



HAL
open science

Importance of metabolic adaptations in intracellular persistence of *Staphylococcus aureus*

Xin Tan

► **To cite this version:**

Xin Tan. Importance of metabolic adaptations in intracellular persistence of *Staphylococcus aureus*. Microbiology and Parasitology. Université Paris Cité, 2019. English. NNT : 2019UNIP5081 . tel-03975099

HAL Id: tel-03975099

<https://theses.hal.science/tel-03975099>

Submitted on 6 Feb 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



UNIVERSITÉ
PARIS
DESCARTES



Université de Paris

Université de Paris

École doctorale Bio Sorbonne Paris Cité (ED562)

INSERM U1151 – Institut Necker Enfants Malades

Importance of metabolic adaptations in intracellular persistence of *Staphylococcus aureus*

Par Xin TAN

Thèse de doctorat de Science de la vie et de la santé

Spécialité : Microbiologie

Dirigée par Alain CHARBIT

Présentée et soutenue publiquement le 07 Octobre 2019

Devant un jury composé de :

Alain CHARBIT Directeur de recherche, Université Paris Descartes

Directeur de thèse

Anne JAMET MCU-PH, Université Paris Descartes

Co-encadrante de thèse

Florent SEBBANE Directeur de recherche, Institut Pasteur de Lille

Rapporteur

Ina ATTREE Directrice de recherche, Université Grenoble Alpes

Rapporteuse

Karen MOREAU PU, Université Claude Bernard Lyon1

Examinatrice

Vincent CATTOIR PU-PH, Université Rennes 1

Examinateur



Except where otherwise noted, this is work licensed under
<https://creativecommons.org/licenses/by-nc-nd/3.0/fr/>

Unité de Pathogénie des Infections Systémiques, INSERM U1151 Equipe 7

Institut Necker Enfants Malades, Université Paris Descartes

Bâtiment Leriche - Porte 9

14 Rue Maria Helena Vieira Da Silva

75014 Paris

路漫漫其修远兮，吾将上下而求索。

屈原

The way ahead is long, I shall search high and low.

Yuan QU

Never give up on what you really want to do. The person with big dreams is more powerful than the one with all the facts.

Albert EINSTEIN

Acknowledgements

Foremost, I would like to express my sincere gratitude to my supervisor Alain CHARBIT, for his patience, immense knowledge, unflagging support and best efforts to teach me how to be a good researcher. I could not have imagined having a better supervisor for my PhD.

Great thanks to my co-supervisor Anne JAMET. Thank you for your helpful advice and insightful comments needed through my thesis. I greatly appreciate it.

Many thanks to Dr. Mathieu COUREIL, for all the support and interesting ideas.

Many thanks to Pr. Xavier NASSIF, to accept me to work in his laboratory.

Thanks to the financial support from China Scholarship Council and INSERM, without their support, it is impossible to accomplish this work.

I also thank the reporter Dr. Florent SEBBANE and Dr. Ina ATTREE, and examiner Dr. Karen MOREAU and Dr. Vincent CATTOIR, for accepting to evaluate this work.

I want to thank the whole team 11 of INEM, the present and former colleagues, for their amity and for the memorable moments with them. Daniel for all your bad jokes, Fabiola for all your help, especially your talent to organize team building meal, Marion for your kindly help and support, Jason for your gossips sharing, Mathilde for your complain and encouragement, Zoé for your talent of violin, and Julie for the “jeux de société”, Jean-Philippe for your expertise of mice, Elodie for your expertise of “les mouches”. I also thank Monique, Emmanuelle, Hervé and Mohamed, especially for the lunch time discussion. I also thank other team members, Elena, Sophia, Eric, Jean, David, Olivier, Christine, Chantal, Mbia, Gaël, Guillaume, Baptiste, Cécile, Héloïse, Manon, Justine and Clémence. I also thank other people who work in INEM, Zhou Zhicheng for your kindly provided nasal scratch samples, Xiangyan, Ivan, Nicolas and Diana for your technical support. Many thanks to the member of APINEM, Manon and Chantal, for organizing the nice afterwork activity.

I want to thank all my friends during my stay in Paris, Diego, Andrea, Mario and Daniel for your company at APPT 211, Yan Jie for the company of joggings, Xiao Xie for your sharing of academic beers, Lao Luo for your company of climbing, Lao Wang for your stories sharing, Gang Ge for your guide on Alpes, Thérèse for your sunshine smile, also to all the friends, and all the other names that I may forget...

Lastly, I would like to thank all my family, especially my parents, Dayi and Jiejie for all your constant support.

Abstract

The Gram-positive human pathogen *Staphylococcus aureus* is a leading cause of severe bacterial infections. Several studies have shown that various cell types could readily internalize *S. aureus* and infected cells have been proposed to serve as vehicle for the systemic dissemination of the pathogen. The general objective of this thesis was to understand the molecular mechanisms involved in the adaptation of *S. aureus* to its intracellular niche. The experimental data obtained during this thesis are presented under the form of articles.

In the first article, we addressed the intracellular behavior of the *S. aureus* strain USA300 in endothelial cells. Time-lapse microscopy revealed that initial intracellular persistence of wild-type bacteria corresponded to distinct single cell events, ranging from active intracellular bacterial proliferation, leading to cell lysis, to non-replicating bacterial persistence even one week after infection. These findings support the notion that endothelial cells might constitute an intracellular persistence niche responsible for reported relapse of infection after antibiotic therapy.

In the second article, we focused on the metabolic adaptation of *S. aureus* during its intracellular life cycle and in particular the role of a key pentose phosphate pathway (PPP) enzyme, transketolase (TKT). We showed that inactivation of the gene encoding TKT activity in *S. aureus* USA300 (*Atkt*) led to drastic metabolomic changes. Using time-lapse video imaging and mice infection, we observed a major defect of the *Atkt* strain compared to wild-type strain in early intracellular proliferation and in the ability to colonize kidneys. Transcriptional activity of the two master regulators Sigma B and RpiRc was drastically reduced in the *Atkt* mutant during host cells invasion and concomitantly RNAPIII transcription increased, suggesting that TKT -or a functional PPP- influences the ability of *S. aureus* to proliferate within host cells by modulating key transcriptional regulators.

In the third manuscript, we studied the adaptation of *S. aureus* during a chronic infection, in cystic fibrosis (CF) patients. We selected pairs of sequential *S. aureus* isolates from 3 patients with CF. We showed that late *S. aureus* isolates from CF patients had an increased ability for intracellular survival in CF bronchial epithelial-F508del cells compared to ancestral early isolates. The increased ability to persist intracellularly was confirmed for one *S. aureus* late isolate within the own-patient F508del epithelial cells. Proteo-genomic and metabolomics analyses of these isolates led us to identify the underlying genetic modifications that induced altered protein expression profiles and notable metabolic changes.

Altogether our results strongly suggest that the intracellular environment might constitute an important niche of persistence and relapse necessitating adapted antibiotic treatments.

Keywords: *Staphylococcus aureus*, transketolase, metabolic adaptation, Pentose Phosphate Pathway, Sigma B, RpiRc, cystic fibrosis, intracellular persistence, proteo-genomic

Abbreviations

Agr: Accessory gene regulator	NADPH: Nicotinamide adenine dinucleotide phosphate
AIM2: Absent in melanoma 2	NPCC: Non-professional phagocytic cells
AMP: Antimicrobial peptides	PEP: Phosphoenolpyruvate
Atg: Autophagy related gene	PI3K: Phosphoinositide 3-kinase
ATP: Adenosine triphosphate	PI3P: Phosphatidylinositol-3-phosphate
BCV: <i>Brucella</i> -containing vacuole	PPP: Pentose phosphate pathway
BHI: Brain heart infusion	PSM: Phenol-Soluble Modulin
CA: Community associated	PTS: Phosphotranferase system
CF: Cystic fibrosis	PVL: Panton–Valentine leukocidin
CFTR: Cystic fibrosis transmembrane conductance regulator	ROS: Reactive oxygen species
Cna: Collagen-binding protein	SaCP: <i>Staphylococcus aureus</i> containing phagosome
EE: Early endosome	SCC <i>mec</i> : <i>Staphylococcal</i> cassette chromosome <i>mec</i>
FnBP: Fibronectin-binding protein	SCV: <i>Salmonella</i> -containing vacuole
HA: Hospital associated	SCV: Small colony variant
HGT: Horizontal gene transfer	SNP: Single nucleotide polymorphism
IL: Interleukin	SpA: Staphylococcal protein A
LAMP: Lysosome-associated membrane <i>proteins</i>	SSTI: Skin and soft tissue infections
LCV: <i>Legionella</i> -containing vacuole	T3SS: Type III secretion system
LC3: Microtubule-associated protein 1 light chain 3	T6SS: Type IV secretion system
LE: Late endosome	TCA: Tricarboxylic acid
LLO: Listeriolysin O	UhpT: Hexose phosphate transporter
MRSA: Methicillin resistant <i>Staphylococcus aureus</i>	VAMP: Vesicle associated membrane protein

Content

PART I INTRODUCTION BIBLIOGRAPHY	9
CHAPTER I: INTRACELLULAR BACTERIA: IMPORTANCE OF METABOLIC ADAPTATION.....	10
1. BACKGROUND	10
2. DIFFERENT BACTERIAL INTRACELLULAR SURVIVAL STRATEGIES	11
3. IMPORTANCE OF BACTERIAL METABOLISM DURING THE INTRACELLULAR LIFE CYCLE	18
4. HOST CELL RESPONSE	28
CHAPTER II: <i>STAPHYLOCOCCUS AUREUS</i>	34
1. GENERAL	34
2. VIRULENCE FACTORS	35
3. VIRULENCE REGULATORS.....	37
4. INTRACELLULAR LIFE CYCLE OF <i>STAPHYLOCOCCUS AUREUS</i>	43
5. IMPORTANCE OF STAPHYLOCOCCAL METABOLISM DURING INFECTION.....	51
CHAPTER III: ADAPTATION OF BACTERIAL PATHOGENS IN THE CYSTIC FIBROSIS MODEL.....	54
1. GENERAL ASPECTS OF BACTERIAL ADAPTATION	54
2. EVOLUTION OF <i>S. AUREUS</i> DURING HUMAN INFECTION	55
3. EVOLUTION OF <i>S. AUREUS</i> IN INDIVIDUAL WITH CYSTIC FIBROSIS	58
OBJECTIVES	65
PART II RESULTS	67
CHAPTER I: INTRACELLULAR SURVIVAL OF <i>STAPHYLOCOCCUS AUREUS</i> IN ENDOTHELIAL CELLS: A MATTER OF GROWTH OR PERSISTENCE.....	68
CHAPTER II: TRANSKETOLASE OF <i>STAPHYLOCOCCUS AUREUS</i> IS INVOLVED IN THE CONTROL OF MASTER REGULATORS OF STRESS RESPONSE DURING INFECTION	80

**CHAPTER III: CHRONIC *STAPHYLOCOCCUS AUREUS* LUNG INFECTION CORRELATES WITH
PROTEOGENOMIC AND METABOLIC ADAPTATIONS LEADING TO AN INCREASED INTRACELLULAR
PERSISTENCE127**

DISCUSSION AND PERSPECTIVES149

REFERENCE.....155

Part I Introduction bibliography

Chapter I: Intracellular bacteria: importance of metabolic adaptation

Bacterial pathogens are a major danger for human health all over the world. In particular, with the rapid emergence of bacteria bearing multiple antibiotic resistances, developing new anti-bacterial approaches is becoming tremendously necessary. An important strategy used by several bacterial pathogens is the invasion of host cells in order to escape attack by the host immune system and evade antibiotic killing. These bacteria called “intracellular bacteria” include obligate intracellular bacteria and facultative intracellular bacteria.

1. Background

Intracellular bacterial pathogens fall into two major groups: obligate and facultative. Obligate intracellular bacteria, such as *Chlamydia*, *Anaplasma*, *Ehrlichia*, and *Rickettsia*, that have lost their capacity to replicate without their host cells, exclusively rely on host resources to enable development and ultimately transmission to a new host. In contrast, facultative intracellular bacteria, like *Francisella tularensis*, *Listeria monocytogenes* or *Legionella pneumophila*, have kept their capacity to replicate in different extracellular environment and this capacity to survive independently from the host cells sometimes is an advantage for their infectious life cycle (Casadevall 2008; Fuchs, Eisenreich *et al.* 2012).

Growing evidence has demonstrated that several prototypical extracellular bacterial pathogens, such as *Staphylococcus aureus*, *Streptococcus pneumoniae* or *Streptococcus pyogenes* also had an intracellular lifestyle in various host cell types, serving for transition or even as reservoir (Osterlund, Popa *et al.* 1997; Hayes, Howlin *et al.* 2015; Shenoy, Brissac *et al.* 2017).

Although intracellular pathogens possess various strategies to overcome host cell defenses, there is a common phenomenon shared by obligate and facultative intracellular bacterial pathogens: they generally display genome reduction. This genome reduction includes loss of genes and appearance of pseudogenes (Andersson and Andersson 1999; Martinez-Cano, Reyes-Prieto *et al.* 2014; Wernegreen 2015). Gene deletion event sometimes include genomic regions encoding entire metabolic pathways. In such cases, the bacterial pathogens have evolved to

acquire the metabolites that they can no longer produce directly from the host. For example, *Rickettsia spp.* have lost large number of genes coding for sugar, purine, and amino acid metabolism (Renesto, Ogata et al. 2005; Merhej, Angelakis *et al.* 2014). Another example, genes loci of bacterial toxin-antitoxin (TA) are lost during the adaption of the intracellular lifestyle, which encode proteins for stress response for free-living prokaryotes (Pandey and Gerdes 2005).

For some intracellular bacterial pathogens, genome reduction may be a selective advantage. This phenomenon, called “pathoadaptation”, is a mechanism where the absence of specific genes could increase bacterial fitness in the host (Pallen and Wren 2007). For example, *Shigella spp.* have lost the gene coding for lysine decarboxylase, probably because the product of this enzyme could inhibit bacterial virulence factors (Maurelli, Fernandez *et al.* 1998). However, in some cases, gain of genes may also occur to favor the intracellular bacterial pathogens. For example, *L. pneumophila* is thought to produce numerous eukaryotic-like proteins, presumably due to a long co-evolution associated with environmental amoeba (Cazalet, Rusniok *et al.* 2004).

Although obligate intracellular bacterial pathogens always have an intimate relationship with their host, they have developed various strategies to escape from host immune responses. In contrast, facultative intracellular bacterial pathogens have more complex relationships with their host and outcome of the interaction of hosts with these bacterial pathogens usually depends on the immunological status of the host. For example, *Mycobacterium tuberculosis* can only cause diseases in a small population of infected hosts.

2. Different bacterial intracellular survival strategies

No matter which bacterial species, the intracellular lifestyle can be divided into three main phases: i) entry, ii) intracellular survival and/or replication and iii) exit. Of note, intracellular replication is not always needed.

The entry process, also called internalization, involves bacterial adherence and subsequent uptake. These two steps depend on both host and bacterial factors. Three main mechanisms of entry are currently described, namely zipper, trigger and looping (Cossart and Roy 2010; Pizarro-Cerda, Charbit *et al.* 2016).

Zipper mechanism. Binding of bacterial proteins with host membrane proteins, leads to bacterial ingestion by a small-scale cytoskeletal rearrangement, which involves recruitment of various host factors. For example, *L. monocytogenes* induces its internalization into non-phagocytic cells via a prototypical zipper mechanism (Pizarro-Cerda, Charbit *et al.* 2016). Internalization of *L. monocytogenes* is mediated by two surface proteins, InlA and InlB, which respectively target E-cadherin and the hepatocyte growth factor receptor Met (Mengaud, Ohayon *et al.* 1996; Shen, Naujokas *et al.* 2000) (**Figure 1A**).

Trigger mechanism. Bacteria activate signaling pathways leading to large-scale cytoskeletal rearrangements by the formation of membrane protrusions named “ruffle” (Cossart and Roy 2010). Then, the extending ruffles fold back and enclose the cell surface, thereby entrapping the nearby bacteria. For example, *Salmonella* induces its internalization into non-phagocytic cells via a trigger mechanism (Pizarro-Cerda, Charbit *et al.* 2016). It injects directly into the host cell cytoplasm various bacterial effectors via one of its two Type III secretion system (T3SS) that trigger cellular responses (Schlumberger and Hardt 2006; Ribet and Cossart 2015) (**Figure 1B**).

Looping mechanism. In the case of looping mechanism, bacterial internalization does not actively promote surface cytoskeletal rearrangements and instead rely on the internalization machineries of host cells. *F. tularensis* is the only intracellular pathogen described thus far to use this entry mechanism. Internalization into macrophages via this looping mechanism involves the initial formation of large loops or pseudopodia (Clemens, Lee *et al.* 2005) (**Figure 1C**).

Of note, in the course of infection, a given pathogen can enter in different cell types and express various sets of virulence genes. In these situations, each infected cell type may have a particular role in the progression of disease.

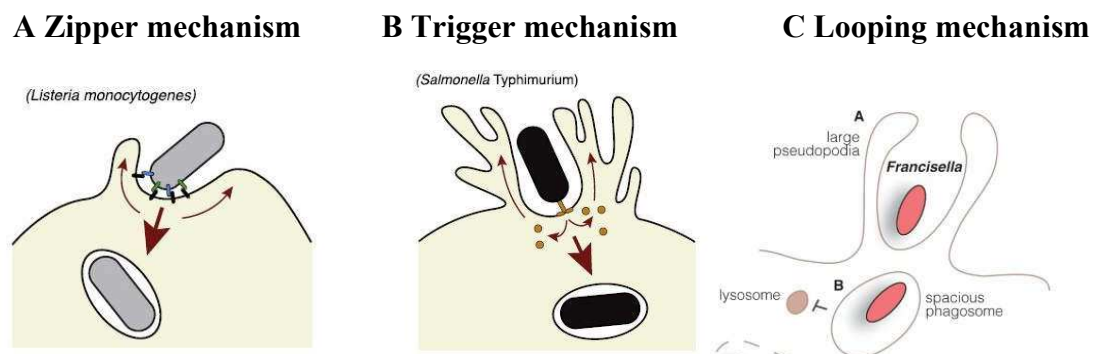


Figure 1: Entry mechanism of intracellular bacterial pathogens. A) Zipper mechanism, the example of *L. monocytogenes*; B) Trigger mechanism, the example of *Salmonella*; C) Looping mechanism, the example of *F. tularensis*. Adapted from Pizarro-Cerdá *et al.* 2016, Ribet *et al.* 2015.

