decades, a variety of covalent electrophiles have been described and utilized in probe scaffolds for serine hydrolases³⁰¹. These include fluorophosphonates (FPs)²⁷³, diphenyl phosphonates³⁰², sulfonyl fluorides³⁰³, β -lactams³⁰⁴, carbamates³⁰⁵, triazole ureas³⁰⁶, and isocoumarins³⁰⁷.

Among these, fluorophosphonate (FP) electrophiles have emerged as particularly attractive for designing ABPs targeting serine hydrolases due to their broad reactivity towards serine hydrolases (SHs), enabling the simultaneous study of numerous enzymes. For instance, examining SH activity in cancer cells through comparative analysis has provided valuable information on enzymes that are dysregulated in eukaryotic systems³⁰⁸. FP probes have also been pivotal in studying bacterial SHs that play vital roles in bacterial physiology and virulence. For example, Ortega et al. explored the activity of SHs in *Mycobacterium tuberculosis* (Mtb), a clinically significant pathogen causing tuberculosis (TB), under both active and dormant conditions³⁰⁹. The persistent nature of TB poses considerable treatment challenges, highlighting the need for innovative approaches. The identification of enzymes that stay active in the Mtb dormant state presents an opportunity to develop new diagnostic markers and anti-TB drugs. SHs are vital at every phase of Mtb's lifecycle, affecting its growth, metabolism, and survival in dormant states. Using FP-based probes to measure SH activity in both replicating and non-replicating forms of Mtb revealed marked activity differences. Notably, certain SHs, such as the essential protease ClpP, were found to be active during dormancy, suggesting they could be valuable targets for developing treatments against dormant Mtb³⁰⁹. In another study, the activity of Mtb esterases was investigated using FP ABPs together with substrate-based probes across active, dormant, and reactivating culture conditions³¹⁰. This research led to the first-time identification of three esterase enzymes that become functional during the early reactivation phase from dormancy, which could play a critical role in pathophysiological phenomena³¹⁰. To

elucidate host-pathogen interactions, FP-based probes (FP-TAMRA and FP-Biotin) were utilized in animal models infected with *Vibrio cholerae*³¹¹. This method enabled the detection of secreted bacterial and active host serine hydrolases (SHs). The study identified four *V. cholerae* proteases active in infected rabbits, and one was active in human cholera stool samples. Lentz et al. investigated serine hydrolase activities within *S. aureus* biofilms utilizing FP-TMR and FP-biotin probes²⁶⁶. Their research led to identifying 12 active serine hydrolase targets, including lipase 1 and 2 (SAL1, SAL2). Remarkably, 10 of these identified targets were previously uncharacterized, with functional studies revealing their potentially significant roles in bacterial virulence²⁶⁶ and stress response³¹².

2.1.3 Glycosidases and carbohydrate-based probes

Glycans, which are covalently attached carbohydrate chains, are essential components in all life forms, playing critical roles from energy storage to facilitating crucial cellular processes such as recognition, communication, and signaling^{313,314}. They are pivotal in structural support within the extracellular matrix, protection in secreted mucus, and in the correct sorting and transport of glycoproteins, thereby ensuring proper cellular function³¹⁵. Glycosylation, the process of attaching glycans to proteins, must be precisely regulated, as alterations in glycosylation patterns can lead to serious health issues, including autoimmune diseases and cancer³¹⁶⁻³¹⁹.

The importance of glycans extends beyond human physiology; they are also involved in microbial interactions with the host³²⁰. Pathogens frequently target glycans to attach to and invade host cells, with examples including the role of glycans in the adhesion of *Helicobacter pylori* to gastric cells³²¹ and influenza A virus to sialic acid-containing glycans³²².

Glycosidases, or glycoside hydrolases (GHs), are a major class of enzymes that cleave the glycosidic bonds in a variety of substrates, including di-, oligo-, and polysaccharides, as well as glycoconjugates such as glycoproteins, glycolipids, and proteoglycans³²³. These enzymes are divided into two main groups based on the site of sugar cleavage. Exoglycosidases are responsible for the hydrolytic cleavage of specifically terminal, non-reducing sugar residues in glycan substrates, while endoglycosidases target the internal glycosidic bonds within the sugar chains³²⁴.

Bacterial glycosidases, on the other hand, play a significant role in the mechanisms of bacterial pathogenesis. They are involved in immune modulation, adherence to host cells, colonization, and nutrient production³²⁵. Many pathogens often secrete glycosidase enzymes that break down

glycans to evade the immune response, aiding their survival and proliferation within the host^{326,327}. Moreover, glycans serve as vital nutrients for pathogens, especially those in the gastrointestinal tract, where bacteria express glycosidases to metabolize glycans from host glycoproteins like mucins^{328,329}. This nutrient acquisition is crucial for maintaining pathogens survival and continued colonization in various host niches^{330,331}.

Glycoside hydrolases (GHs) are categorized into two main types based on the stereochemical outcome of their enzymatic action: inverting or retaining³³². Inverting glycosidases typically carry out hydrolysis through a direct, single-step nucleophilic attack at the anomeric center of the substrate, resulting in the inversion of stereochemistry. On the other hand, retaining glycosidases work via a more complex two-step mechanism. This mechanism is facilitated by two essential catalytic residues: a nucleophile and a general acid/base, facilitate the formation of a covalent glycosyl-enzyme intermediate³³². The strategy of capturing this intermediate is fundamental to the design of most mechanism-based inhibitors and activity-based probes (ABPs) that specifically target retaining glycosidases^{279,333}.

Unlike retaining glycosidases, inverting glycosidases do not form covalent glycosyl-enzyme intermediate during the hydrolysis of their substrate. Instead, these enzymes directly utilize an activated water molecule to break the glycosidic bond in a single step³³⁴. As a result, designing ABPs that directly alter a catalytically active amino acid residue becomes challenging for inverting glycosidases. Conversely, two major classes of irreversible inhibitors that directly target the enzyme's active site nucleophile have been developed to retain glycosidases: cyclitol epoxides and fluorinated glycosides³³⁵.

These inhibitors have been used to analyze various retaining glycosidases, especially in identifying catalytically active carboxylic acid residues. Cyclitol epoxides, such as cyclophellitol, use an epoxide warhead that is activated upon protonation by the enzyme's active site, leading to a fast and specific reaction with the target glycosidase, minimizing off-target interactions. In contrast, fluorinated glycosides, particularly 2-deoxy-2-fluoroglycosides, show lower intrinsic reactivity, as the fluorine substituent destabilizes the transition state. They rely on the presence of a strong anomeric leaving group for activation, ensuring specificity by reacting only within the enzyme's active site. Therefore, both cyclitol and fluorinated glycoside structures are effectively adaptable for creating selective ABPs targeting various retaining β -glucosidases, such as α -glucosidases³³⁶, β -glucosidase³³⁷, α -galactosidase³³⁸, β -galactosidase³³⁹, α -fucosidases^{340,341}, β -glucuronidase³⁴², and α -iduronidases³⁴³, each with its unique mechanism of enzyme interaction.

Most glucosidase ABPs have been developed to study human diseases^{344,345}, including lysosomal storage disorders, Gaucher disease, plant enzymes³⁴⁶, and certain microbial metabolic pathways^{347,348}. However, relatively few glycosidase ABPs have been developed to date to investigate bacterial enzymes that could potentially serve as virulence factors. Due to their conserved mechanism of action, these glycosidase probes are also well-suited for detecting bacterial enzymes.

3. Objectives of the study

The underlying hypothesis of this work was that certain enzymes with their putative roles in bacterial physiology and virulence remain unidentified and/or uncharacterized. The aim of this thesis was to identify and functionally characterize such enzymes in the bacterial pathogens *S. aureus* and *K. pneumoniae*, which may represent potential therapeutic targets.

Specifically, the objectives of this work were:

1. Identify active enzymes in *S. aureus* targeted by the antimicrobial agent carmofur
2. Identify active glycosidases in *S. aureus*
3. Identify active serine hydrolases in *K. pneumoniae*
4. Validate the enzyme function(s) in bacterial physiology and host-pathogen interactions

4. Methodological considerations

In order to achieve the objectives, ABPP was utilized throughout this thesis to identify different classes of active enzymes using a variety of ABPs. The materials and methods used in this work have already been outlined in Papers I, II, and III. Instead of repeating this content, this section will focus on providing a clear and brief overview of the methods used in this work and the rationale behind their selection.

4.1. *S. aureus* and *K. pneumoniae* as model organisms

The choice of *S. aureus*, a Gram-positive bacterium, and *K. pneumoniae*, a Gram-negative bacterium, as model organisms for this study is due to their significant clinical relevance and distinctive characteristics that make them ideal for investigating the mechanisms of bacterial pathogenesis and the development of innovative antibacterial approaches^{27,166}. *S. aureus* is a versatile pathogen known for its capability to cause a wide spectrum of infections, from mild skin infections to life-threatening systemic diseases. Its adaptability and the emergence of MRSA strains underscore the critical need for novel therapeutic strategies. The well-characterized genome of *S. aureus* facilitates genetic modifications, such as gene knockouts, enabling in-depth investigation into gene/protein functions, and insights into virulence factors and resistance mechanisms³⁴⁹.

K. pneumoniae, with its protective outer membrane that poses additional obstacles to antibacterial drug penetration, represents the challenges associated with treating gram-negative bacterial infections. It is particularly known for its role in hospital-acquired infections and its rapid acquisition of resistance to multiple antibiotics, including carbapenems¹⁹¹. Both organisms are pivotal in the study of bacterial virulence and resistance mechanisms. Their extensive use in research is attributed to their relevance in global health, the availability of genetic tools for manipulation, and the transposon mutant libraries^{350,351} which facilitates the follow-up study. In addition, their role in testing the effectiveness of novel antimicrobial agents, makes them exemplary models for advancing the field of infectious disease and therapeutics.

4.2. Profiling of active enzymes by ABPP

Activity-based protein profiling (ABPP) advances beyond the scope of 'omics'-based techniques by focusing on the functional state of enzymes rather than their mere presence. By targeting enzymes in their active states with chemical probes, ABPP offers a precise measure

of enzymatic function, identifying key regulators and druggable targets. This method provides a higher level of validation for protein functionality, surpassing the limitations of transcriptomics and conventional proteomics (reviewed in^{251,253-257}). Serine hydrolases have previously been identified in *S. aureus* using ABPP. This work has been extended to explore other enzyme families in *S. aureus* in Papers I and II. Additionally, serine hydrolases have been explored in *K. pneumoniae* in Paper III using FP probes that were also successfully employed in other organisms^{272,311,352-354}. In general, after the bacterial strains were cultivated under specified conditions, they were exposed to fluorescent-tagged ABPs. Subsequently, the cells were lysed, and fractions were analyzed by SDS-PAGE, which allowed for the identification of labeled targets via fluorescence scanning (**Figure 11**).

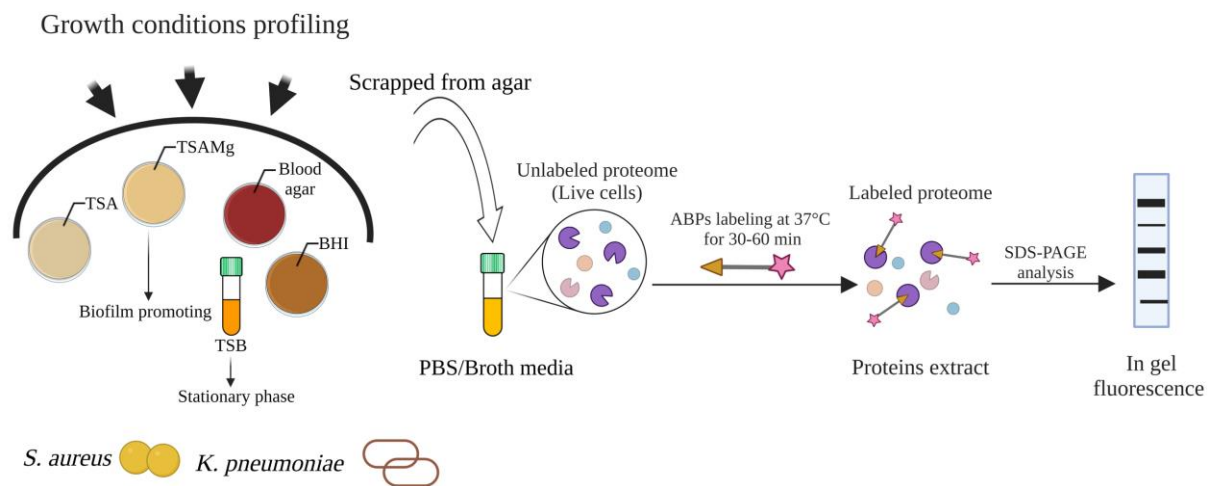


Figure 11. Profiling of active enzymes by ABPP. Bacteria are grown overnight either on agar plates such as Tryptic Soy Agar (TSA), TSA with magnesium chloride (TSAMg), Blood Agar, and Brain Heart Infusion (BHI) or in liquid culture. The bacteria are then scraped into PBS/broth to achieve the desired density before the addition of the ABP. After labeling, the cells are lysed, and labeled proteins are resolved by SDS-PAGE analysis and visualized by in-gel fluorescence scanning.