Intracellular survival. After internalization, intracellular bacteria initially reside within a membrane-bound vacuole. Under normal conditions, the vacuole progressively acidifies, finally it develops into a mature degradative phagolysosome, which contains hydrolytic enzymes and microbicidal oxidative burst. Intracellular bacteria have evolved either to adapt to a vacuolar niche or to thrive freely in the cytoplasmic compartment.

Intravacuolar niche. Some intracellular bacteria have developed various strategies to survive within vacuole, either by preventing phagolysosomal fusion or by modifying the environment within vacuole (e.g. *Brucella*, *Legionella*, *Chlamydia*, *Salmonella*, *Mycobacteria*, ...).

For example, upon phagocytosis, *Brucella* resides within the *Brucella*-containing vacuole (BCV), which undergoes interactions with early endosomes (EE), late endosomes (LE) and partially fuse with lysosomes (Lys) to become the endosomal BCV (eBCV). Following a brief residence in this compartment, *Brucella* transfers eBCV to the endoplasmic reticulum (ER), which makes ER a niche for its replication (Kumar and Valdivia 2009; Celli 2015) (**Figure 2A**).

After internalization into the host cell, *Salmonella* resides within the *Salmonella*-containing vacuole (SCV). The early SCV interacts with EE, LE and fuse with Lys to become the maturing SCV. Later, the maturing SCV progresses into the late SCV which interacts with EE and detoxified lysosomes (Detox. Lys), eventually resulting *Salmonella*-induced modified endosomal maturation. In parallel, lysosomal content is excluded from the maturing SCV, most probably via recycling endosomes (RE). The late SCV locates proximity to the Golgi, hijacks vesicles from this organelle. Meanwhile, the early SCV also interacts directly with the host ER, which might enable coat protein complex (COPII) vesicle association with the SCV. These processes are probably involved in vacuolar rupture and *Salmonella* hyper-replication in the host cytosol (Santos and Enninga 2016) (**Figure 2B**).

After internalization, *Legionella pneumophila* resides within the *Legionella*-containing vacuole (LCV). In phagocytes, LCV rapidly intercepts Rab1-positive ER-derived vesicles. The LCV then matures into an ER-derived compartment that supports bacterial growth. Additionally, the LCV interacts with EE but avoids fusion with Lys (Santos and Enninga 2016) (**Figure 2C**).

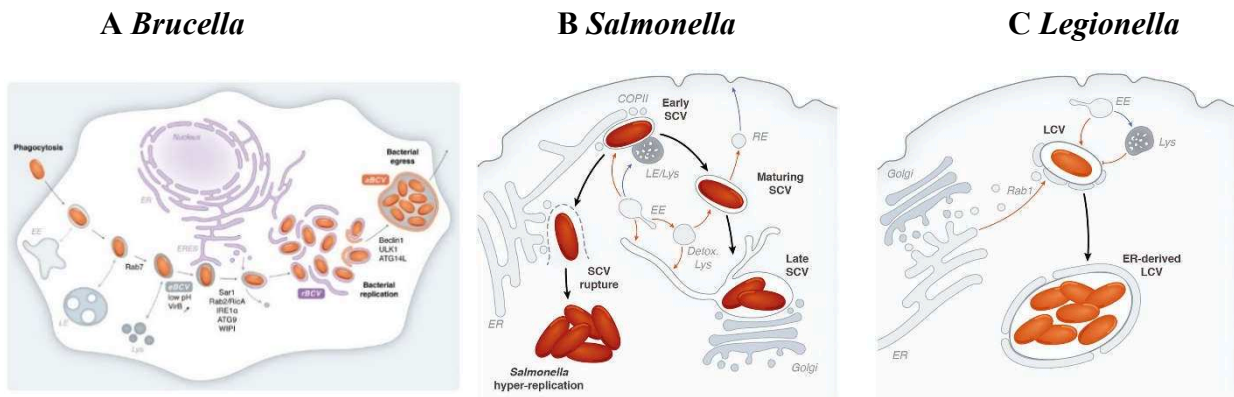


Figure 2: Model of *Brucella* intracellular trafficking in mammalian cells. A) the example of *Brucella*; B) the example of *Salmonella*; C) the example of *Legionella*. Taken from Celli 2015; Santos *et al.* 2016.

Cytosolic niche. In contrast, other bacterial pathogens such as *L. monocytogenes*, *Shigella flexneri* and *F. tularensis* have developed strategies to escape from the phagosome in a very short time, to reside within the host cytosol (Ray, Marteyn *et al.* 2009), see also **Table 1**. Escaping from the vacuole is a bacterial driven process and each cytosolic pathogen has a unique strategy of entry into cytosol that depends on various sets of enzymes or lysins.

For *L. monocytogenes*, phagosomal escape is mediated by the pore-forming cholesterol-dependent cytolysin listeriolysin O (LLO) and two type C phospholipases: phosphatidylinositol specific phospholipase C (PI-PLC) and the broad-range phosphatidylcholine-specific phospholipase C (PC-PLC). LLO transfers to the phagosomal membrane by binding cholesterol and forms pores, which leads to membrane disruption. Eventually this prevents *Listeria*-containing vacuole fusion with lysosomes. PI-PLC is responsible for escape from the vacuole into the cytosol, whereas PC-PLC contributes to disruption of the double-membrane vacuole during cell-to-cell spread (Ray, Marteyn *et al.* 2009).

Phagosomal escape of *S. flexneri* is probably induced by the type III secretion system (T3SS) effectors/translocators IpaB and IpaC, due to their ability to transfer to the host cell membrane for the delivery of bacterial effectors. IpaB and IpaC can disrupt lipid vesicles (liposomes) *in vitro* and are required for contact-mediated hemolysis by *Shigella* as well as bacterial phagosomal escape in macrophages (Mellouk and Enninga 2016). In addition, the T3SS effector IpaH7.8 may promote *Shigella* vacuolar escape both in macrophages and epithelial cells, although its function is not well understood (Fernandez-Prada, Hoover *et al.* 2000; Paetzold, Lourido *et al.* 2007).

F. tularensis escapes from an acidified late-endosome-like vacuole before the fusion with lysosome. Lysis of the vacuole requires the recruitment of vacuolar ATPase pump on the membrane of vacuole, which allows import of protons and acidification (Santic, Asare *et al.* 2008). The type VI secretion system (T6SS) also plays an essential role in *F. tularensis* phagosomal escape. The T6SS assembles a phage tail-like structure across the bacterial envelope and host cell membrane, enabling inject effector proteins (such as VgrG, IglI...) into the cytosol of the host cell (Broms, Sjostedt *et al.* 2010; Celli and Zahrt 2013; Clemens, Lee *et al.* 2018; Ledvina, Kelly *et al.* 2018).

	<i>Shigella flexneri</i>	<i>Listeria monocytogenes</i>	<i>Francisella tularensis</i>
Bacterial gene required	IpaB IpaC T3SS	LLO and type C phospholipases	T6SS, acid phosphatases,...
Host factors required	Unknown	GILT activates LLO by a thiol reductase	Unknown
Vacuole conditions required	Unknown	Acidic vacuole (pH 5.5)	Acidic vacuole
Kinetics of escape	15–30 minutes	17 minutes	30–60 minutes

Table 1: Mechanisms of vacuolar escape by selected intracellular pathogens. Taken from Ray *et al.* 2009.

Exit. Lastly, intracellular pathogens have also developed various strategies to exit the intracellular environment and disseminate to adjacent cells and tissues. Many cytosolic bacteria (*L. monocytogenes*, *Rickettsia* spp, *Shigella* ...) share a common mechanism to spread to neighboring cells. After escape from the phagosome into the cytosol, these bacteria polymerize actin at the bacterial pole, forming a comet-like tails on their surface, leads to the formation of bacteria-containing protrusions and cell-to-cell spread (Weddle and Agaisse 2018) (**Figure 3**).

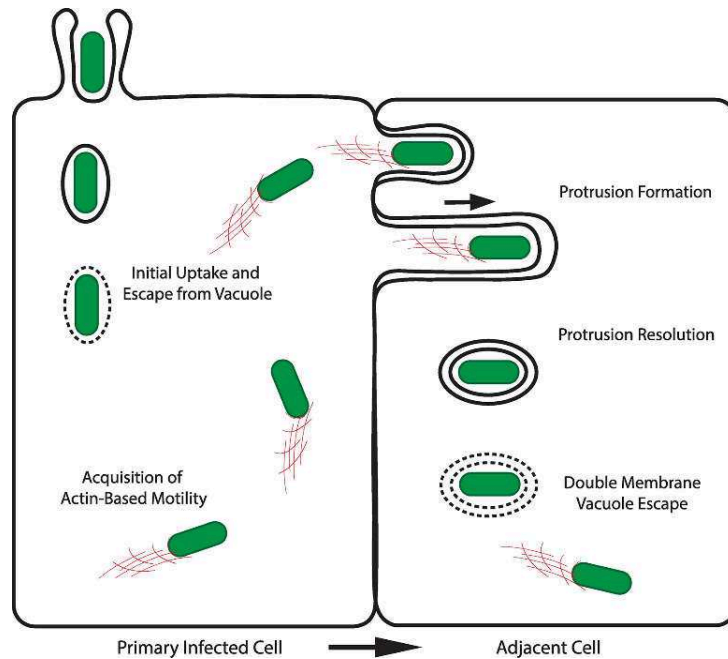


Figure 3: Intracellular bacterial spread from cell-to-cell. Steps supporting the intracellular dissemination of *Listeria monocytogenes*. After escape from the vacuole into the cytosol, these bacteria polymerize actin at the bacterial pole, forming a comet-like tails on their surface, leads to the formation of bacteria-containing protrusions and cell-to-cell spread. Taken from Weddle *et al.* 2018.

Cell-to-cell spread is particularly advantageous because it allows bacteria not to be exposed to the extracellular environment. Like this, pathogens are able to avoid potential detection or destruction by preexisting local inflammatory response (Hybiske and Stephens 2008). However, some pathogens escape from host cells directly by inducing cell death through apoptosis or pyroptosis. The bacterial molecule that is responsible for apoptotic induction remains unknown. Intracellular pathogens can induce proinflammatory pyroptosis via inflammasome activation by sensing of bacterial molecules (flagellin of *L. pneumophila* and *Salmonella* spp. and chromosomal DNA in the case of *F. tularensis*) (**Figure 4**).

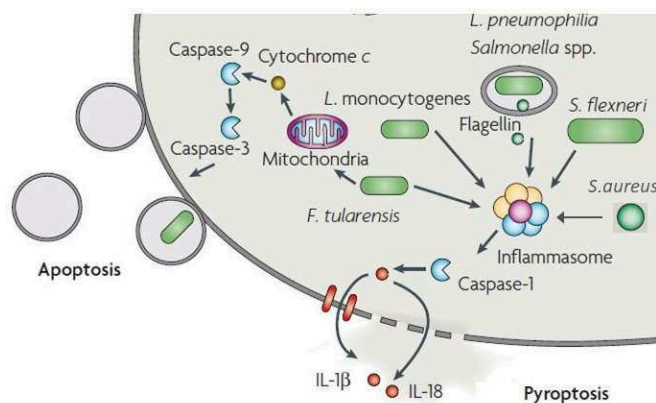


Figure 4: Model of apoptosis and pyroptosis inducing by intracellular bacteria. Apoptosis induced by *F.tularensis* is using the intrinsic pathway of activation: cytochrome *c* release from mitochondria and activation of the initiator caspase-9 and the effector caspase-3. The bacterial molecule that is responsible for apoptotic induction remains unknown. Intracellular pathogens can induce proinflammatory pyroptosis via inflammasome activation by sensing of bacterial molecules, such as: flagellin of *L. pneumophila* and *Salmonella* spp. and chromosomal DNA in the case of *F. tularensis*. The inflammasome activates caspase-1, which leads to interleukin (IL)-1 β and IL-18 activation and secretion. Cytokine secretion occurs initially through a caspase-1-dependent pore, and is then released upon necrotic cell lysis. Taken from Hybiske *et al.* 2008.

For example, *F. tularensis* uses both apoptosis and pyroptosis to lyse infected cells and to infect adjacent cells. Apoptosis induced by *F.tularensis* is using the intrinsic pathway of activation: cytochrome *c* release from mitochondria and activation of the initiator caspase-9 and the effector caspase-3 (Lai, Golovliov *et al.* 2001; Lai and Sjostedt 2003) (**Figure 4**). Intracellular *F. tularensis* induce cell pyroptosis via absent in melanoma 2 (AIM2) inflammasome activation. AIM2 is a key sensor of pathogens that detects the presence of foreign DNA accumulating in the cytosol during the life cycle of intracellular pathogens (**Figure 5**).

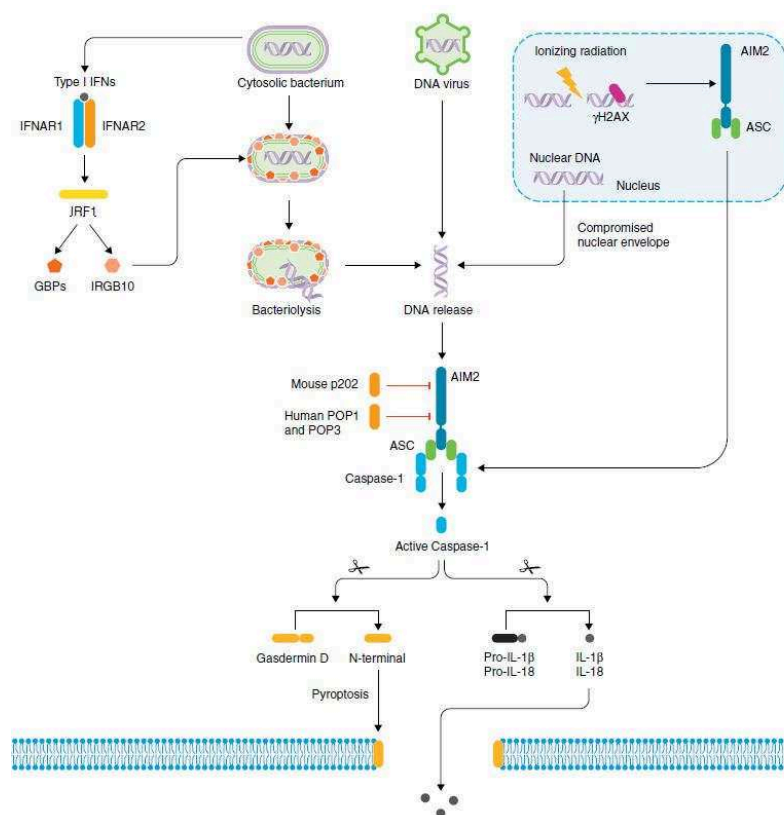


Figure 5: Model of AIM2 inflammasome. AIM2 is a key sensor of pathogens that detects the presence of foreign DNA accumulating in the cytosol during the life cycle of intracellular pathogens. Inflammatory caspases

cleave the pore-forming protein gasdermin D that induces pyroptosis. Gasdermin D is able to oligomerize on the cell membrane. Its N-terminal fragment associates with the inner leaflet of the host cell membrane, where it forms pores of 10–21 nm in diameter. These pores and/or the physical rupture of the cell allow passive release of interleukin (IL)-1 β and IL-18 into the extracellular space which eventually causes cell death. Taken from Mathur *et al.* 2018.

The molecular mechanisms of how pyroptosis is achieved following inflammasome activation are being unraveled. Very briefly, inflammatory caspases cleave the pore-forming protein gasdermin D that induces pyroptosis. Gasdermin D is able to oligomerize on the cell membrane. Its N-terminal fragment associates with the inner leaflet of the host cell membrane, where it forms pores of 10–21 nm in diameter. These pores and/or the physical rupture of the cell allow passive release of interleukin (IL)-1 β and IL-18 into the extracellular space which eventually causes cell death (Franchi, Amer *et al.* 2006; Molofsky, Byrne *et al.* 2006; Mariathasan and Monack 2007; Craven, Gao *et al.* 2009; Soong, Chun *et al.* 2012; Lugrin and Martinon 2018; Mathur, Hayward *et al.* 2018) (**Figure 5**).

3. Importance of bacterial metabolism during the intracellular life cycle

All human bacterial pathogens are heterotrophs. They can subsist on a variety of carbon and nitrogen sources, including carbohydrates, lipids, glycolipids and amino acids. Carbon catabolism provides bacteria with energy in the form of reducing equivalents and ATP, as well as essential biosynthetic precursors including glucose-6-phosphate, fructose-6-phosphate, 3-phosphoglycerate, phosphoenolpyruvate (PEP), acetyl-CoA, propionyl-CoA, oxaloacetate and α -ketoglutarate (Munoz-Elias and McKinney 2006).

The transport and use of nutrients, especially of the major carbon and nitrogen substrates, are affected by multiple specific uptake systems and pathways (**Figure 6**). The genes involved in these pathways are controlled by both global and specific regulators that may directly or indirectly sense key metabolites that are generated in the central carbon and nitrogen metabolic pathways (Deutscher, Francke *et al.* 2006; Mao, Huo *et al.* 2007; Sonenshein 2007; Eisenreich, Dandekar *et al.* 2010). Some of these global regulators may also affect the expression of essential virulence factors according to the nutrient supply in these niches.

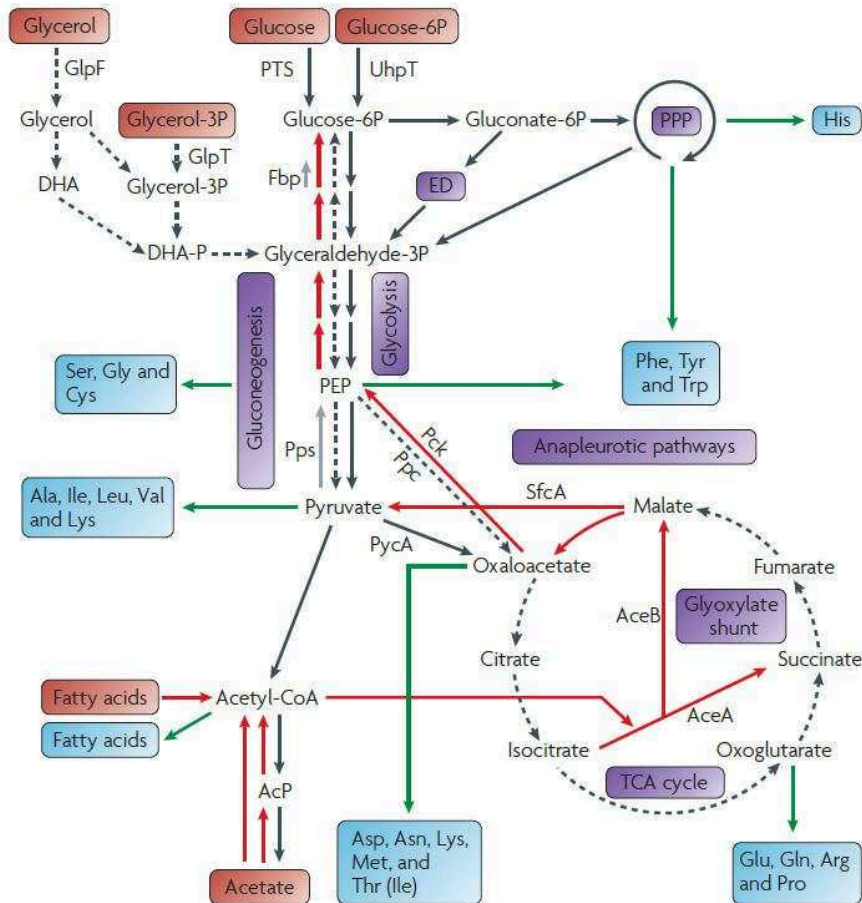


Figure 6: Metabolic pathways of typical heterotrophic and prototrophic bacteria. The Central metabolic pathways (purple boxes) comprise: glycolysis and gluconeogenesis; the variant Entner–Doudoroff pathway; the pentose phosphate pathway; the tricarboxylic acid cycle; and various anapleurotic reactions that replenish arising metabolic gaps, such as the generation of oxaloacetate by pyruvate carboxylase. In particular, carbon metabolism driven by non-glycogenic carbon substrates, such as glycerol, pyruvate, acetate, fatty acids and succinate, requires anapleurotic reactions and gluconeogenesis. Major carbon substrates are in red boxes, and the metabolic fluxes during growth on glucose or glucose-6-phosphate (solid black arrows), glycerol or glycerol-3P (dashed black arrows) and acetate or fatty acids (red arrows) are also shown. Furthermore, the anabolic monomers (amino acids and fatty acids; blue boxes) and the major catabolic intermediates from which they derive (green arrows) are shown. Taken from Eisenreich *et al.* 2010.

3.1 Intracellular nutrients

In order to survive within host cells, intracellular pathogens must adapt their metabolism to the available nutrients and physical conditions (pH, oxygen availability, osmotic pressure...) in these cells, and they must also coordinate their metabolism with their life cycle (Eisenreich, Dandekar *et al.* 2010).

The cytosol contains all of the nutrients that are provided to the host cells from the external medium and catabolic intermediates as well as all of the anabolic compounds that are produced by these cells. Thus, in principle, cytosolic bacteria have direct access to all of these host cell nutrients. However, the accurate constant concentrations of most of these compounds are very variable according to the growth conditions of the cells, their genetic background and they also vary in different cell types. In some instances, host cells and bacteria may use similar nutrients, and so bacteria in the host cell cytosol may develop smart nutrient uptake systems to compete with that of host cell.

Replication in the apparently nutrient-rich cytosol would seem to require fewer metabolic adaptations compared to a vacuolar environment. However, several studies suggest that life in the cytosol might be more complicated than formerly anticipated. Indeed, when bacterial pathogens are microinjected directly into the cytoplasm of mammalian cells, cytosolic intracellular pathogens such as *L. monocytogenes* and *S. flexneri* replicate freely, whereas intravacuolar intracellular pathogens such as *S. typhimurium* and *L. pneumophila* and extracellular pathogens such as *Yersinia enterocolitica* do not replicate at all (Goetz, Bubert *et al.* 2001), suggesting that the host cell cytosol is not simply a nutrient-rich culture medium allowing efficient growth of heterotrophic bacteria. However, the factors responsible for growth restriction of intravacuolar and extracellular bacteria in the cytoplasm have not been identified yet.

3.2 Importance of carbon metabolism for intracellular bacteria

Here, I will briefly introduce the present understanding of intracellular carbon metabolism, using three extensively studied model intracellular pathogens, namely the cytosolically-replicating species *L. monocytogenes* and *S. flexneri* and the mainly phagosomally-replicating species *S. typhimurium*. Each of the three model pathogens seems to undergo specific intracellular metabolic adaptations, which are discussed below.

L. monocytogenes is a Gram-positive bacterium first described in 1926 during an outbreak that affected guinea pigs and rabbits. It was identified in the 1980s as a food-borne pathogen. It mainly affects immunocompromised individuals, pregnant women, and newborns. Severe infections are characterized by bacteremia, meningoencephalitis, abortion, or neonatal sepsis. The most common vehicles of transmission of this saprophytic bacterium to humans are dairy products and other foods including eggs, seafood, and vegetables. Upon ingestion of

contaminated food, after translocating across the gut epithelium, *L. monocytogenes* is capable to resist killing by professional phagocytes and can disseminate either freely or within infected circulating monocytes via the lymph and the blood to the liver and the spleen and even cross the blood–brain or the blood–placenta barrier. As recalled above, inside infected cells, *L. monocytogenes* is capable to rapidly escape from the vacuole by disrupting the phagosomal membrane via the expression of listeriolysin O (LLO) and two phospholipases (PlcA and PlcB). Within the cytosol of the host cell, *L. monocytogenes* multiplies with a generation time of approximately 1 h and spreads into neighboring host cells by co-opting actin-based motility (Fuchs, Eisenreich *et al.* 2012; Radoshevich and Cossart 2018).

Transcriptional analyzes of *L. monocytogenes* in macrophages and epithelial cell lines showed a considerable upregulation of all genes involved in the facilitated uptake and catabolism of glycerol (Chatterjee, Hossain *et al.* 2006; Joseph, Przybilla *et al.* 2006). A series of mutants unable to transport and catabolize glycerol due to a lack of glycerol uptake facilitator (GlpF), glycerol kinase (GlpK), glycerol-3-phosphate dehydrogenase (GlpD), and dihydroxyacetone kinase subunit K (DhaK) were also shown to have impaired intracellular growth (Joseph, Mertins *et al.* 2008), suggesting that glycerol could be a major carbon source for carbon metabolism of intracellular bacteria.

Concomitantly, genes encoding glycolytic enzymes, as well as the *pdhABC* operon genes encoding pyruvate dehydrogenase, were downregulated in the intracellular environment in macrophages (Chatterjee, Hossain *et al.* 2006). During intracellular growth in Caco-2 cells, strong repression of genes of the glycolytic pathway (from glucose to pyruvate) was observed, most likely reflecting the slower growth of *L. monocytogenes* in these cells (generation time about 40 min) compared to that in BHI (25 min) (Joseph, Przybilla *et al.* 2006).

Expression of *uhpT*, which encodes a transporter involved in the exploitation of hexose phosphate from the host cell, was also upregulated in both cell types (Chatterjee, Hossain *et al.* 2006; Joseph, Przybilla *et al.* 2006). Meanwhile, a $\Delta uhpT$ mutant was attenuated *in vitro* and *in vivo* whereas a mutant unable to uptake glucose does not show replication attenuation in macrophages or epithelial cells (Stoll and Goebel 2010). Furthermore, the genes encoding the glucose-specific phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) permeases were not upregulated and expression of the PTS histidine-containing phosphocarrier protein (Hpr) was down regulated inside the host cell cytosol. Altogether these data supported the assumption that glucose-6-phosphate rather than glucose was used for bacterial metabolism within hosts.

Furthermore, Grubmuller *et al.* exploited the technique of ^{13}C -isotopologue profiling to study the carbon metabolism of *L. monocytogenes* in macrophages. They observed that glycerol was catabolized via the lower part of the glycolytic pathway, but not used for gluconeogenesis, and the intermediates generated from glucose 6-phosphate in the upper part of the glycolytic pathway and the pentose phosphate pathway probably serve primarily for anabolic purposes. The intracellular *L. monocytogenes* metabolism which involves at least two major carbon substrates, also named “bipartite metabolism”: glycerol mainly for energy supply and glucose 6-phosphate mainly for indispensable anabolic function (Grubmuller, Schauer *et al.* 2014) (Figure 7). Of note, during intracellular survival, the expression of genes for enzymes involved in the synthesis of branched-chain amino acids (encoded by *ilv* and *leu*) was highly enhanced.

L. monocytogenes is auxotrophic for riboflavin, thiamine, biotin and lipoate and will totally depend on the host cell for the supply of these vitamins when replicating in the host cell cytosol. Indeed, several studies show that the concurrent thiamine uptake and biosynthesis of thiamine precursors is a strategy of *L. monocytogenes* to enable proliferation within the cytoplasm (Schauer, Stolz *et al.* 2009). The importance of biosynthesis of branched-chain fatty acids for cytosolic growth of *L. monocytogenes* has also been demonstrated (Keeney, Colosi *et al.* 2009; Sun and O’Riordan 2010).

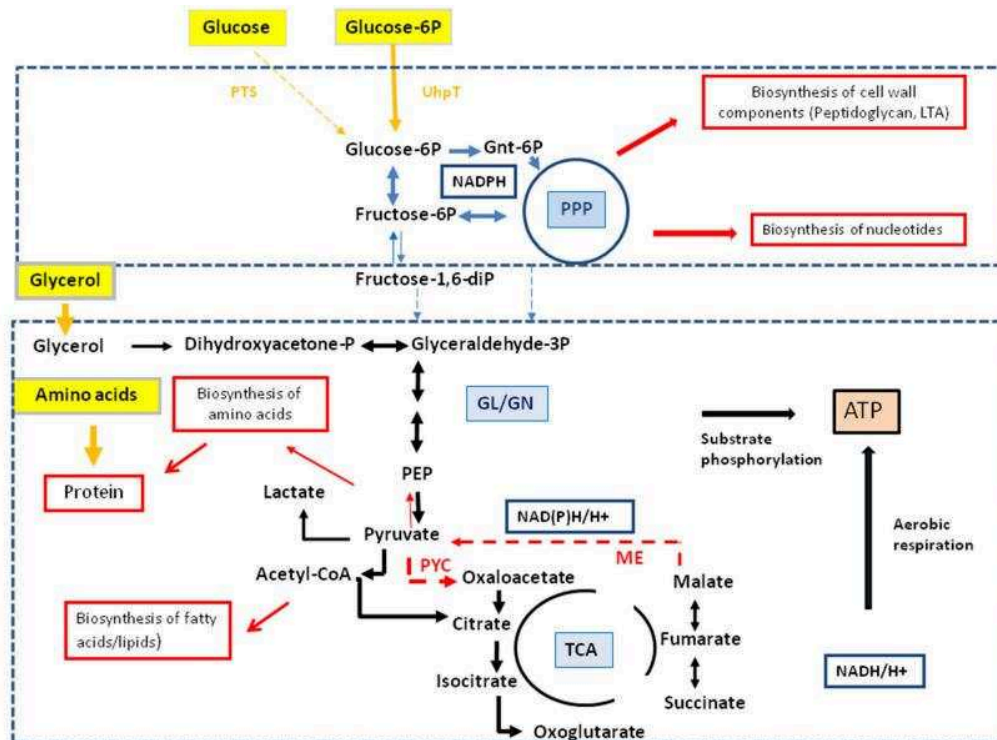


Figure 7: Simplified view of the intracellular metabolism of *L. monocytogenes*. Gray-framed yellow boxes and yellow arrows mark (by color intensity) the major carbon compounds taken up by the intracellular bacteria from

the host cell. Catabolic reactions fed by glucose-6P and glycerol are indicated by blue and black arrows, respectively. Reactions leading to anabolic performances (in red-framed boxes) are indicated by red arrows. Abbreviations: PPP, pentose phosphate pathway; GL, glycolysis; GN, gluconeogenesis; TCA, tricarboxylic acid pathway; PYC, pyruvate carboxylase; ME, malic enzyme (decarboxylating malate dehydrogenase). Taken from Grubmuller *et al.* 2014.

S. flexneri is a Gram-negative pathogen that is responsible for shigellosis. *Shigella* is closely related to *E. coli*, but has evolved specific traits of pathogenicity, physiology and serology. Shigellosis is associated with fever and abdominal cramps; its diagnosis relies on the presence of erythrocytes, polymorphonuclear neutrophils (PMNs), and mucus in patient stools, which stand as diagnostic elements. *S. flexneri* specifically invades the human colon, but not the small intestine. The molecular basis of this specificity remains unknown. Before reaching the epithelial layer, *S. flexneri* has to survive in the gastrointestinal environment, to outcompete the colonic luminal commensal bacteria and to disrupt a thick protective mucus layer. In addition, the inflammatory response induced by *S. flexneri* invasion might result in a perturbation of the composition and the function of these barriers, although these aspects are not well understood. Following ingestion, *S. flexneri* transits to the large intestine, and it uses its T3SS to induce uptake by colonic epithelial cells. *S. flexneri* firstly resides within a vacuole, it quickly lyses the vacuole membrane and replicates within the host cell cytoplasm and spreads from cell-to-cell (Waligora, Fisher *et al.* 2014; Anderson, Sansonetti *et al.* 2016).

Comparing transcriptional profiles from bacteria growing in the cytosol of macrophages with those from bacteria growing in LB (Luria–Bertani) culture show an upregulation of the genes encoding glucose uptake proteins (the glucose-specific PTS component gene *ptsG* and the mannose-specific PTS component genes *manX*, *manY* and *manZ*) and *ugp* gene encoding the glycerol-3P transporter, as well as *uhpT* gene encoding the hexose phosphate transporter (Runyen-Janecky and Payne 2002; Lucchini, Liu *et al.* 2005). The Δ *uhpT* mutant showed reduced growth on glucose 6-phosphate as sole carbon source *in vitro*; however, no reduction in growth was observed in epithelial cells compared with the wild-type strain for *Shigella* (Runyen-Janecky and Payne 2002), suggesting either that glucose 6-phosphate is not the only available intracellular carbon source or that it is transported by other sugar transporters during bacterial intracellular life cycle.

Global proteome analysis of *Shigella* inside host cells identified that mixed-acid fermentation, and metabolism of pyruvate in particular, is required for optimal intracellular growth of *S. flexneri* (Pieper, Fisher *et al.* 2013). In contrast to fermentation pathway enzymes,

components of the tricarboxylic acid (TCA) cycle were less expressed in the intracellular bacteria.

S. flexneri uses three glycolytic pathways to produce pyruvate from glucose: the Embden-Meyerhof-Parnas (EMP) pathway, the pentose-phosphate pathway (PPP) and the Entner-Doudoroff (ED) pathway. Analysis in cell culture shows that the EMP pathway is necessary, while the PPP and ED pathways were dispensable for *S. flexneri* intracellular growth (Waligora, Fisher *et al.* 2014). Importantly, growth defects caused by glycolysis mutants could be overcome by supplementation with pyruvate, showing that this molecule is an essential carbon source for *S. flexneri* intracellular growth (Killackey, Sorbara *et al.* 2016). Furthermore, Kentner *et al.* observed that host glycolytic pathways were not significantly affected by the intracellular pathogen, but infected cells excreted acetate instead of pyruvate and lactate, suggesting that the latter molecules had been captured and used by *Shigella* for its metabolism (Kentner, Martano *et al.* 2014). This study supported the importance of pyruvate for *Shigella* intracellular growth. See also **Figure 8**.

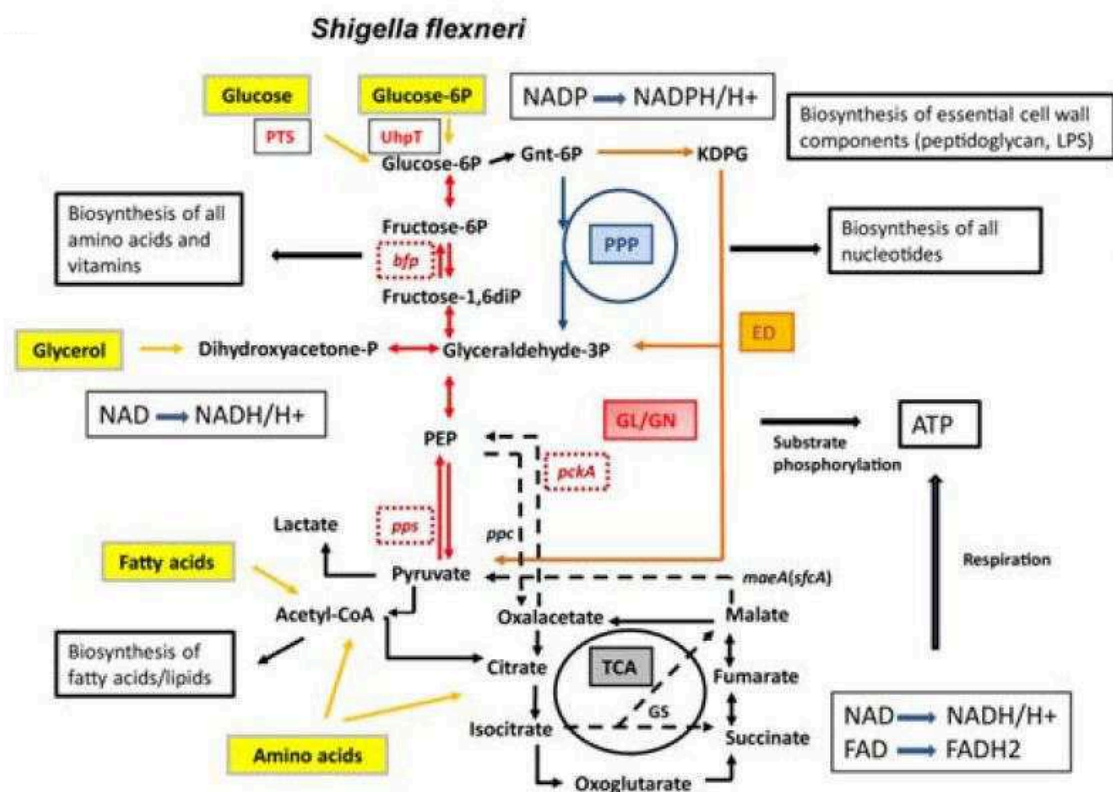


Figure 8: Simplified view of the intracellular metabolism of *Shigella flexneri*. Solid arrows indicate reversible (double-headed arrows) and essentially irreversible (single-headed arrows) reactions of glycolysis and gluconeogenesis (GL/GN, red arrows), pentose phosphate pathway (PPP, blue arrows), Entner-Doudoroff pathway

(ED, orange arrows), the tricarboxylic acid cycle (TCA). The irreversible reactions involved in GN are marked by dotted-framed boxes: *fbp* for phosphofructo-1,6-bisphosphatase, *pps* for PEP synthase, and *pckA* for PEP carboxykinase. Broken black arrows depict anaplerotic reactions: *GS* for glyoxylate shunt, *pycA* for pyruvate carboxylase, *ppc* for PEP carboxylase; *pckA* for PEP carboxykinase, and *maeA* (*sfcA*) for malate enzyme. Yellow boxes and arrows mark major carbon and energy substrates and black-framed boxes mark the biosynthesis of amino acids, vitamins, nucleotides, cell envelope components, and fatty acids/lipids as well as the major sites of ATP production. The major sites for the generation of NADH/H⁺, NADPH/H⁺ and FADH₂ are also shown. Taken from Eisenreich *et al.* 2015.