In Paper I, a carmofur-derived ABP, which acts as both an antineoplastic and antimicrobial agent, was utilized to detect active enzymes, including serine hydrolases, in *S. aureus*. The bacteria were cultivated on tryptic soy agar supplemented with $MgCl_2$ (TSAMg), a condition known to promote biofilm formation in *S. aureus*³⁵⁵. In Paper II, a panel of glycosidase ABPs was used to identify retaining glycosidases in MRSA strain. The bacteria were cultivated on various rich media, including TSA, TSAMg, BHI, and blood agar, and in stationary phase culture in TSB to check whether different growth conditions influence the activity of different retaining glycosidases. Since neither carmofur-derived ABP nor glycosidase ABPs had not

previously been used to detect active enzymes in bacterial pathogens, and their potency for detecting bacterial enzymes was unknown, dose-dependency labeling of these probes was also used to find the optimal labeling concentrations in Papers I and II. Since competitive ABPP is a suitable method for assessing the selectivity of probe labeling, it was employed in Papers I and II. In Paper I, unlabeled parent inhibitors of carboxypeptidase Y (CPY), such as carboxypeptidase Y and 5-fluorouracil, were used, while in Paper II, cyclophellitol-based inhibitors were used to profile the specific labeling of the probes. In Paper III, the global serine-reactive electrophilic probe FP-TMR was utilized to label serine hydrolases in *K. pneumoniae* after growth on blood agar, which served as the standard culture medium.

4.2.1 Chemoproteomics method optimization to detect active enzymes by biotinylated probes

The gel-based method employing fluorescent ABPs provides valuable information but is limited to identifying only the most abundant targets, without revealing their specific identities. Additionally, the method of fluorescence-guided gel band extraction is not precise enough to isolate labeled enzyme(s), especially in highly complex samples or those that contain enzyme(s) migrating together with different specificities³⁵⁶. To overcome these limitations, a strategy involving a biotin-tagged probe combined with a chemoproteomic workflow was adopted. This approach, focusing on affinity-based enrichment, has proven highly effective in selectively identifying ABP targets.

The general workflow for this process includes several key steps: (1) labeling the bacteria with a biotinylated activity-based probe, (2) lysing the bacteria, (3) removing excess probes, (4) enriching for labeled enzymes by capturing them on streptavidin resin, (5) performing on-bead digest of enriched proteins, (6) desalting and concentrating, (7) conducting liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, and (8) data analysis³⁵⁷⁻³⁶⁰. (Figure 12A).

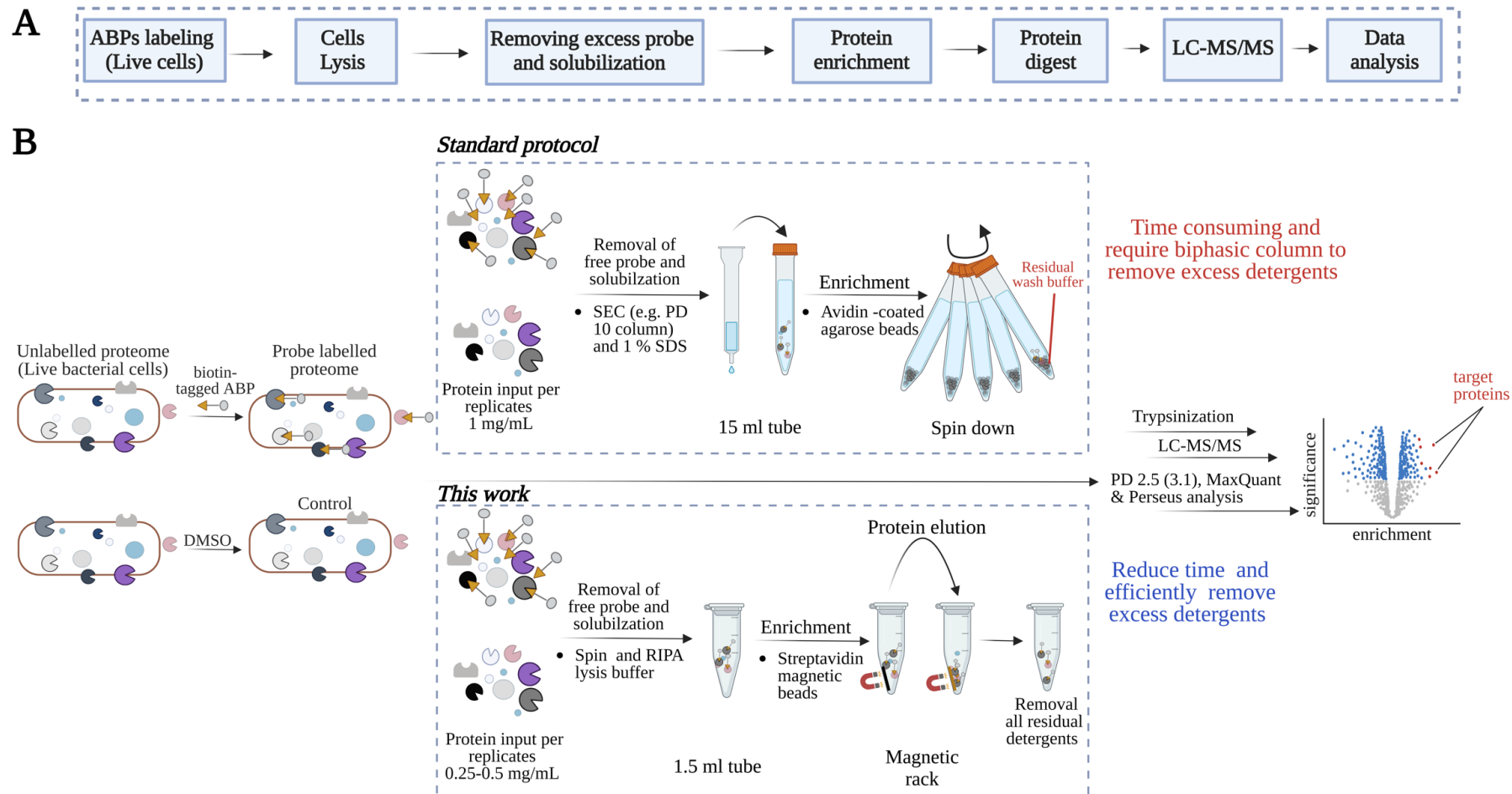


Figure 12. Schematic overview of the chemical proteomic workflow. (A) Essential steps in the standard chemical proteomics protocol and (B) a schematic outline of the workflow used in this thesis, including basic procedural parameters, compared to the traditional method involving avidin-coated agarose beads. PD 2.5 (3.1) stands for Proteome Discoverer 2.5 or 3.1 software. The figure was created with biorender.com.

Following probe labeling, proteins are subjected to size exclusion chromatography (SEC) to remove free probe and non-protein components from the cell lysate. The critical aspect of the full process is to increase the washing steps to remove nonspecifically bound proteins, thereby reducing the complexity of the final MS sample and improving the comparison between control and probe-treated samples^{359,361-363}. In standard chemoproteomic workflows, sodium dodecyl sulfate (SDS) and other detergents are commonly used to solubilize proteins and minimize non-specific binding. However, even at low concentrations, detergents can impede enzymatic digestion and dominate mass spectra due to their high ionization efficiency and high relative abundance compared to peptides^{364,365}. Therefore, the removal of detergent, like SDS is important for effective mass spectrometric analysis in proteomics³⁶⁵. Because the removal of SDS is traditionally thought to be impossible with in-solution digestion, various methods have been developed to eliminate detergents, including the use of protein precipitation in acetone or with trichloroacetic acid (TCA), the use of an S-trap, Oasis columns³⁶⁶⁻³⁷⁰. Alternatively, a biphasic column coupled with strong cation exchange can be integrated into the LC-MS/MS workflow for detergent removal before mass spectrometry analysis^{12,13}. However, these methods require extensive washing steps, including methanol washes, which can lead to significant sample loss³⁷⁰. Unfortunately, the Proteomics and Metabolomics Core Facility (PRiME) lacks the necessary mass spectrometer set up to incorporate a combination of a biphasic column coupled with strong cation exchange resin (SCX) for detergent removal^{367,368,371}.

To address these challenges and minimize sample loss, sample preparation has been optimized by adopting strategies from the single-pot solid-phase-enhanced sample preparation (SP3)³⁷²⁻³⁷⁵ and proximal labeling^{376,377} using magnetic bead-based sample preparation. A detailed comparison is illustrated in **figure 12B**. This optimized protocol uses lower concentrations of biotin probe compared to previous studies, reducing the amount of free probe that can be easily removed through simple centrifugation, thus eliminating the need for desalting columns and common rotary evaporation steps, particularly for live cell labeling. However, for the labeling of protein extracts, the use of a desalting column is required to remove excess probes. RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% (wt/vol) SDS, 0.5% (wt/vol) sodium deoxycholate, 1% (vol/vol) Triton X-100) is also used in sample preparation for its effectiveness in cell lysis and membrane protein solubilization. Importantly, the use of magnetic beads instead of avidin agarose beads offers advantages in sample preparation, such as protein

cleanup or enrichment. Utilizing a magnetic rack simplifies the removal of nonspecific proteins and excess detergents and enhances sample handling.

The MS raw data were analyzed using either Proteome Discoverer 2.5 or 3.1 software, or MaxQuant for protein label-free quantification (LFQ). LFQ was chosen for its simpler experimental setup, which avoids expensive and time-consuming labeling steps, does not require analysis of complex mass spectra, and allows for the addition of more samples to the data set later on^{378,379}. The limitations of label-free quantification are that it heavily depends on very stable liquid chromatography (LC) separation and spray conditions and requires more replicates³⁸⁰. However, it is widely used in shotgun proteomics, and there are many instances of its successful application in conjunction with ABPP or affinity-based chemoproteomics³⁸¹⁻³⁸⁴. While this work protocol only focused on label-free quantification for protein analysis, alternatives such as TMT or SILAC labeling are also possible. The choice of data analysis and the determination of cutoff criteria for enrichment proteins may vary depending on the mass spectrometry instruments and software utilized.

One limitation of this protocol, like other probe-labeled proteomics, is the proper solubilization of membrane proteins, which can be achieved to some degree by boiling the samples in 1% SDS^{381,385}. Another potential shortcoming, as described by others³⁶⁷, is that probe-labeled peptides are either too long or too short for accurate MS characterization. To overcome this limitation, an initial digestion with protease alternative to trypsin needs to be performed to generate peptides of suitable length and properties for MS analysis³⁶⁷. Although this protocol is described for biotinylated-tagged ABP enrichment, the customized protocols can also be used for click chemistry-based ABPP sample preparation, as the immobilized magnetic beads facilitate the removal of excess click chemistry reagents through acetone or methanol precipitation^{361,367}.

4.3. Functional validation of molecular targets

Studying enzymes targeted by ABPs often requires the deletion or heterologous expression of genes encoding these enzymes. Initially, mutants were obtained from the Nebraska Transposon Mutant Library³⁵⁰ for *S. aureus* and the Manoil Lab Transposon Mutant Library³⁵¹ for *K. pneumoniae*. First, SDS-PAGE-based ABPP was performed on both mutant and wild-type strains to confirm the identity of the fluorescent ABP targets using gel-based ABPP. These experiments also revealed any potential compensatory upregulation of other enzymes within

the same family in knockout strains, which could suggest a functional connection between these genes.

Considering the possibility of non-specific effects arising from secondary mutations or polar effects within the transposon mutants, alternative mutant types were adopted in subsequent experiments. Specifically, phage-transduced mutants in the well-characterized *S. aureus* LAC strain were used for the experiments described in Paper I. In Paper II, CRISPR interference (CRISPRi)-based mutant constructs for the selected ABP targets were employed. Additionally, to eliminate concerns about polar effects caused by transposon insertion, other allele-specific mutants for *K. pneumoniae* were used in Paper III.

For further biological validation, the growth fitness of the transposon mutant strains of targeted enzymes was also monitored during *in vitro* growth conditions to determine whether they exhibit any growth defects in regular media.

4.4. Host model systems to study colonization and infection

In Paper III, seven previously uncharacterized or poorly annotated serine hydrolases (SHs) in *K. pneumoniae* were identified using a chemoproteomics strategy. This finding underscored the need for a robust model to study these enzymes roles at the host-pathogen interface. Ideally, the selected model system should replicate the environmental conditions that the bacteria (*K. pneumoniae*) encounter during colonization or infection. However, one challenge with using host model systems is their inability to incorporate every human component and condition that fully mimics bacterial colonization or infection. Since the primary niche of *K. pneumoniae* as a commensal bacterium is the human gut, the growth fitness of the SHs was initially monitored using *in vitro* models that mimic the gut-host interface. Consequently, a co-culture model with HT29-MTX cells, which are rich in mucus and goblet cells, was utilized. Following the identification of phenotypes for three hydrolases, the aim was to further validate these enzymes in models that more accurately reflect the complexity of the human gut. Hence, intestinal organoids, more specifically colon organoids, derived from adult human tissue stem cells, were employed. Besides tissue-derived organoids, intestinal organoids can also be created from human pluripotent stem cells^{386,387}. However, this process takes up to four weeks and does not fully mimic the characteristics of adult tissue^{386,387}.

Colon organoids are three-dimensional, stem cell-derived structures that closely resemble the colonic epithelium architecture and functionality (reviewed in ³⁸⁸). Human-derived organoid models offer several advantages including easy accessibility, higher reliability and relevance

than other models, e.g. immortalized human cell lines and animal models (reviewed in³⁸⁹). Moreover, they provide ample material for study. Animal models, in contrast, often fall short of offering the necessary experimental accessibility to study infection processes with the desired detail and their effectiveness in predicting disease phenotypes is debatable³⁹⁰. The emergence of organoids, self-organizing 3D structures from stem cells that reproduce key features of organ structure and function, presents robust models for studying infections in differentiated human tissues³⁹¹⁻³⁹³. Their value in research, especially in mimicking human conditions for studying diseases, pathogenesis, and host-pathogen dynamics, is increasingly recognized^{391,394,395}. For instance, researchers have used the 3D intestinal organoid model to investigate the mutational patterns of colorectal cancer caused by genotoxic *pks+* *Escherichia coli*³⁹⁶. This model has also been key in studying the effects of *Clostridium difficile*³⁹⁷ toxins and *Salmonella* spp. invasion on the intestinal epithelium³⁹⁸.

However, accessing the luminal side presents challenges in 3D organoids. Intestinal organoids are made up of a single layer of epithelial cells forming a hollow central lumen (the apical side) and introducing microbes into this lumen requires microinjection techniques^{399,400}. While microinjection provides precision, it is labor-intensive, challenging to standardize, and requires a high level of technical skill, resulting in relatively low throughput³⁹⁹. Additionally, the type of analysis being conducted, such as fluorescence, transcriptomic, proteomic, or metabolomic, often requires using multiple organoids to gather enough sample material for testing. Moreover, antibiotics are added to the culture medium of organoid cultures to prevent bacterial growth on the basolateral side, which can alter intestinal function in ways that are independent of the antimicrobial activity^{399,401}. Another approach involves breaking the organoids to facilitate infection, but this exposes both apical and basolateral sides to pathogens^{395,402}.

Considering these challenges and to overcome some of the limitations mentioned above, 2D organoid monolayers have been adopted for studying bacterial pathogens⁴⁰³⁻⁴⁰⁸. This format allows for the direct introduction of pathogens to the apical side of the intestinal epithelium by adding them to the culture medium, facilitating a detailed examination of host-pathogen interactions at the intestinal barrier. Furthermore, 2D organoid monolayers support advanced microscopy techniques and provide a comprehensive model of the *in vivo* intestinal epithelium, including essential intestinal cell types like Paneth cells and stem cells. A limitation of static 2D monolayer models is their failure to sustain the strict anaerobic environment found in the intestine or a consistent nutrient supply. This makes it challenging to maintain long-term stable co-cultures due to the short lifespan of host cells and bacterial overgrowth^{408,409}.

Although air-liquid interface (ALI) cultures of 2D monolayers enhance oxygen supply and promote a more differentiated phenotype of epithelial cells⁴¹⁰, thus favoring bacterial growth, this method was not chosen for use. This decision was based on several factors, such as *K. pneumoniae* being a facultative anaerobe that can grow in low oxygen conditions. Additionally, maintaining monolayer cultures at the ALI requires up to four weeks⁴¹¹, and bacterial overgrowth can occur within a few hours. To facilitate longer testing periods and more detailed observations of phenotypes, the protocol was modified by reducing the use of non-diffusible antibiotics compared to the original protocol⁴¹², aiming to develop a better model for studying targeted enzymes. Despite these efforts, challenges were faced in sustaining co-culturing for longer periods (up to 16 hours) in order to observe pronounced phenotypes between wild-type and mutant strains, due to an insufficient continuous flow of nutrients, which longer cultures require. This limitation could be overcome by integrating a transwell monolayer into a microphysiological system that ensures a steady supply of nutrients to the bacterial compartment⁴¹³. The development of the 2D monolayer system is still ongoing and shows promise for improving the study of bacterial pathogens^{409,414,415}.

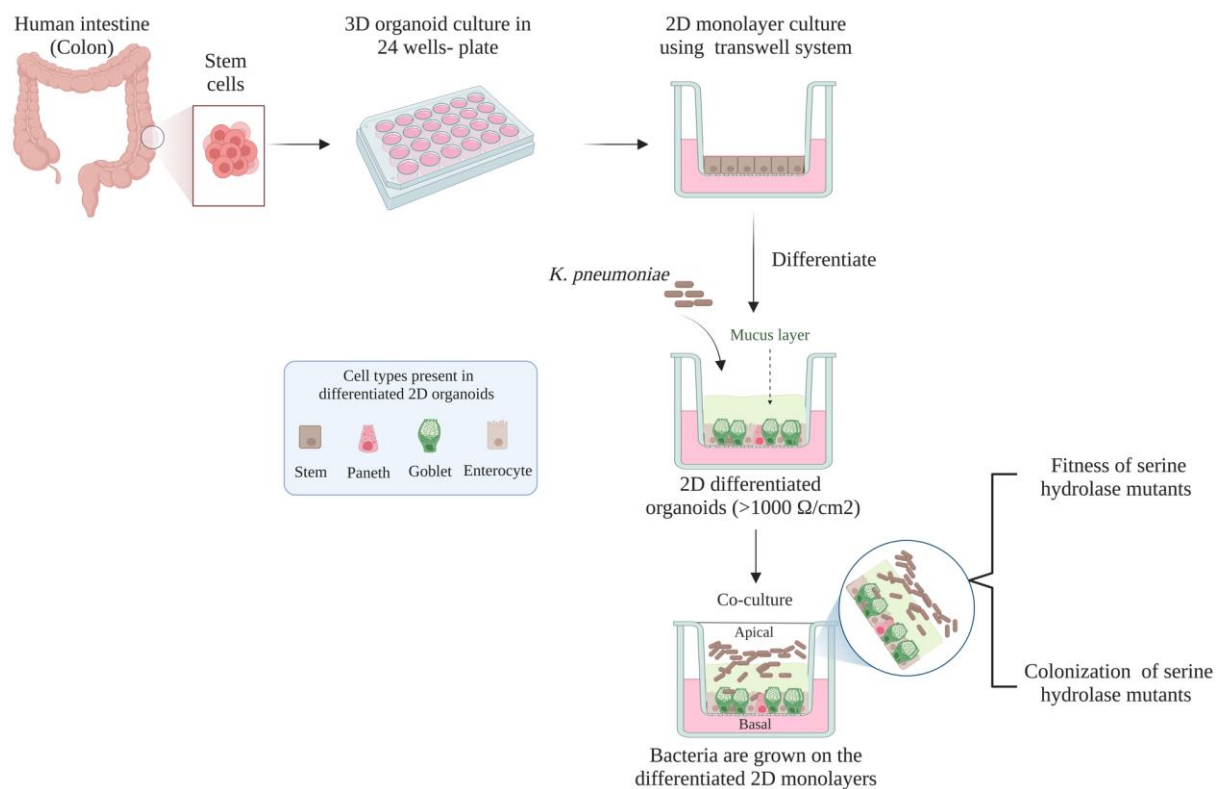


Figure 13. Schematic overview of the co-culture model of *K. pneumoniae* with colonic organoid monolayer. Human colon organoids, derived from stem cells isolated from intestinal biopsies or tissues, are initially cultured as 3D structures in extracellular matrices such as Cultrex Basement Membrane Extracts (BME) in 24-well plates. These 3D organoids are subsequently transferred as single organoid cells to a 2D monolayer format in Transwell systems, allowing them to differentiate into mature intestinal cells. The integrity of the differentiated monolayer barrier is determined by quantifying the transepithelial electrical resistance (TEER). *K. pneumoniae* wild-type and serine hydrolase mutants are then introduced to the apical side of the differentiated 2D organoid monolayers. Bacterial fitness and/or colonization efficacy are monitored by determining colony-forming unit (CFU) over time. The figure was created with biorender.com.

Serine hydrolase mutants exhibited reduced fitness compared to wildtype in organoid co-culture models (paper III), and it was therefore desired to determine their virulence properties in scenarios more similar to *in vivo* infections. In addition, this study identified two previously uncharacterized glycosidases in *S. aureus* that warrant further functional study in relation to host-pathogen interactions. Consequently, wax moth *Galleria mellonella* larvae were utilized as an infection model to study bacterial virulence.

The use of insect larvae, such as *G. mellonella*, for testing pathogen virulence offers a viable alternative to experimental infections in rodents or pigs and is not regulated by legislation⁴¹⁶. This approach is crucial to comply with the 3Rs efforts that aim to reduce, refine, and replace

animal experiments⁴¹⁷. *G. mellonella* larvae, in particular, have been employed as models for studying various significant pathogens⁴¹⁸ and evaluating the effectiveness of antibacterial and antifungal treatments^{419,420}. This is because the innate immune system of the wax moth and higher organisms share general similarities (reviewed in⁴²¹). *G. mellonella* larvae offer several technical advantages over mammalian infection models, such as high throughput, ease of handling, simple housing requirements, and the possibility to purchase large quantities of larvae at a relatively low price, with no legal or ethical issues. Unlike other non-mammalian models such as zebrafish larvae, *C. elegans*, or *Drosophila melanogaster*, experiments with *G. mellonella* can be conducted at the mammalian host's body temperature of 37°C. Thus, findings from insect-based studies can provide foundational insights into disease mechanisms and help formulate hypotheses that can be further explored in vertebrate models.

The organoids used in this study were obtained from the Foundation Hubrecht Organoid Biobank (Utrecht, The Netherlands) under TC-Bio protocol number 14-008 and were used in accordance with informed consent. Human blood was isolated after informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. Approval was obtained from the Medical Ethics Committee of UMC Utrecht, The Netherlands. The human derived organoids and the complement assay using human serum were used during my research stay at UMC Utrecht, Netherlands.

5. Summary of main results

Paper I: Activity-Based Protein Profiling in Methicillin-Resistant *Staphylococcus aureus* Reveals the Broad Reactivity of a Carmofur-Derived Probe

In this paper, carmofur-derived probe, originally developed to target human acid ceramidase (ACase) after recognizing carmofur as a potent ACase inhibitor, was used for activity-based protein profiling in MRSA to identify active enzymes and potential targets of this drug. Carmofur, which is a prodrug of 5-Fluorouracil (5-FU), is a registered anti-neoplastic agent for colorectal cancer treatment in multiple countries and is being explored for various other therapeutic applications. Both carmofur and its parent compound, 5-FU, exhibit broad-spectrum antimicrobial and antibiofilm activity against various bacteria, including MRSA.

- Gel-based ABPP with a fluorescent carmofur probe showed dose-dependent labeling of specific *S. aureus* proteins, with multiple bands labeled at different concentrations.
- Pre-incubation with carmofur led to reduced labeling of specific bands by the fluorescent probe, suggesting specific interactions between the probe and its targets, while pre-incubation with 5-FU did not alter the labeling patterns significantly.
- Treatment with a carmofur-biotin probe resulted in the enrichment of 20 enzymes from diverse families in *S. aureus*, including the NTN hydrolase-related IMP cyclohydrolase PurH and previously uncharacterized fluorophosphonate-binding serine hydrolases (SHs). The enrichment of so many SHs was surprising since carmofur had previously been described to target enzymes with an active-site cysteine.
- This study did not observe the direct link between the enzyme targets of carmofur and the susceptibility of *S. aureus* to the antimicrobial effects of the drug, suggesting that the mechanism of action of carmofur may involve more complex interactions or the cumulative effect of multiple non-essential targets, which might alter cellular processes or metabolic pathways in a manner that contributes to the overall antimicrobial effect.

In conclusion, this paper highlighted the concern regarding the clinical use of carmofur, noting that the drug's broad and somewhat promiscuous reactivity with both human and microbial enzymes could influence its therapeutic effects and potential side effects.

Paper II: Activity-Based Protein Profiling Identifies an Alpha-amylase Family Protein Contributing to the Virulence of Methicillin-Resistant *Staphylococcus aureus*

In this paper, a panel of glycosidase ABPs was utilized to identify and characterize active retaining glycosidase activities in MRSA.

- Gel-based ABPP using fluorescent glycosidase ABPs (**1-7**) in *S. aureus* revealed specific labeling patterns identifying active enzymes. The ABP **1**, which primarily targets alpha glucosidases, labeled a band at 60-70 kDa in both culture supernatants and cell pellets. This band was consistently the most prominent under various liquid and agar-based growth media. Other probes such as ABP**2**, ABP**4**, and ABP**5** also identified specific bands in different growth conditions.
- Tests for dose-dependency revealed that a concentration of 1 μ M was necessary for effective labeling by ABPs **1**, **4**, and **5**. Competitive ABPP with unlabeled inhibitors suggested specific interactions for ABP**1**, while others did not show changes in labeling patterns, indicating potential non-specific interactions or limitations in inhibitor potency.
- The study identified the two previously uncharacterized glycosidase family proteins: the putative 6-phospho- β -glucosidase (BglA) and the α -amylase family protein trehalase C (TreC), using biotinylated analogs of three probe cocktails, through mass spectrometry-based ABPP.
- Gel based validation using transposon mutants and CRISPRi-based mutants indicated that TreC is a target of ABP**1**, and possibly BglA and Atl as well.
- Further functional validation of the newly identified enzymes using CRISPRi gene silencing revealed that *treC*-silenced, but not *bglA*-silenced, led to reduced *S. aureus* virulence, as evident by a significantly higher larval survival rate in a *Galleria mellonella* infection model. However, silencing either gene did not affect bacterial growth in TSB.

In conclusion, this study supported the putative role of the α -amylase family protein, TreC, in bacterial virulence during *G. mellonella* infection.

Paper III: Activity-Based Protein Profiling Identifies *Klebsiella pneumoniae* Serine Hydrolases with Potential Roles in Host-Pathogen Interactions

In this paper, ABPP was used in *K. pneumoniae* to identify and characterize serine hydrolases (SHs), a highly druggable and functionally diverse enzyme family in nature.

- Fluorophosphonate probes were used to identify 10 SHs in *K. pneumoniae* by ABPP. Out of these 10 SHs, 8 were found to possess α,β -hydrolase domains, while the remaining two were linked to serine proteases with trypsin-like peptidase domains. Interestingly, 7 of these SHs were poorly annotated, and their definitive cellular functions are not yet known. Additionally, the majority of the identified SHs displayed limited or no homology with serine hydrolases from other gut commensal bacteria and humans.
- SH-deficient transposon mutants exhibited growth patterns similar to the wild-type strain when cultured in LB liquid media. However, interestingly, three of them demonstrated reduced fitness in an HT 29-MTX cell-based co-culture model.
- Further functional validation using transposon mutants deficient in the serine hydrolases PldB, YjfP, and YchK indicated reduced fitness in co-culture models with human-derived colonic organoids. Notably, the translocation efficacy of PldB and YchK was also low.
- Mechanistically, the transposon mutants of *pldB* and *yjfP* had increased susceptibility to complement and polymyxin B, suggesting that these enzymes have a role in shaping envelope integrity, whereas the putative secreted patatin-like phospholipase YchK had not.
- Additionally, transposon mutants of *yjfP*, *pldB*, and *ychK* were less virulent than the wild-type strain in the *G mellonella* infection model. Moreover, the assessment of virulence among different allele transposon mutants from the Manoil library with insertions in *pldB* (n=3 available strains), *yjfP* (n=3), and *ychK* (n=2) all showed similar phenotypes.
- Biochemical and substrate profiling revealed that both YjfP and PldB possess esterase activity. Structural and biochemical assays suggested that YjfP potentially has deacetylase activity.