S. Typhimurium (*Salmonella enterica* serovar Typhimurium) is a Gram-negative enterobacterium closely related to *E. coli*. The genus *Salmonella* is one of the most common causes of food-borne illness and is a major cause of diarrheal diseases. It mainly affects immunocompromised individuals, young children, and the elderly. Food-borne pathogens usually emerge in outbreaks and may affect a large number of patients. Several outbreaks attributed to different *Salmonella* serovars are reported each year, highlighting the frequency of *S. Typhimurium* and *S. enterica* serovar Enteritidis among the most common causal agents (Fabrega and Vila 2013). After ingestion of contaminated food or water, bacteria reach the intestinal epithelium and trigger gastrointestinal disease. The first obstacle to overcome within the host is the acidic gastrointestinal environment. *S. Typhimurium* activates the acid tolerance response to protect itself against severe acid shock. After entering the small bowel, *Salmonella* must traverse the intestinal mucus layer before adhering to intestinal epithelial cells. In mice, *Salmonella* appear to preferentially adhere to and enter the M cells of the Peyer's patches in the intestinal epithelium, meanwhile invasion of nonphagocytic enterocytes can also occur. After internalization by host cells, bacteria reside within large vesicles called *Salmonella*-containing vacuoles (SCVs), the only intracellular compartment in which *Salmonella* cells survive and replicate (Finlay and Falkow 1988; Fabrega and Vila 2013; Dandekar, Fieselmann *et al.* 2014).

Large-scale transcriptome and proteome data are available for *S. Typhimurium* in host cell intracellular environment and in infected animals (Eriksson, Lucchini *et al.* 2003; Becker, Selbach *et al.* 2006). Comparing transcriptional profiles from bacteria growing in Hela cells with those from bacteria growing in LB culture show up regulation of *ptsG* and *uhpT* (Hautefort, Thompson *et al.* 2008), suggesting that both glucose and glucose phosphates might be available to intraepithelial *Salmonella*.

¹³C-isotopologue profiling data showed that *S. Typhimurium* replicating in the SCV of Caco-2 cells prior to use glucose (but not glucose-6-phosphate) as carbon substrate. However, a mutant, which is impaired in glucose uptake, was still able to replicate in Caco-2 cells, indicating that *S. Typhimurium* can switch to other nutrients as carbon and energy sources in the absence of glucose, most likely to C₃-substrates, such as glycerol, glycerol 3-phosphate... (Gotz, Eylert *et al.* 2010).

Genes encoding enzymes for glycolysis and the ED pathway are upregulated in these bacteria, meanwhile most genes encoding enzymes in the TCA cycle are downregulated. Expression of these genes is more pronounced in Hela cells than in J774 macrophage cell lines (Hautefort, Thompson *et al.* 2008). The ED pathway facilitates growth on sugar acids such as galacturonate that are found in the gastrointestinal tract (Hautefort, Thompson *et al.* 2008). Together these data suggest that *S. Typhimurium* may use host-sugar acids as intracellular carbon sources, in both epithelial and macrophages cells, in addition to host-derived glucose in epithelial cells.

Upon systemic infections in mice, strong attenuation is observed in *S. Typhimurium* mutants which are impaired in the biosynthesis of aromatic amino acids (including histidine) and purines (especially adenine), meanwhile moderate attenuation is found for pyrimidine and methionine mutants, which demonstrated the dependency of *in vivo* growing *S. Typhimurium* on the biosynthesis of these anabolic monomers (Gotz, Eylert *et al.* 2010; Eisenreich, Heesemann *et al.* 2015). The *in vitro* data also supported this assumption: the transcriptome studies showed enhanced expression of the genes encoding enzymes for these anabolic pathways. In addition, the ¹³C-isotopologue profiling results also showed significant *de novo* synthesis of amino acids in *S. Typhimurium* replicating in Caco-2 cells (Gotz, Eylert *et al.* 2010).

In systemically infected mice, *S. Typhimurium* replicates mainly in macrophages, bacterial growth depends on a complete TCA cycle, and mutants unable to convert succinate to oxaloacetate are avirulent (Tchawa Yimga, Leatham *et al.* 2006; Mercado-Lubo, Leatham *et al.* 2009). Removal of malate from the TCA cycle to produce pyruvate requires that TCA cycle intermediates be replenished to keep the TCA cycle operative. However, since it appeared that the glyoxylate shunt was not needed for full virulence, replenishment of TCA cycle intermediates did not seem to occur by this pathway in a systemic *S. Typhimurium* infection model (Tchawa Yimga, Leatham *et al.* 2006). However, this pathway might have a role in persistent infections of animals (Fang, Libby *et al.* 2005). Mutants blocked in gluconeogenesis are still fully virulent in systemically infected mice (Tchawa Yimga, Leatham *et al.* 2006). From

these data, it can be suggested that *S. Typhimurium* grows in mouse macrophages mainly depend on glucose or other C₆ carbohydrates rather than glycolytic carbon sources. Furthermore, Steeb *et al.* measured tissue colonization capabilities of the various *S. Typhimurium* mutants via competitive infections with mixtures of mutant and wild-type *Salmonella in vivo* assay (Steeb, Claudi *et al.* 2013). Results indicated that no single major nutrient alone could support full *Salmonella* virulence. Instead, *Salmonella* depended on utilization of glycerol, fatty acids, N-acetylglucosamine, glucose, gluconate, arginine and lactate. These seven metabolites seemed to supply individual small nutritional contributions, only together enable normal *Salmonella in vivo* growth. See also **Figure 9**.

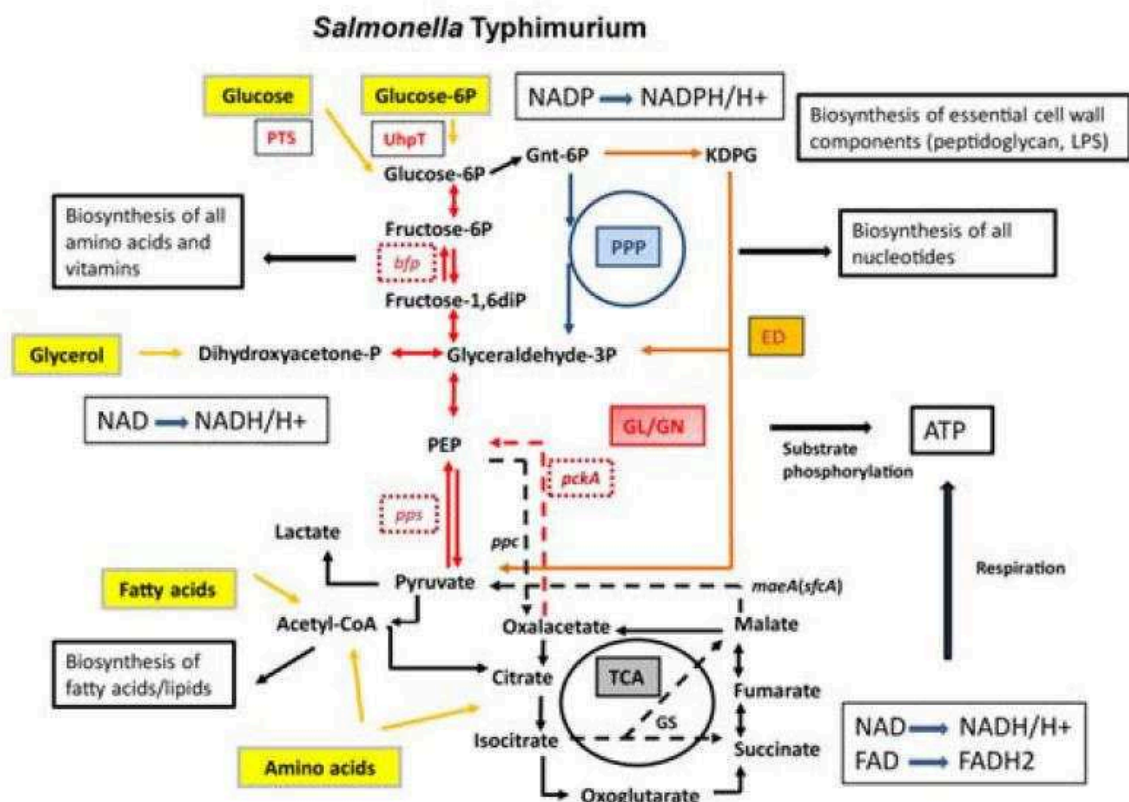


Figure 9: Simplified view of the intracellular metabolism of *Salmonella enterica* serovar Typhimurium. See legend of Figure 8 for further explanations and abbreviations. Taken from Eisenreich *et al.* 2015.

4. Host cell response

On the other hand, host cells develop various defensive strategies against bacterial infections. Here, I will highlight two important defense mechanisms: phagosomal elimination and autophagy.

4.1 Phagosomal elimination

Following phagocytosis by host cells, intracellular bacterial pathogens initially reside in phagosome. Hence, the phagosome is the first defend strategy from the infected host cell and phagosome maturation is critical for macrophages to eliminate the ingested pathogens. There are three major mechanisms of pathogen eradication during this process: i) the oxidative burst, inducing by the production of reactive oxygen species (ROS); ii) phagosomal acidification; and iii) the release of antimicrobial proteins and peptides (AMPs) (Flannagan, Heit *et al.* 2015; Weiss and Schaible 2015).

The process of phagosome maturation comprises a series of strictly coordinated membrane fusion/fission events between the phagosome and compartments of the endo/lysosomal network and eventually with the formation of the phagolysosome, a degradative organelle (Desjardins, Huber *et al.* 1994). It contains three major stages (**Figure 10**).

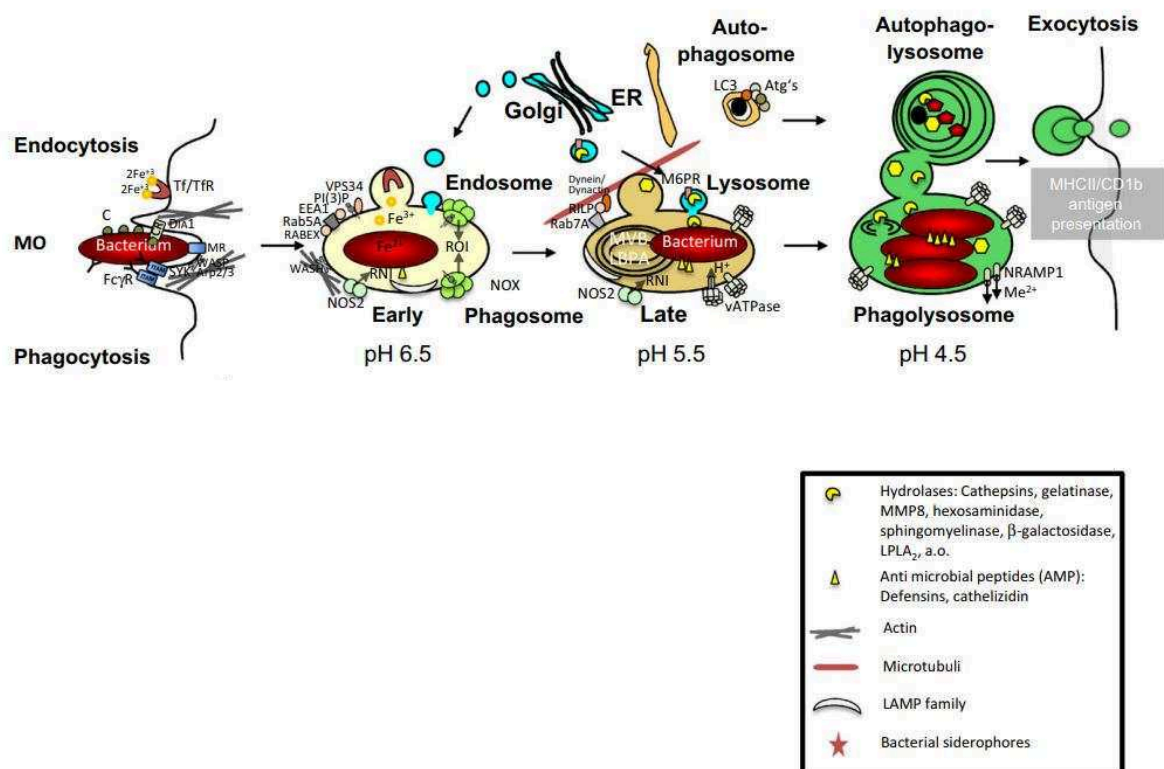


Figure 10: Model of maturation of phagosome and trafficking of intracellular bacteria. Schematic drawing depicts the phagocytic responses of macrophages (MΦs) against bacterial invaders. In MΦs, upon phagosome closure, the maturing phagosome traverses an early and late phagosomal and a phagolysosomal stage paralleling endosomal maturation. Phagosome biogenesis is accompanied by continuous fusion and fission events, including fusion with trans-Golgi transport vesicles, endosomes, lysosomes, and autophagosomes. These interactions cause acquisition and loss of different stage-specific markers. A hallmark of phagosome biogenesis is acidification of the phagosomal lumen by the proton pumping vATPase. A low pH is a prerequisite for optimal enzymatic activity of most late endosomal/lysosomal hydrolases. Phagocytosis and fusion with the lysosomal azurophilic granules is often happening simultaneously. Taken from Weiss and Schaible 2015.

The fusion of the early phagosome and early endosome is the first stage of phagosome maturation. This process, tightly coordinated by a family of proteins, Rab GTPases (Stenmark 2009), is characterized with the acquisition of several specific membrane marker: the small GTPase Rab5, early endosomal antigen 1 (EEA1), the phosphatidylinositol 3 kinase (PI3K) Vps34, and phosphatidylinositol 3-phosphate (PI3P) (Weiss and Schaible 2015). Other molecules are also involved in this process, such as the family of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, vesicle associated membrane proteins (VAMPs), and syntaxins. The early phagosome lacks the proton pumping vesicular ATPase (vATPase) and is therefore characterized by a mildly acidic pH of 6–6.5 (Sturgill-Koszycki, Schlesinger *et al.* 1994). It is also at this stage, the ROS is produced by NADPH oxidase (Nox2), an enzyme complex. When the macrophage is not activated, this complex remains inactive and becomes active when the macrophage is activated. The Nox2 NADPH oxidase complex is comprised of the membranal proteins gp22^{phox} and gp91^{phox} (also known collectively as the cytochrome *b558*) and four regulatory cytosolic subunits: p40^{phox}, p47^{phox}, p67^{phox} and Rac2 (Assari 2006; Lam, Huang *et al.* 2010) (**Figure 11**). This complex can produce a large quantity of ROS which are derived from initial production of superoxide (O₂⁻). O₂⁻ is extremely unstable and weakly bactericidal. It is subsequently transformed into hydrogen peroxide (H₂O₂) and then transforms into hydroxyl radical (OH⁻). The high ROS concentration generated within the phagosome are thought to kill pathogens directly due to their oxidizing capacity. This procedure of ROS production is called the respiratory or oxidative burst (Assari 2006; Lam, Huang *et al.* 2010).

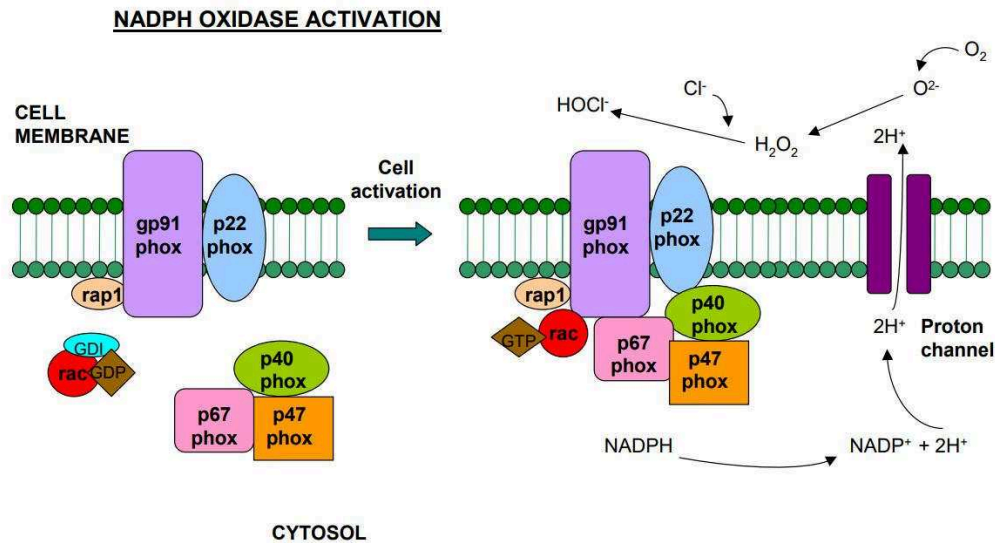


Figure 11: A schematic of Nox2 NADPH oxidase assembly and activation. The Nox2 NADPH oxidase complex is comprised of the membranal proteins gp22^{phox} and gp91^{phox} (also known collectively as the cytochrome *b558*) and four regulatory cytosolic subunits: p40^{phox}, p47^{phox}, p67^{phox} and Rac2. This complex can produce a large quantity of ROS which are derived from initial production of superoxide ($O_2^{\cdot-}$). $O_2^{\cdot-}$ is extremely unstable and weakly bactericidal. It is subsequently transformed into hydrogen peroxide (H_2O_2) and then transforms into hydroxyl radical ($OH^{\cdot-}$). The high ROS concentration generated within the phagosome are thought to kill pathogens directly due to their oxidizing capacity. Taken from Assari 2006.

During the second stage of phagosome maturation, the early phagosome transforms into late phagosome by the fusion with late endosomes. A hallmark of this stage is the progressive acidification of the phagosomal lumen, pH is ~ 5.5 . This drastic drop in pH is catalyzed by the V-ATPase, a proton pump that drives H^+ uptake into the phagosome lumen from the cytosol via using the energy from ATP hydrolysis. The late phagosome is characterized by the acquisition of the Rab7 and Rab9 lysosome-associated membrane proteins (LAMP1 and LAMP2) and the loss of Rab5 and PI3P (Rink, Ghigo *et al.* 2005; Poteryaev, Datta *et al.* 2010).

A large number of AMPs has been found within late phagosomes, such as defensins, cathelicidins, and ubiquitin-derived AMPs, which are responsible for the degradation of bacterial pathogens. The anti-bacterial activity of cathelicidins is specifically effective for *Shigella* spp, *S. aureus*, Group A and B *Streptococci* (Guani-Guerra, Santos-Mendoza *et al.* 2010). The AMPs also play a role of pro-inflammatory and chemotactic activities on immune cells, which may modulate the immune response, serving as a bridge between the innate and adaptive immune responses (Ganz 2003). AMPs probably synergize with other degradative enzymes, including cysteine, serine, and aspartate-proteases (such as the cathepsins B, L, and

D), peptidases, glycosidases (such as N-acetylglycosaminidase, β -glucuronidase, β -galactosidase) as well as (phospho-) lipases.

Other anti-bacterial activity mechanisms also exist, like the sequestration of essential nutrients for bacterial pathogens. For example, lactoferrin sequesters irons in infected macrophages to limit iron availability to intracellular pathogens (Cassat and Skaar 2013). Natural resistance-associated macrophage protein 1 (NRAMP1) is expressed in the late phagolysosome and has been characterized as a transporter for divalent metals (Fe^{2+} , Zn^{2+} , and Mn^{2+}) and protons. For example, Mn^{2+} and Zn^{2+} are needed by microbes as part of the antioxidant defense protein ($\text{Mn}^{2+}/\text{Zn}^{2+}$ -superoxide dismutase) and as a catalytic component of several central proteins (Weiss and Schaible 2015). In addition, these ions are the cofactors for some important enzymes to exert their anti-microbial activity (Hood and Skaar 2012).

Late phagosomes eventually transform into phagolysosome via the fusion with lysosome. This is the last stage for phagosome maturation. This compartment can be defined by its enrichment with an abundance of degradative enzymes including proteases, lipases, nucleases, phosphatases, glycosidases and LAMPs. For example, cathepsin D, a protease implicated with the degradation of protein, is used as a specific marker for immunofluorescence. A drastic drop of pH (~ 4.5) also occurs in this compartment due to the presence of V-ATPase. In addition, the accumulation of H^+ in the maturing phagosome is enhanced by the decreased passive permeability or “proton leak” of the limiting phagosomal membrane, which assists in the maintenance of an acidic pH (Canton, Khezri *et al.* 2014). The phagosome acidification is essential to the function of this organelle. Luminal acidification is not only a consequence of phagosome maturation but is also a prerequisite as indicated by the fact that the presence of weak bases (e.g., NH_4Cl) or agents that specifically inhibit the function of the v-ATPase also interrupt phagosome maturation (Canton, Khezri *et al.* 2014). Furthermore, some lysosomal proteases, such as cathepsins, catalyze optimally at this low pH while the metal transporter NRAMP1 also requires phagosomal H^+ for its activity at low pH (Jabado, Jankowski *et al.* 2000; Flannagan, Heit *et al.* 2015).

4.2 Autophagy

Autophagy occurs constitutively at basal rate under normal cellular conditions to maintain homeostasis for intracellular recycling and metabolic regulation (Ravanan, Srikumar *et al.* 2017). Autophagy is stimulated during various pathological and physiological conditions such as starvation, growth factor deprivation, ER stress, and intracellular pathogen infection

(He and Klionsky 2009). The cells adapt to these conditions by converting these signals into several anabolic and catabolic responses. Autophagy degrades the damaged organelles, long-lived proteins and even invasive bacterial pathogens. Indeed, autophagy has been extensively demonstrated to confer resistance to numerous bacterial pathogens, most notably by the direct destruction of internalized bacteria residing in the cytosol or damaged vesicles, a mechanism also named xenophagy (Bauckman, Owusu-Boaitey *et al.* 2015).

Autophagy is initiated by the formation of double-membrane-bound vesicles, called autophagosomes, which sequester cytoplasmic material in a non-degradative compartment (Rubinsztein, Codogno *et al.* 2012). The outer-membrane of the autophagosome fuses with the lysosomal membrane, and the inner vesicle, together with its cargo, is degraded (Randow and Munz 2012). During autophagosome formation in eukaryotic cells, a membrane is marked with phosphatidylinositol-3-phosphate (PI3P) by a complex of the type III PI3 kinase Vps34 which induces activation of protein Atg5, Atg12, and Atg16L1. At the PI3P marked site membrane elongation results in the formation of an isolation membrane (also known as a phagophore), which eventually closes around the substrate into a completely sealed autophagosome. Then, the ATG5-ATG12-ATG16L1 complex mediates the covalent attachment of ATG8/LC3B to phosphatidylethanolamine (PE) in the isolation membrane (Saitoh and Akira 2010; Randow and Munz 2012) (**Figure 10 and 12**). Once the autophagosome is completed, it rapidly fuses with lysosomes in a Rab7-dependent way, following subsequent degradation.

However, some intracellular pathogens develop various strategies to avoid autophagy. For example, when *L. monocytogenes* escapes into the cytosol, it expresses a surface protein ActA to polymerize actin at the bacterial pole, which mediates bacterial motility. ActA also prevents the coating of *L. monocytogenes* with ubiquitin and thereby interferes with autophagy, possibly because of ActA-recruited host protein (Arp2/3 complex and Ena/VASP) which disguise the bacterial surface (Yoshikawa, Ogawa *et al.* 2009). The phospholipases PlcA and PlcB, which are critical for *Listeria* escape from the double membrane surrounding the bacteria following cell-to-cell spread, also reduce autophagic flux and PI3P levels, causing pre-autophagosomal structure stalling and preventing efficient targeting of cytosolic bacteria (Tattoli, Sorbara *et al.* 2013).

Furthermore, some intracellular bacterial pathogens involve various mechanisms to take advantage of autophagy, converting autophagosomes into a niche that sustains bacterial

replication and allows unorthodox trafficking. For example, *S. aureus* can proliferate in double- or multilamellar LC3⁺ vesicles, which most probably due to canonical autophagosomes failing to acidify and mature (Schnaith, Kashkar *et al.* 2007; Mestre, Fader *et al.* 2010). Schnaith *et al.* observe that *S. aureus* fails to grow in *atg5*^{-/-} mouse embryonic fibroblasts, while autophagy induction by rapamycin enhances the proliferation. However, how these bacteria co-opt autophagy and how autophagy contributes to bacterial growth remains unknown.

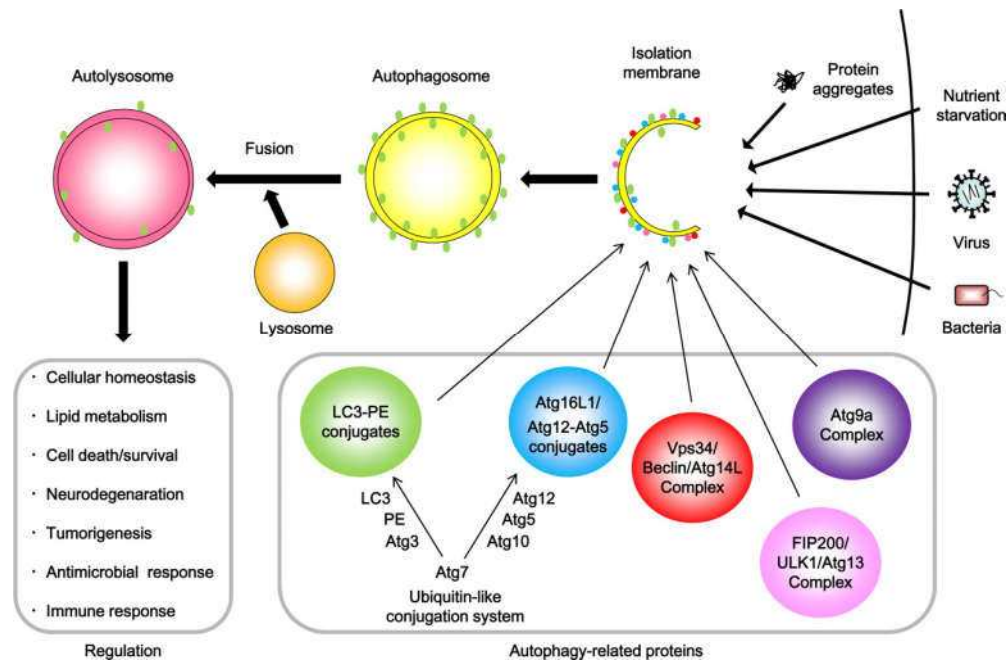


Figure 12: Model of autophagosome formation. Autophagy is stimulated during various pathological and physiological conditions such as starvation, growth factor deprivation, ER stress, and intracellular pathogen infection. Autophagy is initiated by the formation of double-membrane-bound vesicles, called autophagosomes, which sequester cytoplasmic material in a non-degradative compartment. Autophagy-related proteins (Atgs) are recruited to the isolation membrane, a source membrane of autophagosomes, and play a vital role in driving the membrane trafficking necessary for the generation of autophagosomes. Autophagosomes then fuse with lysosomes to become autolysosomes, leading to the degradation of the constituents trapped inside. Taken from Saitoh and Akira 2010.

Chapter II: *Staphylococcus aureus*

1. General

S. aureus is a non-spore forming, non-motile, facultative anaerobic Gram-positive bacterium. *S. aureus* is both a commensal bacterium and a human pathogen. Indeed, approximately 30% of the human population is asymptotically colonized with *S. aureus*. The bacterium is mainly found in the anterior nares, but can also colonize the skin and mucosa (Wertheim, Melles *et al.* 2005; Sivaraman, Venkataraman *et al.* 2009). *S. aureus* can also cause a wide variety of infections, ranging from mild skin infections, to life-threatening diseases such as necrotizing pneumonia and bacteremia. *S. aureus* is a leading cause of bacteremia and infective endocarditis as well as osteomyelitis, skin and soft tissue infections (SSTIs), pneumonia, and device-related infections. Methicillin-resistant *S. aureus* (MRSA) strains were identified in 1961, only two years after Methicillin was used (Boucher and Corey 2008). MRSA strains are the most frequently isolated bacterial pathogens in hospital-acquired infections (DeLeo, Otto *et al.* 2010; Stryjewski and Corey 2014). Hospital associated MRSA (HA-MRSA) infections account for over 170 000 bacterial infections (nearly half of all bacterial pathogen infections), causing 5 400 extra deaths and over a million extra days of hospitalization in Europe each year (Gould, David *et al.* 2012).

The emergence of community associated MRSA (CA-MRSA) infections has dramatically increased. In contrast to HA-MRSA infections, for which there is a predisposing risk factor or condition, CA-MRSA infections can also occur in healthy individuals (Herold, Immergluck *et al.* 1998), suggesting that CA-MRSA strains have enhanced virulence compared with traditional HA-MRSA strains. In particular, *S. aureus* USA300 has now emerged as the predominant cause of CA-MRSA infections in the United States and is continuously spreading around the world (Nimmo 2012).

With the increasing incidence of both community and hospital acquired staphylococcal infections and the prevalence of multidrug resistance strains, *S. aureus* infections remain a huge challenge to clinicians and scientists (Vandenesch, Naimi *et al.* 2003; Tristan, Bes *et al.* 2007).

2. Virulence factors

S. aureus virulence is related to the production of a wide variety of virulence factors that are responsible for adhesion to surface /tissues, lysis of host cells, tissue invasion and destruction (Foster and Hook 1998; Lowy 1998; Dinges, Orwin *et al.* 2000). Importantly, many specific virulence factors produced by *S. aureus* are dedicated to specifically manipulate both innate and adaptive immune responses (Rooijackers, Ruyken *et al.* 2005; Haupt, Reuter *et al.* 2008; Thammavongsa, Missiakas *et al.* 2013; Stapels, Ramyar *et al.* 2014).

We will briefly recall below the main and best studied virulence attributes of *S. aureus* (**Table 2**).

Adhesins. The pathophysiology of *S. aureus* infections usually begins with the colonization in host skin and/or mucosa by its adhesion factors. These factors allow the bacterium to bind to the components of the extracellular matrix of the host, but also to surgical materials (catheter, prosthesis, mechanical valve ...). These factors, also called microbial surface components recognizing adhesive matrix molecules (Foster, Geoghegan *et al.* 2014), include staphylococcal protein A (SpA), fibronectin-binding proteins A and B (FnbpA and FnbpB), clumping factor proteins (ClfA and ClfB) and collagen-binding protein (Cna).

Toxins. *S. aureus* is also able to produce three types of super-antigenic toxins: staphylococcal enterotoxins, the staphylococcal enterotoxin-like proteins, and toxic shock syndrome toxin-1 (Xu and McCormick 2012). So far, more than 20 staphylococcal superantigens (SAGs) have been characterized and at least 80% of clinical strains harbor at least one SAg gene, although most strains encode many (Xu and McCormick 2012).

Panton-Valentine leukocidins (PVL) of *S. aureus* are protein toxins composed of two subunits, named LukF-PV and LukS-PV (32 kDa and 38 kDa, respectively). PVLs can induce cell lysis on target cells. Of note, PVL genes are present in only a small percentage (~5%) of overall *S. aureus* clinical strains, whereas they are strongly associated with CA-MRSA strains (~85%), in particular those causing pneumonia, and skin and soft tissue infections (Naimi, LeDell *et al.* 2003; Yoong and Torres 2013).

Phenol-Soluble Modulins (PSMs) are a family of amphipathic and α -helices peptides (Peschel and Otto 2013; Cheung, Joo *et al.* 2014). In *S. aureus*, there are two major families of PSMs: PSM β (43-45 amino acids) and PSM α (20-25 amino acids) (Wang, Braughton *et al.* 2007). δ -hemolysin (Hld), encoded by the *agr* locus (see below), also belongs to the family of PSM α .

α -hemolysin (Hla) is a secreted 33 kDa protein toxin that is able to lyse cells by forming pores on the target cells via interaction with its receptor ADAM10 (Powers, Kim *et al.* 2012; Berube and Bubeck Wardenburg 2013). This toxin, produced massively by the strains of USA 300, contributes to the pathogenicity of these strains by favoring the bacterial invasion by destruction of the epithelial and endothelial barriers. On the other hand, β -hemolysin (Hlb) is a sphingomyelinase, which does not involve pore-forming in target cells, but causes destabilization of the lipid bilayer and an alteration of its fluidity leading to cell death (Katayama, Baba *et al.* 2013). The majority of *S. aureus* strains possess the *hly* gene encoding Hlb. However, since this gene constitutes a bacteriophage insertion site, more than 80% of human clinical isolates do not express Hlb (Roetzer, Haller *et al.* 2016).

VIRULENCE FACTORS	PUTATIVE FUNCTIONS
CELL SURFACE FACTORS	
<i>Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)</i>	
Staphylococcal protein A (SpA)	Bind to IgG, interfering with opsonization and phagocytosis
Fibronectin-binding proteins (FnbpA and FnbpB)	Mediate attachment to fibronectin and plasma clot
Collagen-binding protein	Adherence to collagenous tissues and cartilage
Clumping factor proteins (ClfA and ClfB)	Mediate clumping and adherence to fibrinogen in the presence of fibronectin
<i>Capsular polysaccharides</i>	Reduce phagocytosis by neutrophils; enhance bacterial colonization and persistence on mucosal surfaces
<i>Staphyloxanthin</i>	Resistance to neutrophil reactive oxidant-based phagocytosis
SECRETED FACTORS	
<i>Superantigens</i>	
Staphylococcal enterotoxins (SEA, B, C, D, E, G and Q)	Massive activation of T cells and antibody presenting cells
Staphylococcal enterotoxin-like proteins (SEL)	
Toxic shock syndrome toxin-1 (TSST-1)	
<i>Cytolytic toxins</i>	
α -hemolysin	Induce lysis on a wide spectrum of cells, mainly platelets and monocytes
β -hemolysin	Hydrolysis of sphingomyelin of the plasmatic membrane of monocytes, erythrocytes, neutrophils and lymphocytes; make cells susceptible to other lytic agents
γ -hemolysin	Induce lysis on erythrocytes and leukocytes
Leukocidin family	
Leukocidins E/D and M/F-PV	Induce lysis on leukocytes
Panton-Valentine leukocidin (PVL)	

<i>Phenol-Soluble Modulins (PSMs)</i>	Induce lysis of red and white blood cells, stimulate inflammatory responses and contribute to biofilm development and the dissemination of biofilm-associated infections
<i>Various exoenzymes</i>	
Lipases	Inactivate fatty acids
Nucleases	Cleave nucleic acids
Proteases	...
Serine proteases (e.g. exfoliative toxins ETA and ETB)	Inactivate neutrophil activity; Activate T cells (only ETA and ETB)
Cysteine proteases (e.g. staphopain)	Block neutrophil activation and chemotaxis
Aureolysin (metalloprotease)	Inactivates antimicrobial peptides
Hyaluronidase	Degrade hyaluronic acid
Staphylokinase (SAK)	Activates plasminogen; inactivates antimicrobial peptides

Table 2: Virulence factors involved in the pathogenesis of *S. aureus* and respective putative functions. From Costa Rita *et al.* 2013.

3. Virulence regulators

Production of *S. aureus* virulence determinants is controlled by several global regulatory loci, which include *agr*, *sar*, *sigB*, *rot*, *arlR/S*, *svrA*, *saeR/S* and the metabolite-responsive regulators (Goerke, Fluckiger *et al.* 2005). The virulence gene regulators could affect the expression of target genes directly, by binding to their promoters, or indirectly, via other regulators. Here, we will describe two important global virulence regulators *agr* and *sigB*, and briefly mention some other metabolite-responsive virulence regulators.

The *agr* operon

The accessory gene regulator system (*agr*) operon was discovered by Recsei *et al.* in 1986 (Recsei, Kreiswirth *et al.* 1986). The *agr* operon constitutes a global regulatory system that controls cell density-dependent virulence factor expression. In acute mice infection models, including skin and soft tissue infections (SSTIs), intracranial abscesses and necrotizing pneumonia, *agr* activity is necessary (Bubeck Wardenburg, Patel *et al.* 2007; Novick and Geisinger 2008; Cheung, Wang *et al.* 2011). On the other side, there is a link between *agr* dysfunction and persistent colonization in nosocomial infections with *S. aureus* (Fowler,

Sakoulas *et al.* 2004; Shopsin, Drlica-Wagner *et al.* 2008; Shopsin, Eaton *et al.* 2010; Kang, Cho *et al.* 2015).