In conclusion, this study provides important insights into the molecular mechanisms that govern the virulence and cell physiology of *K. pneumoniae* at the host-pathogen interface. It highlights PldB, YjfP, and YchK as potential targets for antimicrobial or anti-virulence therapies, the inhibition of which could complement existing antibiotics and human immune defense.

6. General discussion

The increasing prevalence of resistant and multi-resistant bacterial pathogens represents a considerable threat to human health. In light of the increasing antibiotic resistance crisis, there is an urgent need to discover new classes of putative antibacterial targets. A major obstacle in this endeavor is that large portions of many bacterial genomes remain functionally uncharacterized. This gap underscores the necessity for unbiased screening methods to identify promising new drug targets that can be pharmacologically modulated. ABPP offers a robust method by enabling the rapid, direct identification and quantification of enzyme activities within complex biological samples. This technique holds the potential to accelerate the discovery of viable new targets for antibacterial therapy.

The main focus of this study was to use ABPs to identify new enzymatic activities in bacterial pathogens, particularly *S. aureus* and *K. pneumoniae*. This follows a previous study where fluorophosphonate (FP) probe was used to identify previously uncharacterized enzymes in *S. aureus* that have emerging roles in pathogenesis and as potential anti-virulence targets¹⁰⁶. In this work, additional enzyme families that remain underexplored in *S. aureus* were aimed to be identified (Papers I and II) under similar growth conditions that promote biofilm formation. Moreover, general serine-reactive FP probe was used to identify serine hydrolases in *K. pneumoniae* that had also been successfully used previously in other organisms^{272,311,352-354}.

6.1 ABPP in *S. aureus* and *K. pneumoniae*

ABPP is a powerful chemo-proteomics technique that identifies the on-target and off-target of a drug⁴²². Initial efforts were undertaken to profile active target enzymes in *S. aureus* using carmofur-derived ABP (Paper I). Carmofur and its parent compound, 5-FU, are recognized for their broad-spectrum antimicrobial and antibiofilm activity against various bacteria⁴²³. However, the exact mechanism behind the antimicrobial effects of carmofur remains unknown. It is plausible that microbial enzymes targeted by carmofur may directly contribute to its antimicrobial and antibiofilm activities analogous to the role of human acid ceramidase (ACase) inhibition in the therapeutic efficacy of carmofur in humans. Interestingly, multiple enzyme families were identified using carmofur-derived biotin analog coupled with LC-MS/MS including NTN hydrolases, which are targeted by carmofur in humans as well as previously uncharacterized serine hydrolases (Paper I). Due to the promiscuous nature and broad reactivity of the carmofur probe, which may lead to the identification of a broad range of enzyme families,

understanding the functional characteristics of these enzymes can be challenging. Since carmofur is used as a colorectal cancer drug in the clinic, the same reactivity of carmofur with other gut microbiota enzymes and human enzymes, which might potentially relate to the therapeutic and side effects of carmofur, cannot be ruled out. The report has also shown that non-antibiotic drugs including 5FU, a prodrug of carmofur, can inhibit at least the growth of one commensal bacteria by 25%⁴²⁴ (Paper I).

Glycosidases play crucial roles in bacterial pathogenesis, including immune modulation, adherence to host cells, colonization, and biofilm formation³²⁵. Given the high substrate specificity of glycosidases and the absence of a broad-spectrum probe, a panel of glycosidases ABPs has been applied to identify three glycosidase hydrolase family proteins: the alpha-amylase family protein phosphotrehalase (TreC), 6-phospho- β -glucosidase (BglA), and autolysin (Atl), two (TreC and BglA) of which were previously uncharacterized (Paper II). Since serine hydrolases play important roles in bacterial homeostasis, metabolism, and virulence, a broad-spectrum FP probe was used to identify 10 *K. pneumoniae* serine hydrolases, seven of which are poorly annotated and previously uncharacterized (Paper III).

However, in this current study, only simplistic *in vitro* growth conditions were used to profile the active enzymes. Bacteria may express alternative enzymes under other conditions. Future efforts will involve using more complex infection models to identify additional enzymatic activities. Moreover, cell-based ABPP strategies have already been successfully employed to identify active enzymes at the host-pathogen interface and in other physiological environments^{311,425}.

ABPP can identify enzymes that are already known for their potential to contribute to biofilm formation and bacterial pathogenesis. Several enzymes identified in this thesis through ABPP studies had already been recognized previously for their important roles in biofilm formation and bacterial pathogenesis and could serve as potential targets for anti-virulence and putative drug therapies. For instance, the NTN hydrolase family member, IMP cyclohydrolase, PurH have been recognized for their roles in biofilm formation⁴²⁶ and the FphB has recently been characterized as a virulence factor in *S. aureus*¹⁰⁶ (Paper I). In addition, the O-acetyltransferase Oat that features a SGNH-hydrolase-type esterase domain⁴²⁷, is common across a wide variety of bacteria and serves various functions⁴²⁸ (Paper I). The teichoic acid d-Ala esterase FmtA is responsible for the hydrolytic removal of d-alanine esters from wall teichoic acid (WTA), a crucial process that helps regulate the charge and integrity of the bacterial cell wall^{429,430} (Paper I). In Paper II, Autolysin (Atl), also known as peptidoglycan hydrolase, was identified, which

plays crucial roles in cell envelope remodeling, virulence, and biofilm formation^{431,432}. This broad functional spectrum underscores the critical nature of autolysin in bacterial pathophysiology and its potential as a therapeutic target. The HtrA-like serine proteases DegP and DegQ were identified, which demonstrated increased susceptibility to complement-mediated killing and exhibited reduced levels of capsule polysaccharide⁴³³(Paper III). However, the mechanisms through which these proteases exert their effects are not yet fully understood. Additionally, the carboxylesterase BioH, which plays a role in the biosynthesis of biotin⁴³⁴ was enriched (Paper III). In *Mycobacterium tuberculosis*, biotin metabolism is recognized as a viable target pathway for antibacterial drugs^{435 436}. Since ABPP identifies enzyme targets through covalent interactions with a small molecule probe, it confirms that all identified targets are druggable and facilitates the identification of target-specific inhibitors.

Besides glycosidases and serine hydrolases, the MS-based ABPP also detected other hydrolytic enzymes (papers II and III). This is a known limitation of affinity-based pulldown experiments, as they can enrich ribosomal proteins, and endogenously biotinylated proteins and sometimes targets are even enriched due to less stable, non-covalent, reversible interactions with the probe^{437,438}. However, this study excluded those hydrolytic enzymes and specifically focused on glycosidase in *S. aureus* and serine hydrolases in *K. pneumoniae* (Paper II and III).

6.2 Functional validation of uncharacterized enzymes in host-pathogen interactions

Enzymes that are active can be crucial for bacterial virulence or the hosts immune response to infection, thus constituting potential therapeutic targets. Retaining glycosidases and serine hydrolases play crucial roles in numerous biological processes in both bacteria and their hosts and can be selectively targeted by cyclophellitol-aziridine and well-characterized fluorophosphonate (FP)-containing ABPs. However, the understanding of these enzymes in the bacterial pathogens *S. aureus* and *K. pneumoniae* remains limited. Since this study identified two previously uncharacterized glycosidases in *S. aureus* and seven uncharacterized serine hydrolases (SHs) in *K. pneumoniae*, this study aimed to further assess cellular functions in relation to host-pathogen interactions and bacterial physiology. Although the Nebraska Transposon Mutant Library for *S. aureus* JE2 strains is available in the lab, due to the non-specific effects from secondary mutations or polar effects within the transposon mutants, alternative IPTG-induced CRISPRi mutants were used for the functional validation of

glycosidase targets (Paper II). Despite the challenges of IPTG induction *in vivo*, like in the wax moth larvae model, CRISPRi constructs were chosen. CRISPRi constructs specifically block the transcription of genes, thus reducing secondary mutational effects. In addition, they were constructed in the well-characterized *S. aureus* LAC strain. On the other hand, due to the absence of alternative mutants, transposon mutants were utilized in Paper III. Given the critical role of glycosidases in degrading host glycans, which may assist in extracting nutrients for bacteria and facilitate colonization⁴³⁹, CRISPRi gene silencing was employed to investigate the roles of BglA and TreC in wax moth *Galleria mellonella* larvae, as an infection model (Paper II). Interestingly, the results indicated that TreC might contribute to virulence in the *G. mellonella* infection model. The mechanisms by which TreC acts as a virulence factor remain unknown. However, TreC is part of the alpha-amylase family within GH13, one of the largest families of glycoside hydrolases⁴⁴⁰. Additionally, an alpha-amylase family protein has previously been identified as a putative virulence factor in *Streptococcus pneumoniae* through a single signature-tagged mutagenesis (STM) screen⁴⁴¹. Future studies, including biochemical characterization, identification of physiological substrates, and other infection models, including *in vivo* animal studies, are necessary to substantiate the role of this enzyme as a virulence factor.

Since 7 of the identified serine hydrolases (SHs) in *K. pneumoniae* (Paper III) are previously uncharacterized and their cellular functions are poorly defined, their roles at the host-pathogen interface for this critical priority pathogen were also addressed. This thesis also focused on SHs due to their significant impact on microbial pathogenicity⁴⁴²; their frequent activation at the post-translational stage⁴⁴³ makes them ideal candidates for activity-based studies^{444,445}. Additionally, since these enzymes can be targeted through covalent interactions with small molecule probes, they represent promising lead molecules for validating targets for anti-virulence therapy and as potential antibiotic sensitizers.

However, to replicate the environmental conditions bacteria encounter during colonization and infection, several model systems are necessary. Since the primary niche of *K. pneumoniae* is as a commensal in the gut, the HT29-MTX cells, which is rich in goblet cells and mucus, were utilized as an *in vitro* model to mimic the gut environment. Interestingly, pronounced growth defects were exhibited by three SH-deficient transposon mutants, *pldB*, *ychK*, and *yjFP*, in this co-culture model (Paper III). To further validate these mutants, a human-derived colonic organoid model was used (Paper III). The advantage of using organoid models lies in their accessibility, providing faster and more reliable results than animal models and offering a more

accurate representation of human tissue along with greater material availability³⁸⁹. The SH-deficient transposon mutants *pldB*, *yhcK*, and *yjfP* also showed a pronounced fitness loss upon co-culturing with a colonic organoid monolayer. Since these mutants were unable to grow in organoid growth media alone, it was assumed that interactions with host cells are crucial for sustaining the growth of these mutants in the co-culture model. Interestingly, the translocation efficacy was relatively low for PldB and YchK, suggesting these enzymes might be important in hampering the epithelial cell barrier. The mucus layer, primarily composed of glycoproteins, acts as a defense against the bacteria residing in the colon⁴⁴⁶⁻⁴⁴⁸ but also contains phosphatidylcholine (PC), an essential component of the intestinal mucus layer's structure⁴⁴⁹. Bacterial phospholipases in this layer can convert PC to lyso-PC, disrupting the mucus structure and damaging epithelial cells⁴⁴⁹⁻⁴⁵¹. For example, *H. pylori*, secreted lipases can breach mucus-associated phospholipids, aiding this pathogen to invade gastric mucus^{452,453}. A similar mechanism could be plausible for putative secreted patatin-like phospholipases YchK, either accessing nutrients through its hydrolytic activity directly from mucus-associated phospholipids or breaking the defensive barrier for cell invasion and nutrient extraction.

While the specific mechanisms by which the other two enzymes, PldB and YjfP, act on the gut are speculative, maintaining cell envelope integrity might be involved. However, mutants deficient in proteins PldB and YjfP, but not the secreted lipase YchK, showed increased susceptibility to antimicrobial peptide (AMP) antibiotics such as polymyxin B, and to complement-induced killing. Polymyxin B is a membrane-destabilizing AMP that interacts with lipopolysaccharide (LPS) of the outer membrane in gram-negative bacteria and disrupts the bacterial membrane⁴⁵⁴. Complement also targets the outer membrane of Gram-negative bacteria, disrupting it by forming pores⁴⁵⁵. The presence of lysozyme C in colonic organoids model indirectly indicated the presence of Paneth cells, which are typically found in the small intestine⁴⁵⁶ and known for producing antimicrobial peptides and defensins⁴⁵⁷. The initial drop in *K. pneumoniae* growth in organoid models was likely due to the bactericidal effects of AMPs produced by the organoids. Reduced virulence was also observed for these three SH-deficient mutants in *Galleria mellonella* infection models. Given that AMPs are important effectors of innate immunity in larvae, it is possible that *G. mellonella* also produces AMPs⁴⁵⁸.

Biochemical and structural characterization of YjfP indicates that it is a deacetylase, acting on small molecules of peptides and proteins. On the other hand, PldB cleaves a range of saturated lipid esters fluorogenic substrates up to C8 fatty acid chain length. The interesting phenotypes of these SH mutants demand further investigation of their physiological substrates. In addition,

protein BLAST searches with non-redundant protein sequences indicate that these serine hydrolases exhibited little or no homology with those of other gut commensal bacteria or humans. This result suggests that these three serine hydrolases could be important targets for small-molecule drugs, potentially minimizing off-target effects and reducing the inhibition of enzymes in other microbes. This notion also supports the integration of bioinformatics with ABPP to enhance drug development, providing additional insights into their potential as drug targets⁴⁵⁹.

Since transposon mutants might carry secondary mutations affecting these phenotypes, other allelic transposon mutants were also tested in the *G. mellonella* infection model, and similar phenotypes were observed. However, future studies involving gene complementation and gene knockouts through allelic exchange are necessary to fully determine their roles.

7. Concluding remarks and future aspects

Bacterial infections have always posed a significant threat to human health. However, improvements in hygiene practices and the development and use of antibiotics over the past century have significantly reduced the burden and mortality rates associated with these infections. Despite these advances, the extensive use and misuse of antibiotics have led to increased resistance, raising concerns that we are approaching a post-antibiotic era marked by untreatable bacterial infections. This emerging crisis underscores the urgent need for a novel approach to combat antibiotic-resistant bacteria. One promising strategy could involve exploring virulence factors that disarm or neutralize the mechanisms bacteria use to infect and damage their hosts, rather than fully killing the bacteria, which could likely prevent the development of resistance. This thesis has focused on using ABPP to identify previously uncharacterized druggable enzymes and functionally validate them within the context of bacterial physiology and the host-pathogen interface.

Overall, this study identified and functionally validated previously uncharacterized enzyme targets: one glycosidase, TreC, in *S. aureus*, and three serine hydrolases, PldB, YjfP, and YchK, in *K. pneumoniae*. These targets have shown potential as anti-virulence targets. ABPP, which identifies enzyme targets through covalent interaction with small molecule probes, confirms the druggability of the targets and facilitates the development of target-specific inhibitors. Competitive ABPP, in particular, allows for the identification of selective cell-permeable inhibitors. In contrast, traditional target-based antibiotic drug design projects often fail due to the drug's lack of cellular permeability. If performed on live cells, as in this study, cell-based competitive ABPP also pre-validates the accessibility of the targets to the inhibitors. This study provides the groundwork for future investigations into the biological functions of these enzymes, highlighting their potential as promising targets for the development of selective inhibitors and activity-based probes, which will be crucial in characterizing their activities within physiological environments. The initial functional studies of these enzymes suggest that inhibiting these targets, having role in virulence, could potentiate with existing antibiotics and human immune defense to prevent bacterial infection and/or colonization. However, further functional validation of these enzymes in more complex models of infection and host-pathogen interactions is necessary to better understand these newly identified enzymes. This deeper exploration is essential to fully assess their potential as putative antibacterial drugs.

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Paper I

Activity-Based Protein Profiling in Methicillin-Resistant *Staphylococcus aureus* Reveals the Broad Reactivity of a Carmofur-Derived Probe

Md Jalal Uddin,^[a] Hermen S. Overkleeft,^[b] and Christian S. Lentz^{*[a]}

Activity-based protein profiling is a powerful chemoproteomic technique to detect active enzymes and identify targets and off-targets of drugs. Here, we report the use of carmofur- and activity-based probes to identify biologically relevant enzymes in the bacterial pathogen *Staphylococcus aureus*. Carmofur is an anti-neoplastic prodrug of 5-fluorouracil and also has antimicrobial and anti-biofilm activity. Carmofur probes were originally designed to target human acid ceramidase, a member of the NTN hydrolase family with an active-site cysteine nucleophile. Here, we first profiled the targets of a fluorescent carmofur probe in live *S. aureus* under biofilm-promoting conditions and in liquid culture, before proceeding to target identification by liquid chromatography/mass spectrometry. Treatment with a

carmofur-biotin probe led to enrichment of 20 enzymes from diverse families awaiting further characterization, including the NTN hydrolase-related IMP cyclohydrolase PurH. However, the probe preferentially labeled serine hydrolases, thus displaying a reactivity profile similar to that of carbamates. Our results suggest that the electrophilic *N*-carbamoyl-5-fluorouracil scaffold could potentially be optimized to achieve selectivity towards diverse enzyme families. The observed promiscuous reactivity profile suggests that the clinical use of carmofur presumably leads to inactivation of a number human and microbial enzymes, which could lead to side effects and/or contribute to therapeutic efficacy.

Introduction

Activity-based protein profiling (ABPP) uses functionalized active-site directed small molecule probes known as activity-based probes (ABPs) to selectively label active enzymes, which can then be detected, identified and quantified through various analytical methods. A broad variety of probes has been designed to target diverse enzyme families and the application range spans from identification of drug targets and off-targets by chemical proteomics^[1] to in vivo imaging or single-cell imaging studies in diverse organisms as extensively reviewed elsewhere.^[2–6]

Some probes have been designed to target enzyme families in the broadest possible way^[7] and have been applied for profiling studies in diverse organisms, whereas other probes have primarily been designed and validated for interaction with a particular target in a certain organism of interest. Repurposing

these probes and validating their potential interactions with as yet unidentified targets in other biological specimen may provide a short-cut for the discovery and functional validation of previously uncharacterized enzymes.

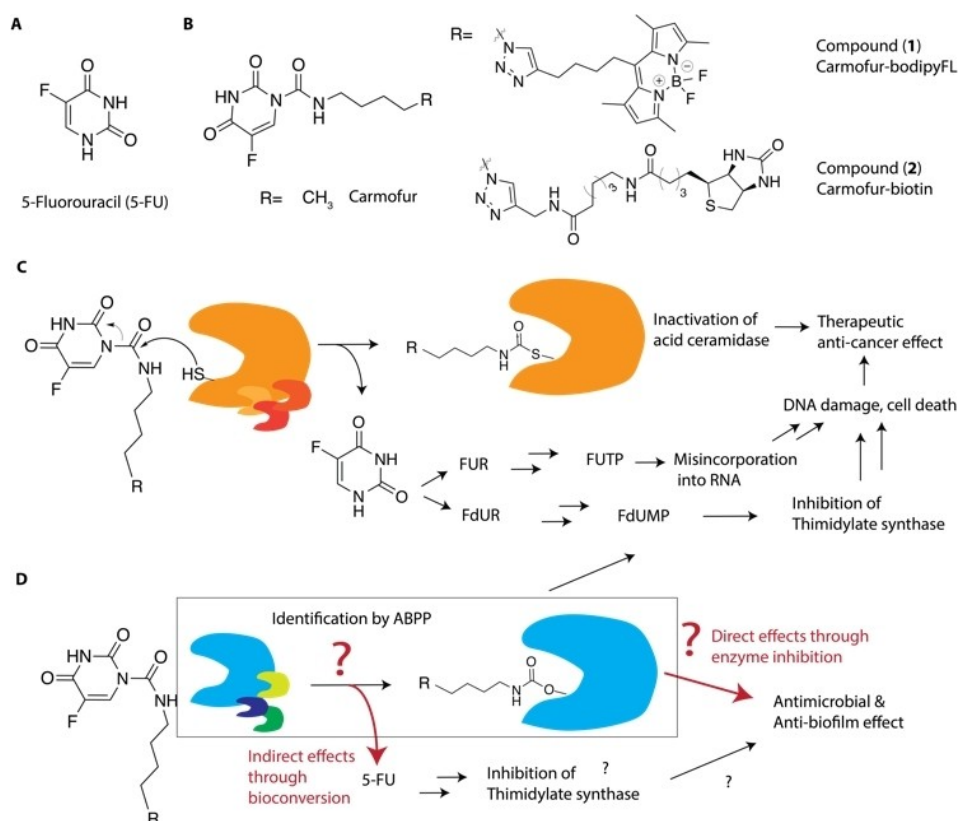
One focus of our research has been the characterization of new enzyme activities in bacterial pathogens. In a previous ABPP-study we have used fluorophosphonate probes to identify ten uncharacterized serine hydrolases in the bacterial pathogen *Staphylococcus aureus* that have emerging roles in pathogenesis and as potential anti-virulence targets.^[8] In an ongoing effort to expand this work we seek to characterize the activity of additional enzyme families that are not well characterized. One interesting candidate for this approach are carmofur-derived probes (Scheme 1) that were generated to target human acid ceramidase (ACase)^[9] after carmofur had been identified as a potent inhibitor of ACase.^[10] Carmofur, or 1-hexylcarbamoyl-5-fluorouracil, is a registered anti-neoplastic drug for treatment for colorectal cancer in several countries^[11,12] and a number of other potential therapeutic applications are under investigation as summarized in a recent review.^[13] Carmofur is a prodrug of the antimetabolite 5-fluorouracil (5-FU) and its bioconversion pathways and known mechanism of action are illustrated in Scheme 1C. In human cells, 5-FU is converted to various bioactive metabolites that, in a complex mechanism, ultimately result in DNA damage and cell death.^[14,15] The bioactive 5-FU-derived metabolites include, for example, fluorodeoxyuridine monophosphate, which inhibits thymidylate synthase, and fluorouridine triphosphate, which is irregularly incorporated into RNA.^[12,15] Whereas carmofur had originally been designed as a prodrug, it has been shown that this compound retains activity against 5-FU-resistant cancers, suggesting involvement

[a] M. J. Uddin, Prof. Dr. C. S. Lentz
 Department of Medical Biology
 UiT- The Arctic University of Norway
 9019 Tromsø (Norway)
 E-mail: christian.s.lentz@uit.no

[b] Prof. Dr. H. S. Overkleeft
 Department of Bioorganic Synthesis
 Leiden Institute of Chemistry, Leiden University
 Einsteinweg 55, 2333 CC Leiden (The Netherlands)

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Scheme 1. Chemical structures of compounds used in this study. A) 5-FU. B) Carmofur and related probes carmofur-bodipyFL (1) and carmofur-biotin (2). C) Bioconversion of carmofur and 5-FU in humans and mechanism of action; FUR: fluorouridine, FdUR: fluorodeoxyuridine, FUTP: fluorouracil triphosphate, FdUMP: fluorodeoxyuridine monophosphate. D) Schematic illustration of the bioconversion pathway and mechanism of action underlying the antimicrobial and anti-biofilm effects of carmofur in *S. aureus*. Enzymes identified by ABPP could directly contribute to the biological effects and/or indirectly have relevance for the bioconversion to 5-FU as indicated by red font and arrows. Whereas (C) shows the presumed inactivation mechanism of carmofur through carbamoylation of the active-site Cys nucleophile of the cysteine amidase ACCase, (D) illustrates the proposed carbamoylation of active-site Ser of serine hydrolases, which this study determined as the major target class of carmofur probes in *S. aureus*.

of additional targets not shared with 5-FU, such as ACCase^[16–18] (Scheme 1C).

ACCase is a cysteine amidase belonging to the N-terminal nucleophile (NTN) hydrolase family and catalyzes the hydrolysis of ceramide to sphingosine and free fatty acids, which is the last step in the lysosomal degradation of (glycol)sphingolipids. Deficiency in ACCase causes the lysosomal storage disorder Farber disease.^[19] In another lysosomal storage disease, Gaucher Disease, a deficiency in glucocerebrosidase leads to an accumulation in glucosylceramide which is a potential substrate of ACCase. Intriguingly, a fluorescent carmofur probe has been used to quantify ACCase levels in human tissue extracts, thus revealing higher levels of ACCase activity in splenic tissues from Gaucher disease patients compared to tissues from a healthy control group.^[9]

Interestingly, carmofur and its parent drug 5-FU have been shown to exhibit general antimicrobial as well as antibiofilm activity against various bacteria including methicillin-resistant *S. aureus* (MRSA).^[20,21] The mechanism behind the antimicrobial activity of 5-FU in bacteria is not well understood, but believed to be connected to inhibition of thymidylate synthase and a resulting blockade of DNA synthesis.^[22] The anti-biofilm and virulence-attenuating activities in several species have been

connected with competition with regulatory functions of uracil^[23] and interference with quorum sensing.^[24,25] The mechanism underlying the antimicrobial activity of carmofur has not been investigated. We hypothesized that, in analogy to the contribution of ACCase inhibition to the therapeutic effect of carmofur in human, microbial enzymes targeted by carmofur might directly contribute to the antimicrobial and anti-biofilm effects of the drug and/or contribute to the bioconversion of carmofur to 5-FU.

In this study, we therefore applied carmofur-derived ABPs to profile enzymatic targets of this drug in MRSA. Because members of the NTN hydrolases family, which carmofur targets in human, are poorly characterized in bacteria, we hypothesized that this approach would be suitable to identify novel enzymes of this family with relevant biological functions. Our study revealed that, in addition to NTN hydrolases, carmofur interacts with a diversity of bacterial enzymes, particularly serine hydrolases. We demonstrate that lack of several individual target enzymes does not affect the susceptibility of *S. aureus* to carmofur and propose that these enzymes collectively contribute to the bioconversion of this drug to 5-FU and might also play a role for its anti-biofilm activity. Our study also has important implications regarding the use of carmofur in the

clinic, since it suggests that the drug might inactivate a similarly broad range of enzymes in humans as well as the human gut microbiome, which may affect the therapeutic outcome and cause side-effects.

Results and Discussion

Gel-based activity-based protein profiling by using a fluorescent carmofur probe

We conducted initial ABPP studies with the fluorescent carmofur-derived ABP (1) on live *S. aureus* cells (clinically relevant methicillin-resistant strain USA300 JE2) that were grown under biofilm-promoting growth conditions on tryptic soy agar supplemented with 100 mM MgCl₂ (TSAMg) in analogy to previous studies.^[6] After labeling, cells were lysed and labeled proteins resolved by SDS-PAGE analysis and visualized by in-gel fluorescence scanning. We observed that the carmofur probe indeed led to dose-dependent labeling of certain *S. aureus* proteins (Figure 1). Two species corresponding to a molecular weight of >52 kDa were most potently labeled (with bands apparent already at a probe concentration of 100 nM). Several additional bands with a molecular weight of around 31, 38, 54, 58, and 76 kDa, respectively, were labeled at 1 μM (Figure 1A). Since bacteria are expected to produce different enzymes depending on their growth environment, we compared the labeling profile of bacteria grown on TSAMg with that of bacteria grown to stationary phase in liquid culture using a standard rich cultivation medium (tryptic soy broth, TSB; Figure 1B). To differentiate secreted proteins that accumulate over the course of liquid cultivation from cell-associated

proteins, we separated cells from the culture media that contains secreted proteins by centrifugation for differential analysis. We observed three dominant bands in the culture supernatant, whereas several proteins were detected in the cell pellet (Figure 1B).