The *agr* operon consists of two divergent promoters, P2 and P3. Expression from P2 produces the components of a quorum-sensing system (AgrB, D, C, A) and expression from P3 produces the *agr* effector molecule RNAIII (**Figure 13**). RNAIII is responsible for the regulation of various virulence factors, leading to a switch from expression of cell-surface associated proteins, such as staphylococcal protein A (SpA) and fibronectin-binding proteins A and B (FnBPA and FnBPB), to secreted toxins such as α -haemolysin (Hla) and δ -haemolysin (Hld, encoded within the RNAIII transcript), phenol-soluble modulins (PSMs), and Pantone–Valentine leucocidin (PVL) (Novick and Geisinger 2008; Queck, Jameson-Lee *et al.* 2008; Kane, Carothers *et al.* 2018). At low cell density, the *agr* genes are continuously expressed at basal levels (Recsei, Kreiswirth *et al.* 1986). A signal molecule, the autoinducing peptide (AIP), produced and secreted by the bacteria, accumulates outside of the cells. When the cell density increases and the AIP concentration reaches a threshold, it activates the *agr* response by increasing RNAIII expression. AgrB and AgrD are essential for the production of AIP that functions as a signal for quorum sensing. AgrB is a transmembrane protein, involved in the proteolytic processing of AgrD and facilitating the export of the processed AgrD peptide.

There are four allelic variants (types I–IV) of *agr*, each encoding a distinct AIP molecule, which functions as a specific ligand for the corresponding sensor histidine kinase AgrC, but as an inhibitor of other AgrC variants. AgrC possesses an extracellular N-terminal domain and seven trans-membrane domains. Binding of AIP induces a conformational change within the cytoplasmic helix that links the sensor and kinase domains of AgrC, activating its autophosphorylation (Novick and Geisinger 2008; Wang, Quan *et al.* 2014). However, binding with heterologous AIP (from *S. aureus* expressing one of the three other allelic variants of Agr) causes the helical linker to twist in the opposite direction, preventing auto-kinase activity (Wang, Zhao *et al.* 2014).

AgrC and AgrA act as a classical two-component system (TCS), where AgrC transfers the phosphoryl group, carried on a Histidine residue to an Aspartate residue of the conserved response regulator AgrA. Phosphorylated AgrA dimers bind to the *agr* intergenic region (*agr*-IR) between P2 and P3 and up-regulate expression of these promoters (Novick and Geisinger 2008; Painter, Krishna *et al.* 2014).

Recently Sully *et al.* identified a small molecule, designated Savirin (for *S. aureus* virulence inhibitor), which inhibits *agr*-associated virulence gene expression by preventing AgrA binding

to the *agr*-IR (Sully, Malachowa *et al.* 2014). ω -Hydroxyemodin (OHM), a small compound isolated from *Penicillium restrictum* is also able to inhibit the quorum sensing function of the *agr* operon, by binding to the *agrA* promoter and preventing interaction with DNA (Daly, Elmore *et al.* 2015). Another inhibitor of the Agr activity has been developed, named Solonamide B, which prevents AgrC activation in all allelic variants of *agr*, hence inhibits quorum-sensing and subsequent virulence gene expression via *agr* locus (Nielsen, Mansson *et al.* 2014).

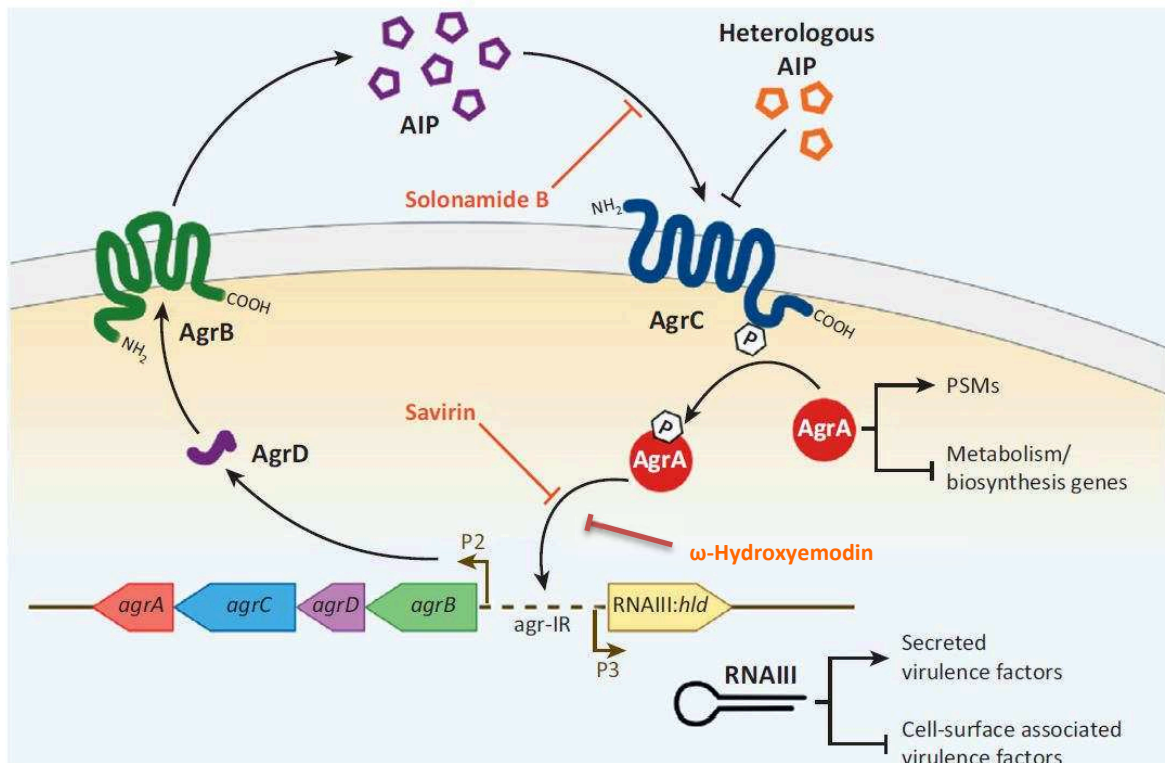


Figure 13: The model of Agr quorum-sensing accessory gene regulator system. From Painter *et al.* 2014. The *agr* operon consists of two divergent promoters, P2 and P3. Expression from P2 produces the components of a quorum-sensing system (AgrB, D, C, A) and expression from P3 produces the *agr* effector molecule RNAIII. RNAIII is responsible for the regulation of various virulence factors, leading to a switch from expression of cell-surface associated proteins to secreted toxins. A signal molecule, the autoinducing peptide (AIP), produced and secreted by the bacteria, accumulates outside of the cells. When the cell density increases and the AIP concentration reaches a threshold, it activates the *agr* response by increasing RNAIII expression. AgrB and AgrD are essential for the production of AIP that functions as a signal for quorum sensing. AgrB is a transmembrane protein, involved in the proteolytic processing of AgrD and facilitating the export of the processed AgrD peptide. Savirin, which inhibits *agr*-associated virulence gene expression by preventing AgrA binding to the *agr*-IR. ω -Hydroxyemodin, a small compound isolated from *Penicillium restrictum* is also able to inhibit the quorum sensing function of the *agr* operon, by binding to the *agrA* promoter and preventing interaction with DNA. Another inhibitor of the Agr activity has been developed, named Solonamide B, which prevents AgrC activation in all allelic variants of *agr*, hence inhibits quorum-sensing and subsequent virulence gene expression via *agr* locus.

SigB

The alternative transcription factor σ^B (SigB) is known to affect the expression of numerous genes (including virulence factors and regulatory elements), upon changes in the environment (starvation, heat shock, osmotic shock, etc...). Wu *et al.* first described and sequenced a chromosomal cluster of four *S. aureus* genes comprising *sigB* (*rsbU*, *rsbV*, *rsbW*, *sigB*) (Wu, de Lencastre *et al.* 1996) (**Figure 14B**). The regulation of the σ^B activity is modulated by the *rsbU*, *rsbV*, and *rsbW* gene products. RsbW acts as an anti- σ^B factor. RsbV is an anti-anti- σ^B factor, which competes with σ^B for RsbW. RsbV is modulated by both the phosphatase RsbU and the phosphorylase RsbW (Senn, Giachino *et al.* 2005; Pane-Farre, Jonas *et al.* 2009). In *S. aureus*, σ^B is activated via the RsbU-dependent pathway (Pane-Farre, Jonas *et al.* 2009). RsbW inactivates σ^B through the complex RsbW- σ^B , and thus suppresses the aggregation of the σ^B subunit with the RNA polymerase (RNAP) core enzyme. During the exponential growth phase, RsbV is phosphorylated and inactivated by RsbW. Consequently, the σ^B factor is sequestered in a RsbW- σ^B complex. In response to environmental stress, RsbV is dephosphorylated by RsbU, leading to the formation of the highly specific RsbV-RsbW complex and the release of activated σ^B factor, which is able to bind to the RNAP core enzyme to form an active σ^B holoenzyme (Bronner, Monteil *et al.* 2004). Nevertheless, the *rsbU* natural mutants (e.g. RN6390) can still produce a functional σ^B protein, suggesting that the activity of σ^B is also dependent on factors other than RsbU. Thus, it has been suggested that a second σ^B activation pathway that is independent of RsbU but dependent on RsbV exists (Palma and Cheung 2001) (**Figure 14A**).

Various *S. aureus* genes, most of which are involved in stress response, have been shown to be controlled by σ^B (Gertz, Engelmann *et al.* 2000; Pane-Farre, Jonas *et al.* 2009). Notably, SigB is involved in bacterial aggregation by modulating the expression of the genes encoding clumping factor or other adhesins. Of interest, SigB was also described to down-regulate the *agr* system (Bischoff, Entenza *et al.* 2001), which is most likely responsible for the enhanced inflammatory activity. The down-regulated *agr* system helps the bacteria to form biofilm and silences bacterial aggressiveness for persistence within host cell (Boles and Horswill 2008; Grundmeier, Tuchscher *et al.* 2010). Remarkably, Tuchscher showed that SigB also played a crucial role during bacterial adaptation during long-term persistence (Tuchscher, Bischoff *et al.* 2015).

Initially description of persistence phenomenon is almost 60 years ago, Bigger *et al.* observed a subpopulation of staphylococci is able to survive with treatment of penicillin without acquiring resistance-conferring genetic changes, these cells regrow a new population that is sensitive to the antibiotic (Bigger 1944). It has since been observed in many bacterial species. These microorganisms, called persisters, are dormant phenotypic variants of bacterial cells that are tolerant to killing by antibiotics (Lewis 2010). Persisters are associated with chronic infections and antibiotic treatment failure. It exists another operational definition for persistence: a subpopulation of microorganisms can persist under certain stressful condition, such as antibiotics treatment, starvation, carbon source limitation and internalization by host cells (Helaine and Kugelberg 2014), which is more widely used.

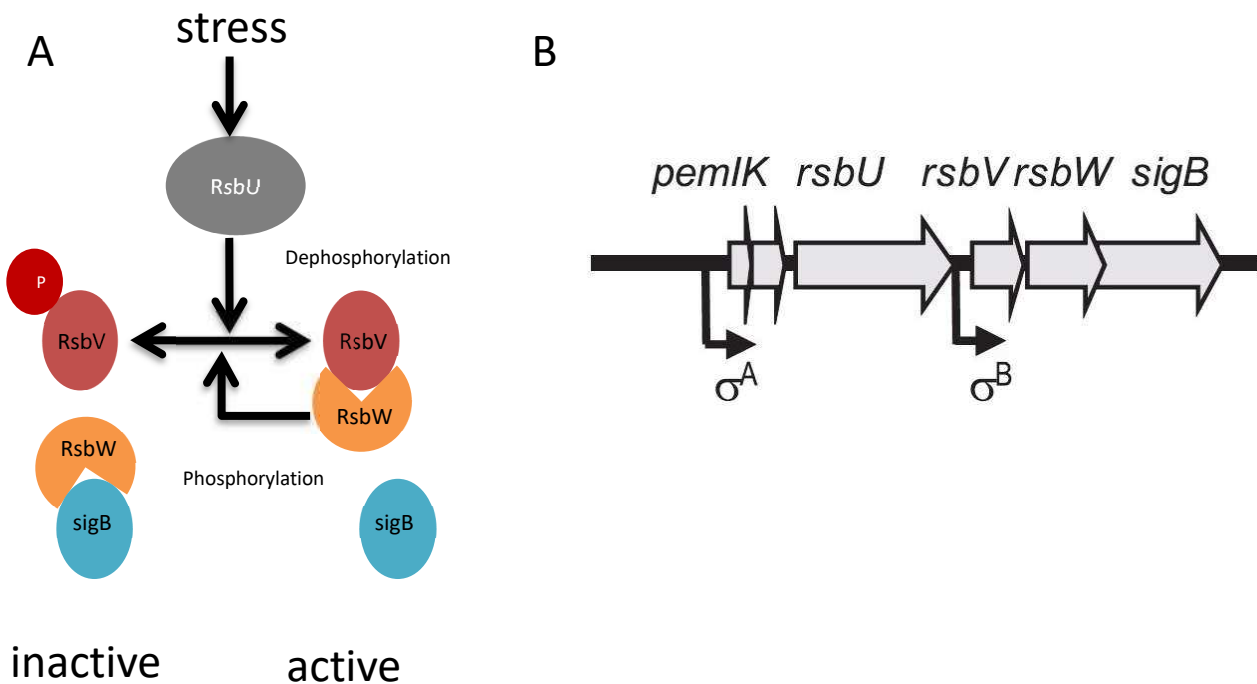


Figure 14: Model of SigB activation and *sigB* operon structures in *S. aureus*. **A)** Model of SigB activation of *S. aureus*. The regulation of the σ^B activity is modulated by the *rsbU*, *rsbV*, and *rsbW* gene products. RsbW acts as an anti- σ^B factor. RsbV is an anti-anti- σ^B factor, which competes with σ^B for RsbW. RsbV is modulated by both the phosphatase RsbU and the phosphorylase RsbW. In *S. aureus*, σ^B is activated via the RsbU-dependent pathway (Pane-Farre, Jonas et al. 2009). RsbW inactivates σ^B through the complex RsbW- σ^B , and thus suppresses the aggregation of the σ^B subunit with the RNA polymerase (RNAP) core enzyme. During the exponential growth phase, RsbV is phosphorylated and inactivated by RsbW. Consequently, the σ^B factor is sequestered in a RsbW- σ^B complex. In response to environmental stress, RsbV is dephosphorylated by RsbU, leading to the formation of the highly specific RsbV-RsbW complex and the release of activated σ^B factor, which is able to bind to the RNAP core enzyme to form an active σ^B holoenzyme. **B)** *sigB* operon structures in *S. aureus*.

Metabolite-responsive regulators

Modification of central metabolic pathways, such as the tricarboxylic acid (TCA) cycle, can affect virulence determinant biosynthesis by feedback and feedforward alteration of enzymatic activities or by activating metabolite-responsive regulators via the alteration of intracellular concentrations of specific metabolites (Chatterjee, Herrmann *et al.* 2007; Somerville and Proctor 2009). Several metabolite-responsive regulators have been identified in *S. aureus* (e.g., CcpA, CcpE, CodY, and Rex) that are involved in the regulation of both metabolism and virulence. For examples, the carbon catabolite protein A (CcpA) responds to glucose-associated metabolic signals (Deutscher, Kuster *et al.* 1995) and inactivation of *ccpA* results in a lower level of RNAIII. The carbon catabolite protein E (CcpE) responds to citrate and inactivation of *ccpE* results in increased staphyloxanthin production and enhanced bacterial virulence in a mouse model of systemic infection (Ding, Liu *et al.* 2014). CodY responds to GTP and branched-chain amino acids and inactivation of *codY* results in derepressed synthesis of the *agr* locus enhanced bacterial virulence of USA300 strain in the mouse model (Majerczyk, Sadykov *et al.* 2008; Pohl, Francois *et al.* 2009; Montgomery, Boyle-Vavra *et al.* 2012; Gaupp, Wirf *et al.* 2016) (**Figure 15**).

Finally, the PPP-responsive regulator RpiR, which catalyzes the reversible isomerization of ribulose-5-phosphate and ribose-5-phosphate, was also suggested to down-regulate expression of RNAIII (Gaupp, Wirf *et al.* 2016). Members of the RpiR family have been identified in both Gram-negative and Gram-positive bacteria, including *E. coli* and *Bacillus subtilis* (Yamamoto, Serizawa *et al.* 2001; Sonenshein 2007). As sugar-responsive regulators, members of the RpiR family of proteins have N-terminal helix-turn-helix DNA binding motifs and C-terminal sugar isomerase binding domains (Zhu, Nandakumar *et al.* 2011). Two of the three homologues (RpiRB and RpiRC) of *S. aureus* positively influence the transcription of the PPP genes *rpiA* and *zwf* (Zhu, Nandakumar *et al.* 2011). The significance of RpiRc was demonstrated in an acute pneumonia model and a systemic abscess formation model, where inactivation of *rpiRc* markedly increased mortality and the bacterial burden in mice (Gaupp, Wirf *et al.* 2016).

cells, osteoblasts, fibroblasts, keratinocytes ... (Strobel, Pfortner *et al.* 2016; Moldovan and Fraunholz 2019). The intracellular environment could protect staphylococci from host's immune system and antibiotics. Infected cells can thus constitute reservoirs likely to play an important role for bacterial persistence in the host and relapses of infection.

Like other intracellular bacteria, the *S. aureus* intracellular lifestyle can be divided into three main phases: entry, survival or replication and exit. See also (Figure 16).

4.1 Entry

Invasion of NPCCs by *S. aureus* is mediated by a zipper-type mechanism and involves several bacterial adhesins. The most dominant staphylococcal adhesins participating to bacterial entry into NPCCs are FnBPs (Dziewanowska, Patti *et al.* 1999; Jevon, Guo *et al.* 1999; Edwards, Potter *et al.* 2011), which can recruit $\alpha_5\beta_1$ integrins by a fibronectin bridge. This interaction is sufficient to induce zipper-type uptake of staphylococci (Sinha, Francois *et al.* 2000). However, FnBP-independent invasion was also observed in *S. aureus*. For example, it has been shown that *S. aureus* Newman strain invaded epithelial cells and fibroblasts by using extracellular adherence protein (Harraghy, Hussain *et al.* 2003). Zapotoczna *et al.* found that the protein IsdB directly bound to β_3 -containing integrins and that this interaction could promote bacterial adhesions to, and internalization by, host cells (Zapotoczna, Jevnikar *et al.* 2013). In addition, the wall teichoic acid (WTA) has been shown to play an important role for nasal colonization, by promoting binding to a type-F scavenger receptor (Weidenmaier, Kokai-Kun *et al.* 2004; Baur, Rautenberg *et al.* 2014). The staphylococcal autolysin was also identified as an adhesion mediating binding to its host cell receptor, the heat shock cognate protein Hsc70 (Hirschhausen, Schlesier *et al.* 2010). Finally, ClfB has been shown to bind to cytokeratins as well as to the α -chain of fibrinogen (Ni Eidhin, Perkins *et al.* 1998; Ganesh, Barbu *et al.* 2011), and Cna to the collagen triple helix (Zong, Xu *et al.* 2005). The internalization process of the pathogen mediated by WTA, ClfB, Cna and other molecules remains unknown so far.