To address whether the observed labeling profile achieved under biofilm-promoting conditions is the result of specific active-site directed interactions, we investigated if pre-incubation with the unlabeled parent inhibitor carmofur could block labeling of the fluorescent carmofur probe 1 in a competitive ABPP setup. Indeed, pre-incubation with carmofur (3–30 μM) led to reduced labeling of three of the most pronounced bands at 31, 38, and 54 kDa, suggesting that carmofur can compete with ABP (1). In contrast, pre-incubation with 5-FU, which is not expected to irreversibly interact with any of the targets, did not lead to alterations in the ABP-labeling patterns up to a concentration of 30 μM.

We suspected that the alterations in the labeling profile at high concentrations of 5-FU might be due to nonspecific effects related to its antimicrobial activity rather than competition labeling. We therefore determined the Minimum inhibitory concentrations (MICs) of the inhibitors carmofur and 5-FU against *S. aureus* USA300 JE2, which were both 5 μM. Thus, specific competition of carmofur with the targets of 1 becomes evident at sub-MIC concentrations, supporting the specific nature of these interactions. For 5-FU, in contrast, the changes in the labeling profile are seen at concentrations six times higher than its MIC (Figure 1C) and therefore most likely result nonspecifically from its antimicrobial activity rather than from specific competition with the probe.

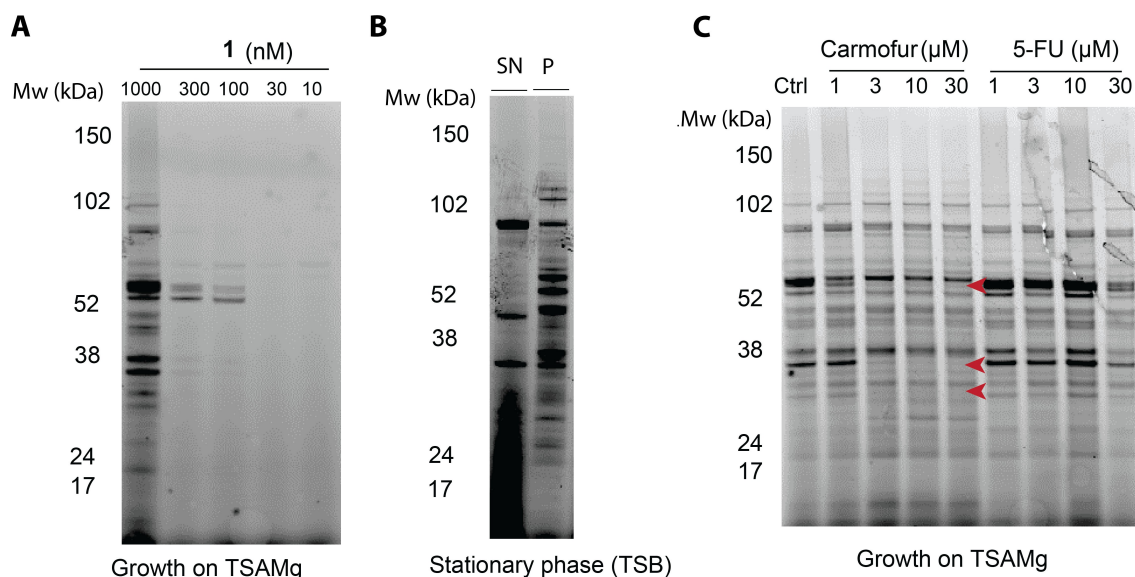


Figure 1. Fluorescent ABP-labeling profiles of *S. aureus* USA300 JE2 with carmofur probe 1. Live bacteria were labeled with different concentrations of probe 1 for 60 min at 37 °C before cells were lysed, and samples were subjected to SDS-PAGE analysis. All graphs show fluorescent scans in the Cy2 (488 nm) channel on an Amersham™ Typhoon™ 5 (Cytiva). A) Dose-dependent labeling profile of *S. aureus* USA300 JE2 cells harvested from TSAMg with probe 1 and B) labeling profile of *S. aureus* USA300 JE2 cell fractions from the stationary phase in liquid culture with probe 1 (1 μM); SN: culture supernatant, P: cell pellet. C) Competitive ABPP. Cells were pre-incubated with carmofur or 5-FU at the indicated concentrations for 60 min before the addition of probe 1 (1 μM). Arrowheads indicate bands with consistently reduced labeling after pre-incubation with carmofur.

Target Identification

A limitation of the gel-based approach using fluorescent ABPs is that only the most abundant targets are detected and that their identity remains undefined. To overcome these shortcomings, we switched to using the biotin-tagged probe (2) in combination with a chemoproteomic workflow. It should be noted, however, that also this approach focuses on covalently bound targets and less stable, reversible interactions may be overlooked. In brief, live bacteria were grown under biofilm-promoting conditions on TSAMg before labeling with the biotin-tagged ABP (2) or carmofur as a control, lysed, and enriched for biotinylated proteins using a streptavidin resin. Samples were then analyzed by LC-MS/MS. Although the probe was used at 2 μ M, which is lower than the MIC of carmofur (5 μ M), we reasoned that its biological effects may still induce nonspecific changes to the general proteome that could translate to changes in the enrichment and detection of nonspecifically enriched “background” proteins in the probe-treated versus untreated chemoproteomic dataset. To account for such false positives, we decided to treat the unlabeled control sample with an equal concentration of carmofur. Treatment with carmofur-biotin (2) resulted in significant enrichment (p value < 0.05, enrichment > 1.5-fold) of 23 proteins compared to the carmofur-treated control dataset (Supporting Dataset 1, data are available from ProteomeXchange with identifier PXD043275). These 23 enriched proteins included 20 putative enzymes with diverse annotations (12 hydrolases or transferases, three oxidoreductases, three lyases, one isomerase, one putative chaperone-like protein; Table 1) as well as three non-enzymatic proteins that are likely false positives.

We identified one enzyme with structural similarity to the NTN hydrolase family,^[29,30] the multifunctional PurH.^[26] Featuring inosine 5'-monophosphate (IMP) cyclohydrolase and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) formyltransferase domains, this enzyme has a dual role in de novo purine synthesis.^[26] Canonical members of the NTN hydrolase superfamily undergo self-processing to eliminate an N-terminal polypeptide, resulting in the active enzyme with a Ser, Thr or Cys nucleophile at the new N-terminus.^[27,30] The bifunctional PurH however possesses an NTN-like fold, but the putative binding site with the corresponding nucleophile responsible for covalent binding to the carmofur probe remains to be identified.

The enzyme family for which the highest number of members were enriched by the carmofur probe 2 were serine hydrolases. This includes 3 α,β -hydrolases, the fluorophosphate-binding hydrolases (FphB, FphC, and FphF) that we have described recently and which share a conserved Ser-His-Asp catalytic triad with serine as the active site nucleophile.^[8,28] We also identified additional hydrolases/transferases that are annotated with a serine in the active site: the putative O-acetyltransferase Oat with an annotated SGNH-hydrolase-type esterase domain and the teichoic acid D-Ala esterase FmtA, which harbors a β -lactamase domain with a conserved catalytic triad of Ser-Lys-Asp. FmtA catalyzes the hydrolytic removal of D-

alanine esters on wall teichoic acid (WTA),^[29,30] thereby regulating charge and integrity of the bacterial cell wall.

Among these serine hydrolase targets of carmofur, the only enzyme for which a substrate-bound crystal structure is available that could give indications on the molecular basis of binding of carmofur, is FphF.^[28] FphF is a serine carboxylesterase with a broad substrate-selectivity profile against synthetic fluorogenic substrates. The enzyme had peak activity against a heptanoate-based substrate and the crystal structure showed that the C7 acyl chain fully occupied the hydrophobic acyl binding pocket of FphF.^[28] We propose that carmofur binding leads to carbamylation of the active site serine and assume that the hexyl-carbamoyl group of carmofur, which has a similar chain length to the C7 acyl of the preferred substrate, fits well into the acyl binding pocket. Since the partially open architecture of the acyl binding pocket of FphF can also accommodate substrates with longer acyl chains (although with poorer fits in docking studies) with terminal atoms pointing out of the pocket,^[28] this provides a molecular basis for binding of the longer carmofur-derived probes. The observed promiscuity and reactivity toward serine hydrolases concurs with a recent report identifying carmofur as an inhibitor of another N-terminal cysteine hydrolase (*N*-acylethanolamine acid hydrolase), as well as of a human serine hydrolase (fatty acid amide hydrolase).^[31] We therefore conclude that the carbamoyl-5-fluorouracil electrophile elicits a similar reactivity profile to carbamates, which are known inhibitors of serine hydrolases.^[32]

The molecular basis and specificity of the interactions of the carmofur probes with the diverse other enriched enzymes such as lyases, oxidoreductases or metal-dependent hydrolases with in part unknown or even without predicted active site nucleophiles (as indicated in Table 1), remains to be determined.

Gel-based target validation

To validate the chemoproteomic results and to assign the bands identified in gel-based ABPP, we performed gel-based labeling studies using transposon mutants deficient in targets identified by MS, with a focus on the NTN and serine hydrolases. As we detected several Fph enzymes in the MS dataset, we first tested the entire panel of Fph A–H mutants and the secreted lipases SAL1 and –2 which was available from previous studies^[8] in strain USA300 LAC. This allowed for a clear assignment of SAL2, FphB, FphE, FphF as labeled targets of the carmofur probe 1 (Figure 2A). Among the α,β -hydrolases some differences were apparent in the gel-based and MS-based studies: FphC was not detected by the gel-based approach, whereas SAL2 and FphE were detected on the gel, but not by MS. These differences could result from differences in permeability and activity of the fluorescent versus biotinylated probe. In the case of FphC this might also be due to limited resolution and lower sensitivity of gel-based ABPP. Interestingly, α,β -hydrolases also accounted for all fluorescently labeled bands that were detected after growth in liquid culture. SAL2 was found as the two dominant bands in the supernatant at around

Table 1. Target enzymes of carmofur probe (2) in *S. aureus* USA300 JE2.

Accession no.	Protein	Description	Active-site nucleophile/putative site of attachment	Gene name	Unique peptides	Mol weight [kDa]	Abundance ratio: biotin/ctrl	Abundance ratio p value
QPB88705.1	FphF	carboxylesterase ^[28]	Ser	<i>estA/fphF</i> ^[8,28]	2	29.1	100.0	1×10 ⁻¹⁷
WP_001146763.1	AhpD	alkyl hydroperoxide reductase	Cys	<i>ahpD</i>	5	16.5	10.0	7.34368×10 ⁻¹²
QPB88396.1	YndB	SRPBCC domain-containing protein	unknown	<i>YndB</i>	2	20.1	5.8	0.0009
WP_001184005.1	AcuC	acetoin utilization protein	Lys	<i>acuC</i>	5	44.6	4.7	0.0036
WP_000379821.1	OatA	acetyltransferase	Ser	<i>oatA</i>	7	69.1	2.9	0.0008
QPB87247.1	PyrF	orotidine-5'-phosphate decarboxylase	Lys	<i>pyrF</i>	7	25.6	2.8	0.0257
WP_000058383.1	MaeB	NAD-dependent malic enzyme 4	Lys	<i>maeB</i>	3	44.2	2.6	0.0108
QPB88680.1	BetB	betaine-aldehyde dehydrogenase	Cys	<i>betB</i>	3	54.6	2.2	0.0132
QPB86902.1	Eno	phosphopyruvate hydratase	Lys, Glu	<i>eno</i>	6	47.1	1.9	0.0243
QPB88514.1	ApbA	2-dehydropantoate 2-reductase	Lys, Glu	<i>apbA</i>	4	34.4	1.9	0.0322
WP_001178942.1	CshA	DEAD/DEAH box helicase	unknown	<i>cshA</i>	24	56.9	1.9	0.0099
QPB87344.1	FphC	hydrolase, α/β hydrolase fold family	Ser	SAUSA300_1194/ <i>fphC</i> ^[8]	9	35.2	1.7	0.0023
WP_001248939.1	YvcK	YvcK family protein	unknown	<i>yvcK</i>	5	36.2	1.7	0.0341
QPB87084.1	GNAT	GNAT family N-acetyltransferase	unknown	SAUSA300_0943	6	21.3	1.7	0.0294
QPB87049.1	YjbK	CYTH domain-containing protein	unknown (metal-dependent)	<i>yjbK</i>	2	23.4	1.7	0.0326
QPB86868.1	PepT	peptidase T	unknown (metal-dependent)	<i>pepT</i>	12	45.8	1.6	0.0224
WP_001281145.1	Alr	alanine racemase	Lys	<i>alr</i>	3	42.8	1.6	0.0105
QPB88602.1	FphB	Carboxylesterase ^[8]	Ser	SAUSA300_2473/ <i>fphB</i> ^[8]	10	36.8	1.5	0.0388
QPB87101.1	FmtA	teichoic acid D-Ala esterase	Ser	<i>fmtA</i>	3	46	1.5	0.0390
QPB87118.1	PurH	IMP cyclohydrolase	Ser, Thr or Cys	<i>purH</i>	26	54.3	1.5	0.0114

76 and >38 kDa (Figure 2B). SAL1 was associated to the cell pellet and appeared as diverse species of various molecular weight (Figure 2C). The only relevant remaining band in both fractions was assigned to the 31 kDa FphE (Figure 2B, C).

To account for the unidentified bands that can be detected in cells grown on TSAMg, we tested additional JE2-based mutants from the Nebraska Transposon Mutant Library (Figure 2D).^[33] The *purH* transposon mutant, showed an altered labeling profile where several bands around and below the expected size of 54 kDa are missing (Figure 2D). It remains to be determined if the bands in question are all different fragments of PurH or if some of these changes may be attributed to secondary effects of the knock-out (Figure 2D). Certain ambiguity also remains regarding identification of the

46 kDa FmtA on the gel, as a band of this size was clearly reduced, but not absent (Figure 2D). In conclusion, these data confirm that the majority of the targets of the fluorescent carmofur probe in gel-based ABPP are indeed serine hydrolases.