4.2 Survival

After internalization within host cells, *S. aureus* either resides in a vacuolar compartment or in host cytosol, subsequently replicates intracellularly and eventually causes host cell death, leading to bacterial release and dissemination.

4.2.1 Within vacuole

S. aureus has evolved various mechanisms to survive phagocytosis by host cells. Following phagocytosis, *S. aureus* initially resides in a phagosome. Few minutes after uptake, the *S. aureus* containing phagosome (SaCP) acquires early endosomal markers such as the small GTPase Rab5 (Flannagan, Heit *et al.* 2016). Then, SaCP quickly acquires the late phagosomal marker LAMP-1. Other lysosomal markers such as LAMP-2 have been identified in SaCP. However, a fully functional phagolysosomal microenvironment is absent for the internalized *S. aureus*. The reduced acidification of the phagolysosome, associated with failure of phagolysosomal maturation and reduced activation of cathepsin D, β -glucuronidase, and lysosomal integral membrane protein (LIMP-II), were discovered in *S. aureus*-infected macrophages (Jubrail, Morris *et al.* 2016; Tranchemontagne, Camire *et al.* 2016). Jubrail *et al.* suggested that macrophages kill the ingested bacteria at early time post infection. However, these macrophages eventually lose their ability to eliminate *S. aureus* which can then replicate within such “exhausted” macrophages. Other studies have supported this observation (Tranchemontagne, Camire *et al.* 2016; Lacoma, Cano *et al.* 2017).

Of note, *S. aureus* is able to tolerate in acidic environments, which may contribute to its survival in phagolysosomes (Weinrick, Dunman *et al.* 2004). For this, *S. aureus* has evolved various mechanisms that lead to alkalinization of its environment. For example, *S. aureus* can generate ammonia through arginine deiminase, urease, nitrite and nitrate reductase. Inside host phagocytes, the bacteria also suffer the oxidative burst provoked by the rapid release of reactive oxygen species (ROS) and reactive nitrogen species (RNS). *S. aureus* has developed strategies to resist ROS and RNS by producing the carotenoid staphyloxanthin and by expressing protective enzymes such as catalase, superoxide dismutase and peroxiredoxins (Karavolos, Horsburgh *et al.* 2003; Clauditz, Resch *et al.* 2006; Cosgrove, Coutts *et al.* 2007; Richardson, Libby *et al.* 2008). *S. aureus* has also adapted to increase its resistance against antimicrobial peptides (AMPs) like defensins and lysosome by modifying its cell surface wall via up-regulation expression D-alanyl-lipoteichoic acid (DltA, responsible for D-alanine esterification of teichoic acids) and *O*-acetyltransferase A (OatA, responsible for *O*-acetylation of the peptidoglycan) (Peschel 2002; Bera, Herbert *et al.* 2005).

Interestingly, a low phagosomal pH is required for survival and replication of the CA-MRSA *S. aureus* strain USA300 in macrophages (Tranchemontagne, Camire *et al.* 2016) and a functional glycopeptide resistance-associated (GraRS) two-component system has been shown to be crucial for USA300 adaptation and replication with in acidified phagolysosomes

(Flannagan, Kuiack *et al.* 2018) as well as for a stronger resistance to cationic AMPs via up-regulation with the multiple peptide resistance factor (MprF)(Joo and Otto 2015; Flannagan, Kuiack *et al.* 2018).

S. aureus survival in motile phagocytes may be responsible for dissemination of the infection into deeper tissues and infected phagocytes have been considered as possible “Trojan horse” for *S. aureus* dissemination (Thwaites and Gant 2011). During bloodstream infections, *S. aureus* is efficiently sequestered in the liver where bacteria are captured by liver macrophages, Kuffer cells. In these cells, *S. aureus* replicate within acid vesicles, decorated with LAMP-1, for several days, without being detected by innate immune system (Surewaard, Deniset *et al.* 2016). Furthermore, infected neutrophils from the liver can re-enter the circulation and disseminate the pathogen to secondary infection sites (Pollitt, Szkuta *et al.* 2018).

4.2.2 Within cytosol

S. aureus phagosomal escape has been observed in different epithelial cell lines: A549 (human alveolar basal epithelial cells), Hela (human cervix epithelial cell), HEK923 (human embryonic kidney epithelial cell), S9 (upper airway epithelial cell), CFBE (Cystic fibrosis bronchial epithelial cell) as well as in endothelial cell-like line (Eahy.926), NIH/3T3 (fibroblast cell) or keratinocytes lines (such as HaCaT or RHEK-1), suggesting that these cell types are probably a comfortable niche for subsequent cytoplasmic bacterial replication (Jarry and Cheung 2006; Giese, Glowinski *et al.* 2011; Chi, Lin *et al.* 2014; Grosz, Kolter *et al.* 2014; Neumann, Bruns *et al.* 2016; Strobel, Pfortner *et al.* 2016). Of note, phagosomal escape seems to be a strain-dependent process. Generally, the highly cytotoxic strains can translocate to cytoplasm environment whereas non-cytotoxic remain stuck in the phagosome. However, the non-cytotoxic strain Cowan I is also able to induce phagosomal escape in A549 cell line (Strobel, Pfortner *et al.* 2016).

S. aureus phagosomal escape has been linked with *agr* activity (Jarry and Cheung 2006; Kubica, Guzik *et al.* 2008). Jarry *et al.* suggested that the *agr* regulated α -toxin was involved in *S. aureus* phagosomal escape because α -toxin is a pore forming toxin which induces phagosomal membrane disruption (Jarry and Cheung 2006). The role of α -toxin in phagosomal escape has been reported in epithelial cells with cystic fibrosis phenotype as well as in macrophages (Jarry and Cheung 2006; Jarry, Memmi *et al.* 2008; Kubica, Guzik *et al.* 2008). However, in the LCSFN cell line complemented with the wild-type CF transmembrane conductance regulator (CFTR), α -toxin had no effect (Jarry and Cheung 2006).

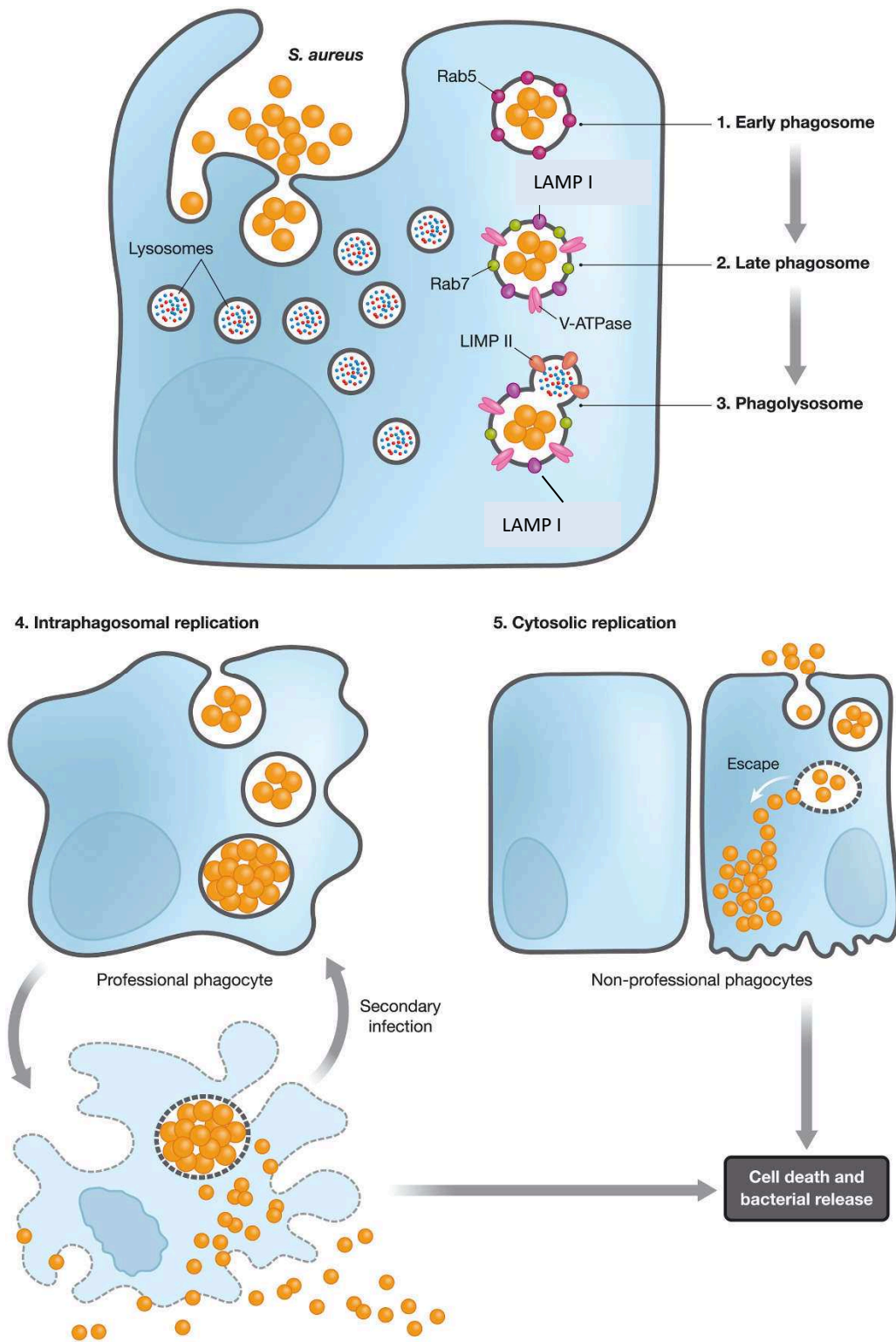


Figure 16: Model of *S. aureus* life cycle in professional and non-professional phagocytes. From Moldovan *et al.* 2019. *Staphylococcus aureus* is internalised by professional and non-professional phagocytes by phagocytosis. (1) Shortly after internalisation, *S. aureus* resides in Rab-5 positive early phagosomes, which quickly mature into

(2) late phagosomes. These are lysosome-associated membrane protein-1 (LAMP-I) and Rab7-positive, and pH is continuously decreasing by v-ATPase activity. (3) The SaCP may fuse with lysosomes forming a phagolysosome. The phagolysosome maturation may be incomplete (no cathepsin D or beta-glucuronidase activity) or delayed. Following phagocytosis, *S. aureus* faces a different fate, depending on the host cell type: (4) in macrophages, *S. aureus* stays confined to the SaCP and replicates in the acidic compartment. The exhausted macrophage dies by a mode of death, which has both apoptotic and necrotic hallmarks. Intracellular bacteria are released and are phagocytosed again. Functional *agr*, *graRS* and *sigB* operons as well as α -toxin (Hla) and the protease aureolysin (Aur) are necessary for survival and persistence in macrophages. (5) In non-professional phagocytes, bacteria do not replicate in the SaCP. Cytolytic strains can escape from the phagosome and replicate in the cytosol. The host cell is killed from within. A functional *agr* system is essential for phagosomal escape. Several bacterial factors such as phenol soluble modulins PSM α and the nonribosomal peptide synthase AusAB or Pantone-Valentine leukocidin (in keratinocytes only) appear to be required.

Another *agr*-regulated virulence factor, PSM α , was identified as a key mediator of *S. aureus* phagosomal escape (Grosz, Kolter *et al.* 2014). As mentioned earlier, PSM is a family of amphipathic and α -helices peptides (Peschel and Otto 2013; Cheung, Joo *et al.* 2014) and δ -toxin, encoded by the *agr* locus also belongs to the family of PSM α . Inactivation of *psma* dramatically decreased the translation of *S. aureus* clinical strain to the cytosol in epithelial cells (Grosz, Kolter *et al.* 2014). Blattner *et al.* also showed that inactivation of PSM-specific ABC-transporter component *pmtC* (Chatterjee, Joo *et al.* 2013) drastically reduced phagosomal escape of *S. aureus* (Blattner, Das *et al.* 2016). Proteomic analysis showed that all phagosomal escape strains were PSM producers (Strobel, Pfortner *et al.* 2016). Of note, whereas overexpressing δ -toxin or PSM β in *S. aureus* RN4220 (non-cytotoxic laboratory strain) led staphylococci phagosomal escape, overexpressing PSM α did not translocate *S. aureus* RN4220 to the cytosol (Giese, Glowinski *et al.* 2011). In addition, membrane disruption by δ -toxin and PSM β depend on the presence of the staphylococcal β -toxin (a sphingomyelinase). However, *S. aureus* strain USA300, an expert for phagosomal escape, does not code for a functional β -toxin, due to the integration of prophage ϕ SA3usa within the β -haemolysin gene (Diep, Gill *et al.* 2006). Alternatively, prophage might be lost (for example, during exposure to phagosomal ROS) and then the strain can produce β -toxin, eventually contributing to phagosomal escape. These results support the importance of PSMs for *S. aureus* phagosomal escape in NPPCs. However, the mode of action of PSM during this process remains currently unknown.

Recently Blattner *et al.* identified two genes, *ausA* and *ausB*, that were required for efficient phagosomal escape (Blattner, Das *et al.* 2016). These two genes encode subunits of the non-ribosomal peptide synthase (NRPS) AusA and a phosphopantetheinyl transferase AusB, which is involved in AusA activation (Zimmermann and Fischbach 2010). The NRPS AusAB

produces three cyclic dipeptides: phevalin, tyrvalin, and leuvalin. During infection in HeLa cells, both *ausA* and *ausB* transposon-insertion mutants showed significant decreased phagosomal escape rate compared with the parent *S. aureus*. The lower escape rates correlated with lower cytotoxicity of the mutants. The mode of action of AusAB or its dipeptide products are still unresolved.

Most current studies suggest that cytosolic replication after phagosomal escape is the predominant fate of *S. aureus* in NPCCs. However, in cystic fibrosis-derived pulmonary epithelial cells, *S. aureus* start replicating 9 hours after their internalization and 24 hours after infection, *S. aureus* were found in vacuoles which had not fused with lysosomes (Kahl, Goulian *et al.* 2000). Thus, another compartment may exist in host cells for bacterial replication.

Role of autophagy in S. aureus intracellular survival. It has been demonstrated that SaCP is targeted by autophagy (Schnaith, Kashkar *et al.* 2007; Mestre, Fader *et al.* 2010; Neumann, Bruns *et al.* 2016). However, the molecular mechanisms for autophagosome formation, autophagosomal escape and intracellular survival of *S. aureus* are still controversial. Notably, Schnaith *et al.* report that *S. aureus* use autophagosomes as a replicative niche, whereas Mestre *et al.* observe replication of *S. aureus* in the cytosol (Schnaith, Kashkar *et al.* 2007; Mestre, Fader *et al.* 2010). Mestre *et al.* notably showed that an α -toxin-deficient mutant failed to recruit the autophagic marker microtubule-associated protein 1 light chain 3 (LC3B) to the SaCP (Mestre, Fader *et al.* 2010), suggesting that α -toxin plays an important role for induction of autophagy. α -toxin may induce SaCP membrane disruption in NPCCs. Autophagy would be then induced to remove these leaky vesicles (Maurer, Reyes-Robles *et al.* 2015).

Neumann *et al.* observed that more *S. aureus* survived inside *atg5* (encoding ATG5 protein which involves in autophagy flux) knockout (KO) than ATG5-reconstituted mouse embryonic fibroblast (MEFs), indicating that autophagy is not essential for intracellular survival (Neumann, Bruns *et al.* 2016). The higher replication of *S. aureus* in *atg5* KO MEFs suggested that bacterial replication inside the host cells can occur without autophagosomes. In addition, transmission electron microscopy of ultrathin sections revealed infected NIH/3T3 fibroblast cells containing dividing bacteria residing in the cytoplasm without a surrounding membrane.

Neumann *et al.* suggested that replication of *S. aureus* inside non-professional phagocytes was independent from autophagy induction. These authors employed the strain SH1000, which is devoid of prophages and has a repaired *rsbU* gene resulting in normal SigB activity (Neumann, Bruns *et al.* 2016). The community acquired methicillin-resistant *S. aureus* (MRSA)

strain MW2 and USA300, which were used by Schnaith *et al.*, contain a *mecA* cassette conferring methicillin resistance and often also the Pantone–Valentine leukocidin (PVL) pore-forming toxin (Neumann, Bruns *et al.* 2016). In conclusion, the different strains used in the study are likely to express virulence factors and pore-forming toxins at different levels which probably affects the interplay between *S. aureus* and the host cell.

4.3 Exit

Following *S. aureus* intracellular replication, host cells die by programmed cell death (including apoptosis, pyroptosis and necroptosis) and necrosis, thereby releasing *S. aureus* to extracellular environment (Munoz-Planillo, Franchi *et al.* 2009; Flannagan, Heit *et al.* 2015; Flannagan, Heit *et al.* 2016; Man, Karki *et al.* 2017).

Apoptosis is a programmed cell death procedure that relies on an active cascade of cysteine endopeptidases called caspases (Taylor, Cullen *et al.* 2008). Various virulence factors of *S. aureus* are responsible to host cell death. For example, Haslinger-Loffler *et al.* showed that when overexpress α -toxin in strain Cowan I, which is barely hemolytic, it marked more potent for induction of apoptosis in HUVEC than the parental strain (Haslinger-Loffler, Kahl *et al.* 2005). α -toxin causes leukocytes cell death either by apoptosis or necrosis (Essmann, Bantel *et al.* 2003; Haslinger, Strangfeld *et al.* 2003) and low doses of α -toxin induce apoptosis accompanied by a breakdown of the mitochondrial membrane potential (Haslinger, Strangfeld *et al.* 2003). In contrast, high doses of α -toxin induce cell necrotic death (Essmann, Bantel *et al.* 2003; Haslinger, Strangfeld *et al.* 2003). Meanwhile, PVL induces a rapid caspase-9/caspase-3-dependent cell death in PMN (Genestier, Michallet *et al.* 2005). Besides from apoptosis *S. aureus* is also able to induce pyroptosis. Pyroptosis is induced by the sensing of bacterial molecules through the host inflammasome. The inflammasome activates caspase-1, which leads to interleukin (IL)-1 β and IL-18 secretion and causes cell death (Hybiske and Stephens 2008). *S. aureus* can function as a stimulus for NOD-like receptor protein 3 (NLRP3) inflammasome, however, the molecular mechanism of the stimulating signal is yet known so far.