Transposon mutations in abundant carmofur targets do not affect antimicrobial susceptibility to carmofur

Because the mechanism underlying the antimicrobial activities of carmofur is not properly documented, we aimed to determine if the enzymes enriched by the carmofur probe are relevant for the susceptibility to carmofur. Carmofur-binding to these targets could contribute to the antimicrobial effect

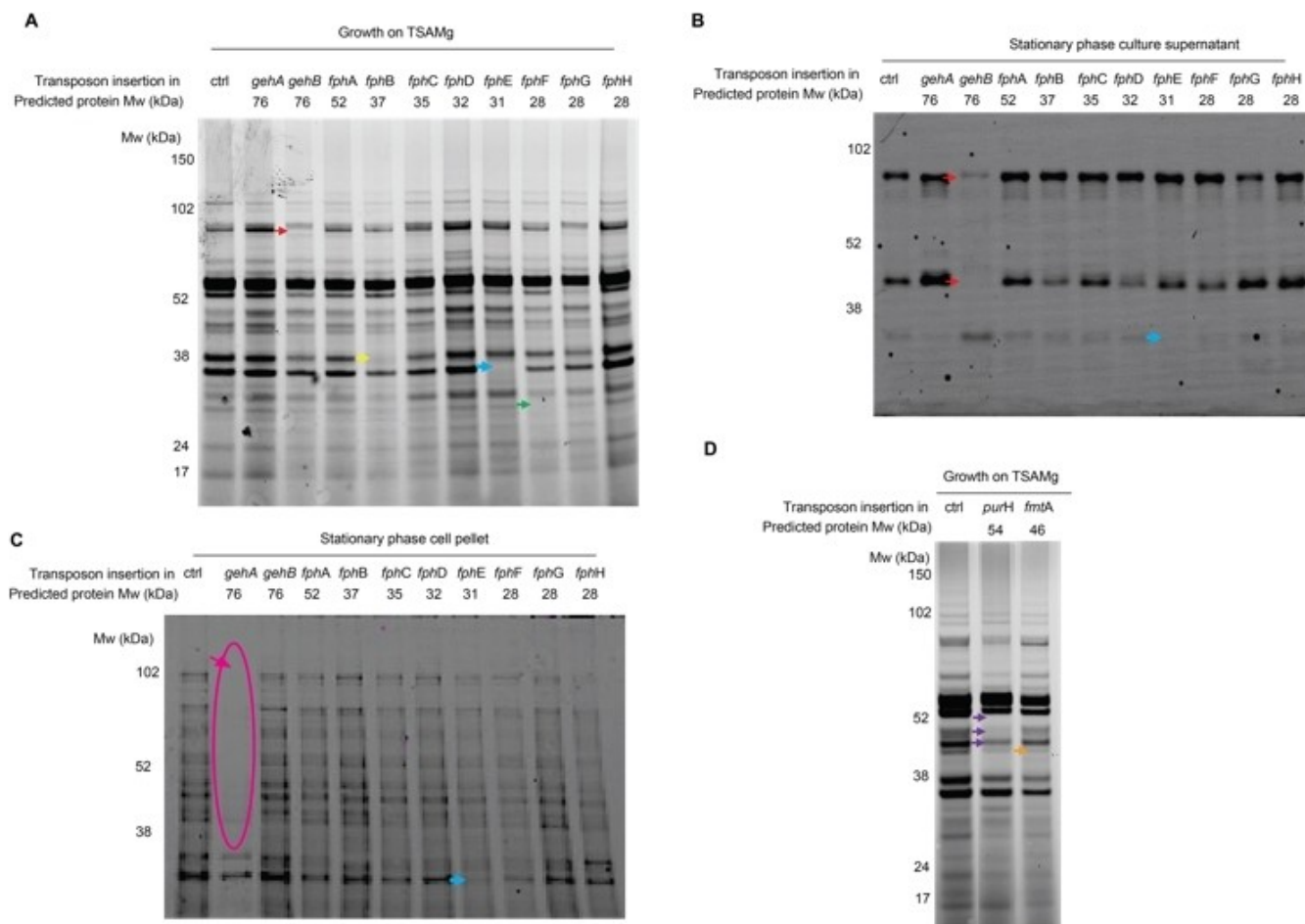


Figure 2. Fluorescent carmofur probe 1 labeling profiles of *S. aureus* USA 300 LAC transposon mutant strains with insertions of fluorophosphonate-binding hydrolases (Fphs) A–H and the secreted lipases SAL1 and –2 genes. The cells were labeled under two different conditions: A) cells grown in TSAMg and cells in stationary phase culture in TSB, fractionated into B) supernatant (SN) and C) cell pellet (P). D) Labeling profiles of *S. aureus* USA 300 JE2 transposon mutant strains with insertions of purine biosynthesis protein (*purH*) and teichoic acid D-Ala esterase (*FmtA*) genes. The labeled proteins disappearing in individual mutant strains are indicated by arrowheads.

directly (through target inhibition), but this is unlikely since none of the targets are essential. Alternatively, carmofur-binding can affect the antimicrobial activity indirectly by means of releasing 5-FU (Scheme 1D). Assuming that both the carmofur probes 1 and 2 and their parent drug lead to carbamylation of the active site nucleophile (similar to ACase^[9,18]) and the mode of action of carbamates on serine proteases,^[32] these events will lead to release of 5-FU. We reasoned that, if activation of the prodrug was required, this could be reflected by slower bacterial killing by carmofur compared to 5-FU.

However, time-kill curve analysis revealed no differences in the kinetics of bactericidal effects elicited by 5-FU and carmofur (Figure 3). This suggests that either no conversion of carmofur to 5-FU is required, or more likely, that this conversion occurs very quickly. Unfortunately, there are – to the best of our knowledge – no available data on the biotransformation kinetics of carmofur to 5-FU in either bacteria, mammalian cells, humans, or animals that could help put these results into perspective.

We proceeded to determine the antimicrobial susceptibility of transposon mutant strains deficient in probe targets SAL1,

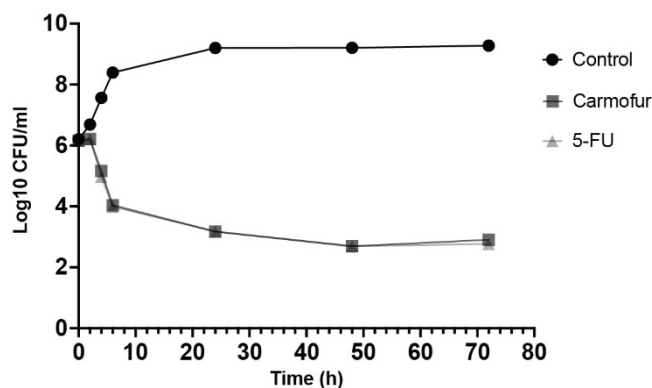


Figure 3. Time-kill curve analysis of carmofur and 5-FU. Stationary-phase *S. aureus* USA300 JE2 were treated with 4x the MIC of carmofur and 5-FU at 37 °C for 72 h. At various timepoints, aliquots were analyzed for CFU determination.

SAL2, FphB, FphC, FphE, FphF, PurH, or FmtA and their corresponding WT strains LAC or JE2 by broth microdilution testing. All strains showed an identical MIC of 5 μ M. Our data

suggest that the activity of these enzymes individually does not affect the bactericidal activity of carmofur. Because the carmofur probe interacts promiscuously with a wealth of targets, it could be expected that they contribute collectively to the conversion of carmofur to 5-FU and that hence single knock-outs have only limited effects. It is also possible that other bacterial enzymes can hydrolyze carmofur to release 5-FU without being inactivated and labeled by the carmofur probes.

In contrast to the bactericidal activity, it seems plausible that inactivation of some of the identified enzymes may contribute to the anti-biofilm activities of carmofur.^[21] PurH, for example, has already been attributed an important role in biofilm formation^[34] and a putative involvement of other targets of the carmofur probe in biofilm formation merits further investigations.

Conclusions

This study has revealed the surprisingly broad reactivity profile of carmofur- and activity-based probes in the bacterial pathogen *S. aureus* and revealed the activity of a number of previously uncharacterized enzymes under biofilm-promoting conditions that merit further functional characterization. In addition to one expected member of the NTN hydrolase family, the IMP cyclohydrolase PurH, carmofur probes showed a similar reactivity to carbamates, in that they were able to interact with a number α,β -hydrolases and other serine hydrolases. Future studies might alter the electrophilic *N*-carbamoyl-5-fluorouracil scaffold in carmofur in order to generate more selective probes or inhibitors for some of the underexplored enzyme families targeted. In light of the clinical use of carmofur as a drug in colorectal cancer (as reviewed in ref. [13]) it must be considered that this drug might exhibit a similarly broad reactivity profile against human enzymes that could be potentially be related to both therapeutic and side effects. It is also plausible that carmofur reacts promiscuously with enzymes from human-associated microbiota. Considering the emerging knowledge of the impact gut microbiota have on human physiology, microbial (off-)targets could even be implicated in the mechanisms leading to its therapeutic effects. A prominent study has reported the impact of non-antibiotic drugs, including 5-FU, on human gut microbiota, even though the study concluded that the body concentration of 5-FU (in plasma) is lower than the concentration that reduces growth of at least one commensal bacteria by 25%.^[35] However, in contrast to 5-FU, which is administered intravenously, carmofur is administered orally, thus making it very plausible that treatment leads to a concentration of carmofur in the human gut that is high enough to affect microbial enzymes.

Experimental Section

Bacterial strains and culture conditions: This work used *S. aureus* strains USA300 LAC, USA300 JE2 and their isogenic mutants as summarized in Table 2. All strains were routinely cultured on tryptic soy agar (TSA) or TSA with 100 mM MgCl₂ (TSAMg) or Difco tryptic

Table 2. Bacterial strains used in this study.

Strain	Description	Ref./ source
<i>S. aureus</i> USA300 LAC	wild-type USA300 Los Angeles County (LAC) clone; multilocus sequence type 8, SCCmec type IV cured of antibiotic resistance plasmid	[41]
LAC_gehA: Tn	transposon insertion mutant in LAC SAU-SA300_2603 (SAL1); Ery ^R , Linc ^R	[8]
LAC_gehB: Tn	transposon insertion mutant in LAC SAU-SA300_0320 (SAL2); Ery ^R , Linc ^R	[8]
LAC_fphA: Tn	transposon insertion mutant in LAC SAU-SA300_2396 (<i>fphA</i>); Ery ^R , Linc ^R	[8]
LAC_fphB: Tn	transposon insertion mutant in LAC SAU-SA300_2473 (<i>fphB</i>); Ery ^R , Linc ^R	[8]
LAC_fphC: Tn	transposon insertion mutant in LAC SAU-SA300_1194 (<i>fphC</i>); Ery ^R , Linc ^R	[8]
LAC_fphD: Tn	transposon insertion mutant in LAC SAU-SA300_2148 (<i>fphD</i>); Ery ^R , Linc ^R	[8]
LAC_fphE: Tn	transposon insertion mutant in LAC SAU-SA300_2518 (<i>fphE</i>); Ery ^R , Linc ^R	[8]
LAC_fphF: Tn	transposon insertion mutant in LAC SAU-SA300_2564 (<i>fphF</i>); Ery ^R , Linc ^R	[8]
LAC_fphG: Tn	transposon insertion mutant in LAC SAU-SA300_1733 (<i>fphG</i>); Ery ^R , Linc ^R	[8]
LAC_fphH: Tn	transposon insertion mutant in LAC SAU-SA300_0763 (<i>fphH</i>); Ery ^R , Linc ^R	[8]
<i>S. aureus</i> USA300 JE2	a plasmid-cured derivative of USA300 LAC and Parent strain of Nebraska Transposon Mutant Library	[33]
JE2_purH: Tn	transposon insertion mutant in JE2 SAU-SA300_0975 (<i>purH</i>); Ery ^R , Linc ^R	[33]
JE2_fmtA: Tn	transposon insertion mutant in JE2 SAU-SA300_0959 (<i>fmtA</i>); Ery ^R , Linc ^R	[33]

soy broth (TSB). All bacterial strains were incubated at 37 °C and liquid cultures were aeriated by shaking at 180 rpm unless indicated otherwise.

Probes and inhibitors: The carmofur probes 1 and 2 were available from a previous study and their synthesis had been described.^[9] Carmofur and 5-FU were purchased from Sigma.

Labeling with fluorescent ABP (1): After overnight growth on TSAMg plate or in liquid culture as indicated, bacteria were suspended to the desired density in TSB and added to microtubes in a final volume of 50–100 μ L. For competitive ABPP experiments, the inhibitors (carmofur and 5-FU) were added from 100x-concentrated stock solutions in DMSO and pre-incubated with the cells for 60 min (37 °C, 300 rpm) prior to ABP-labeling. Compound 1 (1 μ M) was added from a 100x stock solution in DMSO and cells were incubated for 60 min, at 37 °C, 300 rpm. After probe labeling, bacterial suspensions were transferred to 2 mL screw-cap tube filled with 30–50 μ L of 4x SDS-Loading buffer and ca. 60–100 μ L of 0.1 mm glass beads and lysed by bead-beating.

SDS-PAGE analysis of fluorescently labeled proteins: After adding the 4x SDS sample buffer (40% glycerol, 240 mM Tris-HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 5% β -mercaptoethanol), lysates of probe-labeled bacteria were boiled at 95 °C for 10 min and separated by SDS-PAGE gel. The gels were scanned for fluorescence in the Cy2 (488 nm) channel on a Amersham™ Typhoon™ 5 (cytiva).

Labeling with biotinylated probes and sample preparation for mass spectrometry: *S. aureus* USA300 JE2 cultures were grown on TSAMg for 24 h and resuspended to an OD₆₀₀ ~20 in 3 mL TSB. For each biological replicate, 1 mL aliquots were transferred to a 1.5 mL tube and either carmofur–biotin (probe 2; 2 μM) or an equal concentration of carmofur (as a control for nonspecific biological effects of the probe) were added, and cells were incubated for 60 min at 37 °C, 700 rpm before samples were spun down at 4500 g for 5 min at 4 °C, and the supernatant was aspirated. The cell pellets were resuspended in 1.2 mL RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) and lysed by bead-beating. Samples were centrifuged for 5 min at 10000 g at 4 °C. Protein concentration in the supernatant was adjusted to 1.0 mg mL⁻¹. Proteins were stored at -20 °C prior to sample preparation. For each sample, 50 μL streptavidin magnetic beads were washed twice with 1 mL RIPA lysis buffer and incubated with 1 mg protein from each sample with an additional 500 μL RIPA lysis buffer at 4 °C for overnight at 18 RPM rotator. After enrichment, beads were pelleted using a magnetic rack, and washed twice with RIPA lysis buffer (1 mL, 2 min at RT), once with 1 M KCl (1 mL, 2 min at RT), once with 0.1 M Na₂CO₃ (1 mL, ~10 s), once with 2 M urea in 10 mM Tris-HCl (pH 8.0; 1 mL, ~10 s), and twice with RIPA lysis buffer (1 mL per wash, 2 min at RT). After the final wash, the beads were transferred in 1 mL RIPA lysis buffer to fresh protein Lo-Bind tubes. Then, beads were washed three times in 500 μL 2 M/4 M urea, 50 mM ammonium bicarbonate (Ambic) with shaking for 7 min. Finally, beads were washed 3x with 500 μL of 50 mM Ambic with shaking for 7 min, each time transferring the samples to a new tube in-between washes. For on-bead digestion, 150 μL 50 mM Ambic, 3 μL 1 mM CaCl₂, 0.75 μL 1 M DDT, 4.5 μL 500 mM IAA and 6 μL MS grade trypsin solution were added and samples were incubated at 37 °C overnight at 800 rpm. Tryptic peptide digests were separated, and beads were washed with 70 μL 50 mM Ambic. For each sample, to the combined eluates received 20 μL of formic acid were added, and the samples were kept at -20 °C until analysis via LC-MS/MS.

LC-MS analysis: Varian's OMIX C18 tips were employed to perform sample cleanup and concentration. Peptide mixtures that comprised 0.1% formic acid were loaded onto a Thermo Fisher Scientific EASY-nLC1200 system (C₁₈, 2 μm, 100 Å, 50 μm, 50 cm) and subjected to fractionation by using a 5–80% acetonitrile gradient in 0.1% formic acid at a flow rate of 300 nL min⁻¹ for a duration of 60 min. The peptides that were separated were examined using a Thermo Scientific Orbitrap Exploris 480. Data was acquired in a data-dependent mode with the aid of a Top20 method. The raw data were processed by using the Proteome Discoverer 2.5 software, and the fragmentation spectra were searched against (*S. aureus* 300 LAC). Peptide mass tolerances of 10 ppm and a fragment mass tolerance of 0.02 Da were employed during the search. Peptide ions were filtered using a false discovery rate (FDR) set at 5% for peptide identifications. To ensure accuracy, the filter criterion of two unique peptides was used, and three replicates were conducted for all samples. The protein abundance obtained from Proteome Discoverer was averaged across replicates, and the ratio of the ABPP-enriched sample versus the control tryptic digestion sample was calculated. Proteins enriched more than 1.5-fold by the biotinylated probe were selected. ABPP-enriched and other pull-down experiments are significantly limited by nonspecific binding.^[36,37] The enrichment of these nonspecific proteins may be due to various reasons, such as naturally biotinylated proteins (e.g., carboxylase family proteins) and ribosomal proteins being enriched by streptavidin beads, as well as proteins with an affinity for hydrophilic beads being enriched. However, proteins exhibiting the latter two types of nonspecific binding were excluded. The mass spectrometry proteomics data

have been deposited to the ProteomeXchange Consortium via the PRIDE^[38] partner repository with the dataset identifier PXD043275.

Antimicrobial activity testing: To determine the MIC, stock solutions of carmofur and 5-Fluorouracil were prepared at a concentration of 200 μM in sterile distilled water. These solutions were then diluted 1:2 in 96-well microtiter plates and inoculated with *S. aureus* strains at approximately 5 × 10⁵ CFU mL⁻¹ in 100 μL. The 96-well microtiter plates were incubated overnight (18 h) to determine the MIC.^[39]

Time-kill assay of carmofur/5-fluorouracil: A time-kill study was performed according to the Clinical and Laboratory Standards Institute guidelines following a method previously described.^[40] In brief, 4 × MIC (20 μM) of carmofur/5-fluorouracil was used to detect differences in time-dependent killing. Overnight cultures of *S. aureus* USA 300 JE2 were grown in Mueller–Hinton (MH) broth at 37 °C until the exponential phase was reached, indicated by OD₆₀₀ = 0.4. The cells were then diluted to yield a final concentration of approximately 2 × 10⁶ cells per mL and transferred to a 50 mL tube containing 20 mL of MH broth, followed by the addition of appropriate concentrations of carmofur and 5-fluorouracil. Tubes were then incubated at 37 °C, and aliquots were removed at 0, 2, 4, 6, 24, 48, and 72 h for the determination of viable counts. Serial dilutions were prepared in sterile PBS and plated according to the method previously described. Colonies were counted after incubation of TSA plates at 37 °C for 16–22 h, with a detection level of 1 × 10² CFU mL⁻¹.

Supporting Information

The authors have included Supporting Dataset 1 in the Supporting Information.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD043275.

Keywords: *S. aureus* · activity-based protein profiling · biofilm carmofur · 5-fluorouracil

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Supplementary Dataset 1. Target proteins of carmofur-probe (2) in *S.aureus* USA300 JE2

#	Accession	Protein	Description	Protein family	Protein family/molecular function	active site nucleophile	Gene Name	Mol weight [kDa]	Unique Peptides	Abundance: biotin R1	Abundance: biotin R2	Abundance: biotin R3	Abundance: ctrl R1	Abundance: ctrl R2	Abundance: ctrl R3	Average Abundance: biotin	Average Abundance: Ctrl	Abundance Ratio: (biotin) / (ctrl)	Abundance Ratio Value	P-
1	QPB88705.1	FphF	Carboxylesterase ³¹	α/β hydrolase	Hydrolase, α/β hydrolase fold family	Ser	<i>estA/fphF</i> ^{8, 31}	29.1	2	66473.3	217223.1	435598.7				239765.0	1.0	100.0	1E-17	
2	WP_001146763.1	AhpD	Alkyl hydroperoxide reductase	Oxidoreductase	Peroxidase, Acting on a peroxide as acceptor	Cys	<i>ahpD</i>	16.5	5	1028771.2	1722907.3	1699798.8	114322.8	126236.9	203441.4	1483825.7	148000.3	10.0	7.344E-12	
3	QPB88396.1	YndB	SRPBCC domain-containing protein	Chaperone-like protein	Activator of Hsp90 ATPase homolog 1-like protein	Unknown	<i>YndB</i>	20.1	2	210257.2	178770.2	243453.0	38925.9	44698.6	26171.6	210826.8	36598.7	5.8	0.0008609	
4	WP_001184005.1	AcuC	acetoin utilization protein	Hydrolases	Hydrolases, Acting on carbon-nitrogen bonds, other than peptide bonds	Lys	<i>acuC</i>	44.6	5	938998.9	726315.4	1069997.0	133308.7	137501.2	315150.5	911770.4	195320.1	4.7	0.0035692	
5	WP_000379821.1	OatA	acetyltransferase	SGNH_hydrolase	SGNH_hydrolase_yrhL_like, the active site resembles the typical Ser-His-Asp(Glu) triad from other serine hydrolases	Ser	<i>oatA</i>	69.1	7	701670.5	276402.8	985381.2	278818.1	213157.6	189722.7	654484.8	227232.8	2.9	0.000793	
6	QPB87247.1	PyrF	orotidine-5'-phosphate decarboxylase	Lyase	Lyase, carbon-oxygen lyases	Lys	<i>pyrF</i>	25.6	7	2596459.3	1516035.2	2944252.0	951521.4	835070.6	765111.4	2352248.8	850567.8	2.8	0.025706	
7	WP_000728763.1	SpA	staphylococcal protein A	Non-enzymatic	Gram-positive cocci surface proteins LPxTG/LYSM	none	<i>spa</i>	55.5	12	4095135.2	5092316.0	13947671.5	2990051.9	3767970.5	1833766.1	7711707.6	2863929.5	2.7	0.001222	
8	WP_000058383.1	MaeB	NAD-dependent malic enzyme 4	Lyase	Decarboxylase	Lys	<i>maeB</i>	44.2	3	213673.0	120221.7	572089.0	181421.5	83256.0	82236.6	301994.6	115638.0	2.6	0.0108281	
9	QPB88680.1	BetB	betaine-aldehyde dehydrogenase	Oxidoreductase	Oxidoreductase, Acting on the aldehyde or oxo group of donors	Cys	<i>betB</i>	54.6	3	275429.1	207907.1	404351.6	209148.1	112809.0	82387.1	295896.0	134781.4	2.2	0.013202	
10	QPB86902.1	Eno	phosphopyruvate hydratase	Lyase	Lyase, carbon-oxygen lyases	Lys, Glu	<i>eno</i>	47.1	6	1371007.7	602889.0	1277412.9	760894.4	404141.2	509625.1	1083769.9	558220.2	1.9	0.0243337	
11	QPB88514.1	ApbA	2-dehydropanoate 2-reductase	Oxidoreductase	Oxidoreductase, acting on the CH-OH group of donors	Lys, Glu	<i>apbA</i>	34.4	4	993749.9	353182.7	1121246.3	361368.0	577090.9	374541.9	822726.3	437666.9	1.9	0.0322089	
12	WP_001178942.1	CshA	DEAD/DEAH box helicase	Hydrolase	Hydrolase, acting on acid anhydrides	water/unknown	<i>cshA</i>	56.9	24	36539823.0	17762673.2	44969009.0	19276498.6	19577795.4	14422330.6	33090501.7	17758874.9	1.9	0.0098863	
13	QPB87344.1	FphC	hydrolase, α/β hydrolase fold family	α/β hydrolase	Hydrolase, α/β hydrolase fold family	Ser	<i>SAUSA300_1194 / fphC⁸</i>	35.2	9	2149048.0	1862392.8	1768078.3	1063110.4	1166949.5	1082514.6	1926506.4	1104191.5	1.7	0.0022939	
14	QPB87474.1	CvfC	virulence factor C	Non-enzymatic	Contributes to the production of a protease and nuclease via an agr-independent pathway	none	<i>cvfC</i>	43	3	269888.6		359833.3	184058.5	180657.3		314860.9	182357.9	1.7	0.0018011	
15	WP_001248939.1	YvcK	YvcK family protein	Transferase	Binds UDP-GlcNAc, a precursor of peptidoglycan and also called called a gluconeogenesis factor	Unknown	<i>yvcK</i>	36.2	5	1799491.0	1241971.9	1581613.1	863095.7	1130954.3	741161.8	1541025.3	911737.3	1.7	0.0340661	
16	QPB87084.1	GNAT	GNAT family N-acetyltransferase	Transferase	Transferases, transferring groups other than amino-acyl groups	Unknown	<i>SAUSA300_0943</i>	21.3	6	2122604.9	1366731.6	2312459.0	762990.7	1681667.7	989260.2	1933931.8	1144639.5	1.7	0.0294157	
17	QPB87049.1	YjbK	CYTH domain-containing protein	Hydrolase	CYTH-like phosphatases 1	metal dependent	<i>yjbK</i>	23.4	2	425156.6	405312.4	94215.8	399540.4	74374.6	82627.2	308228.3	185514.1	1.7	0.0326473	
18	WP_001549158.1	Map	extracellular adherence protein	Non-enzymatic	Extracellular adherence protein	none	<i>Map</i>	65.5	17	11938012.0	10183990.6	15474997.0	7793899.7	10646533.6	4876968.4	12532333.2	7772467.2	1.6	0.0175591	
19	QPB86868.1	PepT	peptidase T	Hydrolase	Hydrolase, acting on peptide bonds	metal dependent	<i>pepT</i>	45.8	12	4730594.9	3647833.0	4817123.6	2212161.6	2996053.9	3007430.6	4398517.2	2738548.7	1.6	0.0224451	
20	WP_001281145.1	Alr	alanine racemase	Isomerase	Isomerases, acting on amino acids and derivatives	Lys	<i>alr</i>	42.8	3	298519.4		303010.3	220528.7	177614.0	174990.9	300764.8	191044.5	1.6	0.010524	
21	QPB88602.1	FphB	Carboxylesterase ⁸	α/β hydrolase	Hydrolase, α/β hydrolase fold family	Ser	<i>SAUSA300_2473 / fphB⁸</i>	36.8	10	9323874.3	8311485.5	9412396.7	7565700.7	5784317.3	4133797.0	9015918.8	5827938.3	1.5	0.0387642	
22	QPB87101.1	FmtA	teichoic acid D-Ala esterase	Hydrolase	Hydrolase, acting on ester bonds	Ser	<i>fmtA</i>	46	3	95695.2	39416.0	151284.2	63980.7	64403.9	60139.4	95465.1	62841.3	1.5	0.0389844	
23	QPB87118.1	PurH	IMP cyclohydrolase	NTN hydrolase	Hydrolase, acting on carbon-nitrogen bonds, other than peptide bond	Cys	<i>purH</i>	54.3	26	94656309.3	96966807.0	111214235.2	78563253.4	61363890.4	64096856.2	100945783.8	68008000.0	1.5	0.0114217	

Paper II

Paper III