Since releasing lysosomal protease cathepsin B into the cytoplasm contributes to NLRP3 activation (Bergsbaken, Fink *et al.* 2009), thus, it seems that lysosomal rupture or permeabilization by *S. aureus* toxins releases cathepsin which then activates the

inflammasome. Indeed *S. aureus* α , β , and γ - toxins have been shown to be important for activation of the NLRP3 inflammasome (Munoz-Planillo, Franchi *et al.* 2009).

5. Importance of staphylococcal metabolism during infection

S. aureus is a typical heterotrophic and essentially prototrophic microorganism (Fuchs, Eisenreich *et al.* 2012) which can survive in diverse host environments. This versatility depends on its ability to acquire and utilize nutrients from different sources, but glucose seems to be the preferred one. Glucose uptake is achieved by the glucose-specific phosphotransferase system (PtsG). *S. aureus* is also able to import glucose-6-phosphate by a hexose phosphate transport protein (UhpT) (Park, Kim *et al.* 2015). The central carbon metabolism of *S. aureus* contains the complete glycolytic pathway, TCA cycle and PPP.

S. aureus possesses a quinone-dependent malate oxidoreductase encoded by *mgo2* that may replace the missing TCA cycle enzyme under certain growth conditions (e.g. upon growth on pyruvate or lactate as carbon source). Among the anaplerotic pathways, pyruvate kinase (PYC) and PEP-carboxykinase (PckA) are present. The glyoxylate shunt (GS) is absent, whereas the malic enzyme converting malate to pyruvate is present (Fuchs, Eisenreich *et al.* 2012). In the exponential growth phase, where is a nutrient-rich environment (especially in presence of glucose), the TCA cycle is repressed, and acetyl-CoA is converted predominantly via acetyl-phosphate (acetyl-P) to acetate with ATP generation by substrate-level phosphorylation. Together with carbon substrates of lower energy (e.g. pyruvate or TCA cycle intermediates), acetyl-CoA enters the active TCA cycle. This is accompanied by an increased oxidative phosphorylation activity through the electron transfer chain to remove NADH generated in the TCA cycle.

At the end of the exponential phase, the concentration of an essential nutrient (e.g., glucose) decreases to a level where it can no longer sustain rapid growth. If conditions are favorable, *S. aureus* can enter into post-exponential growth, where it can catabolize of non-preferred carbon sources such as acetate (Somerville, Chaussee *et al.* 2002). The post-exponential catabolism of non-preferred carbon sources requires TCA cycle activity and coincides with a large increase in TCA cycle enzymatic activity (Somerville, Said-Salim *et al.* 2003). Because the TCA cycle provides biosynthetic intermediates, ATP, and reducing potential, the transition to TCA cycle-

driven metabolism dramatically alters the staphylococcal metabolome (Somerville and Proctor 2009).

Growth of *S. aureus* can occur under aerobic and anaerobic conditions, and oxygen, nitrite or nitrate can be used as the final electron acceptors in respiration. During aerobic growth, electrons enter into the electron transport chain from NADH and are transferred to menaquinone by the NADH dehydrogenase complex (Bayer, McNamara *et al.* 2006). Menaquinone transfers the electrons to oxidized cytochrome *c*, generating the reduced form of cytochrome *c*. The electrons are then transferred to oxygen by cytochrome *c* oxidase (this process can also generate ROS), then ATP is produced via ATP synthase complex. The inactivation of the electron transfer chain, either by genetic inactivation (Kohler, von Eiff *et al.* 2003) or by extreme iron restriction (Friedman, Stauff *et al.* 2006), results in a growth phenotype similar to that of anaerobic/fermentative growth. The inactivation of the electron transfer chain produces a clinically relevant phenotype in staphylococci known as the small-colony variant (SCV) phenotype (Proctor, von Eiff *et al.* 2006).

During acute infection, *S. aureus* grows predominantly extracellularly and the above described metabolic capabilities allow a perfect adaption to the varying nutrient conditions that this pathogen may encounter. With the failure of antibiotic treatment or inefficient host response, the staphylococci acute infections will develop to a chronic infection. The intracellular life cycle of *S. aureus* may be involved in persistence and dissemination of the bacteria in the infected host (Garzoni, Francois *et al.* 2007; Kubica, Guzik *et al.* 2008). During some persistent and recurrent *S. aureus* infections, such as osteomyelitis, implant infections and airway infections in cystic fibrosis patients, a SCV phenotype was observed. SCV was also observed from intracellular persistence assay within non-phagocytic host cells such as epithelial cells, endothelial cells and bone cells (von Eiff, Heilmann *et al.* 1997; Atalla, Wilkie *et al.* 2010; Tuchscher, Bischoff *et al.* 2015). SCV appears to express high level of *sigB* and low level of *agr*, and seems to represent a general adaptation response (Mitchell, Brouillette *et al.* 2010).

The presence of SCV may be an important mechanism for chronic infections. Thus, *S. aureus* SCVs have been particularly used as a model to study persistence (Proctor, von Eiff *et al.* 2006; Garzoni and Kelley 2009; Sendi and Proctor 2009; Mitchell, Gattuso *et al.* 2011; Tuchscher, Medina *et al.* 2011; Kalinka, Hachmeister *et al.* 2014; Strobel, Pfortner *et al.* 2016). As recalled earlier, the SCV phenotype is generally associated with the formation of tiny colonies on plates, increased biofilm capacity, antibiotic resistance and culture supernatants of SCVs generally show reduced cytotoxicity on cell cultures. Furthermore, live cell imaging shows that SCVs persist intracellularly mainly in late endosomal compartments without significant replication

(Schroder, Kland *et al.* 2006). A number of “Omics” approaches have been carried out, which show that *S. aureus* SCVs have a reduced metabolic activity (Seggewiss, Becker *et al.* 2006; von Eiff, McNamara *et al.* 2006; Kriegeskorte, Konig *et al.* 2011). Several advances studies have provided further support for the close linkage between metabolism and virulence synthesis (Somerville and Proctor 2009). The reduced metabolic activity of SCV could explain the persistence.

Besides, recent works shed light on lipid pathways of *S. aureus* with its persistent infection. The potential of further deciphering lipid pathways is broad since in addition of being major energy sources and important component of membranes, lipid related molecules released by bacteria are signal molecules sensed by other bacteria (as in quorum sensing) or sensed by host immune cells. Indeed, bacterial lipoproteins and lipopeptides are pathogen-associated molecular pattern (PAMP), which are major agonists for Toll-like receptor (TLR) signaling during innate immune response. In *S. aureus*, lipoproteins are triacylated and bind to TLR1 and 2. Grayczyk *et al.* found that a mutation in the lipoic acid synthetase gene (*lipA*) elicits a hyper-inflammatory response of macrophages (Grayczyk, Harvey *et al.* 2017). Lipoic acid (LA) is a fatty acid related cofactor covalently linked to proteins such as the pyruvate dehydrogenase (PDH) complex yielding lipoyl-PDH (Grayczyk, Harvey *et al.* 2017). Wild type *S. aureus*, but not *lipA* mutant, releases nanomolar quantities of lipoyl-PDH in the culture supernatant, which is able to reduce TLR2-dependent activation of macrophages. It is likely that lipoyl-proteins antagonize lipoprotein activation of macrophage by a competition for TLR binding, inducing immune evasion, which ultimately promote persistence.

More recently, Chen *et al.* demonstrated that, in addition to its known nutrient acquisition function, the Geh secreted lipase is also a potent immunomodulator (Chen and Alonzo 2019). Indeed, Geh hydrolyzes environmental long chain FAs including *S. aureus* own lipoproteins released in the milieu. Thus, by hydrolyzing self-released lipoproteins, *S. aureus* Geh prevents their recognition by host immune cells and fulfills an elegant immune evasion function, which ultimately promotes persistence (Chen and Alonzo 2019).

Chapter III: Adaptation of bacterial pathogens in the cystic fibrosis model

Patients with cystic fibrosis are highly susceptible to chronic colonization by various pathogens including *S. aureus*, which can cause recurrent and persistent infections. By now, *S. aureus* adaptation in the long-term cystic fibrosis infection is still poorly understood.

1. General aspects of bacterial adaptation

When a bacterium is moved from one environment to another, such as from soil to water, or from an external environment to a mammalian host, adaptation is an absolute necessity. The adaptation to a new environment is guided by the selective pressures found in the environment. Host adaptation allows bacteria to switch from an intermittent colonization to a chronic colonization or infection (Sokurenko, Hasty *et al.* 1999). Identification of the selective forces shaping this evolution can inspire new therapeutic for combating bacterial infections. Furthermore, an understanding of how bacteria diversify as they adapt can illuminate the molecular mechanisms of pathogenesis and is crucial for tracking transmission networks.

In general, adaptation includes two aspects: phenotypic acclimation and genetic adaptation. Phenotypic acclimation is based on alterations in gene regulatory mechanisms caused by environmental factors, such as toxins or nutrients, resulting in a phenotypic change without genotypic alterations. This initial adaptive mechanism is quickly brought into play. On the other hand, genetic adaptation is mediated by either acquisition of exogenous genetic material acquired from the environment, a phenomenon known as horizontal gene transfer (HGT), or changes to the pre-existing genetic content by mutation (Nielsen, Bohn *et al.* 2014). Phenotypic acclimation and genetic adaptation have been found in laboratory experiments with *E. coli* and in CF model for *P. aeruginosa* (Cooper and Lenski 2000; Yang, Jelsbak *et al.* 2011).

By now, the study of genomic evolution holds enormous potential for research. By tracking genomic changes that facilitate a pathogen to persist within our bodies, we can identify its weaknesses and design therapeutic strategies against these infections. Additionally, even if within-patient bacterial evolution generates diversity, related sequential isolates recovered from

the same patient are separated by relatively few mutations, enabling straightforward analysis for identifying the genomic basis of phenotypic changes (Rolain, Diene *et al.* 2013). Furthermore, we can use these genomic mutations to track the progression and transmission of pathogens throughout a population (Lieberman, Michel *et al.* 2011; Yang, Jelsbak *et al.* 2011). With the speed and cost of genomic sequencing becoming more and more available and affordable (a few days and less than \$100 per genome, including sample preparation), the potential for rapid insight is enormous.

2. Evolution of *S. aureus* during human infection

The primary niche of *S. aureus* is the human anterior nares but the skin, and mucosa have also been identified as secondary niches (Wertheim, Melles *et al.* 2005; Sivaraman, Venkataraman *et al.* 2009). It is reasonable to assume that *S. aureus* is highly adapted towards asymptomatic carriage in the human nasopharynx, which leads to its long term co-evolution within the human host (van Belkum, Melles *et al.* 2009). In addition to carriage, *S. aureus* can cause opportunistic acute infections at different anatomical sites, or persistent chronic infections such as osteomyelitis, endocarditis or respiratory infections (especially of cystic fibrosis patients). In particular, CF patients are often persistently colonized with *S. aureus* and the majority of CF *S. aureus* infections are caused by single clones as determined by genotyping analysis of sequential isolates (Branger, Gardye *et al.* 1996; Kahl 2010). However, polyclonal infections may occur and periods of strain replacement have been observed over time (Branger, Gardye *et al.* 1996; Kahl 2010). Several evidences for genetic diversification during infection have been reported such as variation of the polymorphic region of the gene encoding staphylococcal protein A (SpA) (Kahl, Mellmann *et al.* 2005) and loss or acquisition of mobile genetic elements (MGE) (Goerke, Matias y Papenberg *et al.* 2004).

Since the emergence of penicillin-resistant strains of *S. aureus* over a half century ago (Kirby 1944), the ability of *S. aureus* to readily acquire resistance to antibiotics has made it complicated to control infection. In particular, methicillin-resistant strains which are resistant to all β -lactam antibiotics are causing a global health problem (DeLeo and Chambers 2009).

β -lactam resistance is mediated by the *mecA* gene which encodes the protein PBP2A. *mecA* is found on a family of MGE known as staphylococcal cassette chromosome *mec* (SCC*mec*) (Witte, Cuny *et al.* 2008). In fact, acquisition of methicillin resistance during infection has not been observed frequently, probably due to the fact that most *S. aureus* infections are

monoclonal. Since different clonal lineages have distinct restriction modification systems, this may eventually limit horizontal gene transfer (HGT) (Waldron and Lindsay 2006). However, several studies have reported the HGT of SCC mec during infection, which eventually leads to the emergence of new methicillin-resistant *S. aureus* (MRSA) strains (Berglund and Soderquist 2008; Bloemendaal, Brouwer *et al.* 2010). The first case report of a vancomycin-resistant *S. aureus* (VRSA) which is also resistant to methicillin, was from a diabetic patient with a co-infection by *S. aureus* and *Enterococcus faecalis* (Chang, Sievert *et al.* 2003). MRSA strains have been shown to acquire vancomycin resistance via transfer of the Tn1546 transposon encoding the vancomycin resistance gene (*vanA*) from a 57.9 Kb multi-resistance conjugative plasmid from the *E. faecalis* strain (Weigel, Clewell *et al.* 2003).

Besides the acquisition of MGE encoding antibiotic resistance, several studies have confirmed, using comparative genomic approaches, that the acquisition of specific mutations were also responsible for the emergence of antibiotic resistance during infection. For example, Mwangi *et al.* investigated the development of multidrug resistance in *S. aureus* during a persistent infective endocarditis infection (Mwangi, Wu *et al.* 2007). The whole genome sequencing (WGS) of isolates obtained during infection, revealed the acquisition of 35 mutations in 31 loci in a time-dependent manner. These events correlated with the emergence of resistance to antibiotics which were used to treat the infection. Mutations associated with the development of resistance to rifampicin, β -lactam antibiotics, and vancomycin have been identified (Mwangi, Wu *et al.* 2007). Overall, this work demonstrated the tremendous power of using WGS of sequential isolates to explore the evolution of bacteria during infections. An understanding of the molecular mechanisms of the emergence of antibiotic resistance during infection, may help therapeutic decisions. Besides the gain of antibiotic resistance, *S. aureus* adapt infections via other mechanisms, such as alteration of *agr* activity and switch to SCV phenotype (see also chapter II above).

Agr. The accessory gene regulator system (*agr*) operon in *S. aureus* was discovered by Recsei *et al.* in 1986 (Recsei, Kreiswirth *et al.* 1986), and *agr* has been shown to play a critical role in virulence in several animal models of infection (Abdelnour, Arvidson *et al.* 1993; Cheung, Eberhardt *et al.* 1994). Interestingly, some clinical isolates of *S. aureus* evolved a dysfunctional *agr* activity during nosocomial infections (HA-acquired) (Fowler, Sakoulas *et al.* 2004). In fact, 15–60% of *S. aureus* isolates from nosocomial infections have been reported to have lost *agr* activity (Shopsin, Drlica-Wagner *et al.* 2008; Traber, Lee *et al.* 2008). In contrast, *agr*-negative community-associated *S. aureus* strains (CA-acquired) have been rarely identified. Of note, Mwangi *et al.* identified a mutation in *agr* which was acquired during exposure to prolonged

antibiotic treatment (Mwangi, Wu *et al.* 2007). Paulander *et al.* showed that *agr*-negative strains have a fitness advantage over *agr*-positive strains in the presence of sublethal concentrations of some antibiotics (Paulander, Nissen Varming *et al.* 2013). Accordingly, the exposure to antibiotics in the hospital setting may select for the loss of function of *agr* and could explain the frequent identification of *agr* mutants from nosocomial infections. Overall, these selected studies reveal a trend whereby antibiotic treatment of hospital infections enhances the selection of mutations which mediate antibiotic resistance. In parallel, as expected during chronic infections, a transition towards a reduced virulence, persistent infection type is observed in *agr*-negative strains.

SCVs. A prototypical reduced virulence state has been recognized by the study of small colony variants (SCVs), which is often associated with chronic osteomyelitis and cystic fibrosis lung infections (Proctor, von Eiff *et al.* 2006; Kahl 2010). By definition, SCVs form small colonies on nutrient agar due to their slow growth and reduced metabolism. SCVs also show reduced sensitivity to antimicrobial killing (Proctor, von Eiff *et al.* 2006). Furthermore, it is well established that SCVs express less toxins, but more surface proteins which could favor long term colonization and persistence (Proctor, von Eiff *et al.* 2006; Tuchscher, Heitmann *et al.* 2010).

In most of the cases, spontaneous SCV are unstable and SCV phenotype is readily reversible after growth in nutrient-rich conditions (Tuchscher, Medina *et al.* 2011), suggesting that the emergence of SCV may be a phenotypic acclimation without genotypic alterations. Indeed, it was shown that *S. aureus* phenotype switching occurs via a constitutive mechanism which is linked to replication (Edwards 2012). A large-scale reversible intra-chromosomal rearrangement is a possible mechanism for emergence of SCV (Cui, Neoh *et al.* 2012).

Besides, stable SCVs have also been identified such as SCV mutants auxotrophic for thymidine, menadione or hemin because of mutations arising in *thyA*, *menD* or *hemB* genes respectively (von Eiff, Heilmann *et al.* 1997; Proctor, von Eiff *et al.* 2006; Chatterjee, Kriegeskorte *et al.* 2008; Lannergard, von Eiff *et al.* 2008).

3. Evolution of *S. aureus* in individual with cystic fibrosis

3.1 The cystic fibrosis disease

Cystic fibrosis (CF) is a fatal autosomal recessive disease that affects many organs including the lungs, pancreas, and sweat glands. CF is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, encoding a chloride ion channel (Riordan, Rommens *et al.* 1989). The mutation of the Cl⁻ channel causes a dysfunction that can be more or less severe resulting in an accumulation of thick dehydrated mucus layer on all mucociliary surfaces in the body (Cheung and Deber 2008). If both CFTR genes are defective (homozygous mutation), there will be no functioning CFTR protein, and the patient will exhibit the symptoms of CF (Derichs 2013). A single defective CFTR gene (heterozygous mutation) is generally asymptomatic (Cheung and Deber 2008). There are more than 1,000 possible changes that can occur in *CFTR* to cause cystic fibrosis, but approximately 70% of all patients with cystic fibrosis have the same mutation ($\Delta F508$), corresponding to a deletion of a single amino acid (a phenylalanine residue at site 508) (Zielenski 2000). CF was not recognized as a separate disease until 1938. Before this time, it was grouped together with Celiac disease (Andersen 1938). CF is the most common autosomal recessive lethal disease in the Caucasian population. The incidence varies by race and ethnicity and is estimated to be 1:3,200 in whites, 1:15,000 in people of African descent, 1:35,000 in people of Asian descent, and 1:9,200 to 1:13,500 in Hispanics (Hamosh, FitzSimmons *et al.* 1998; Rohlf, Zhou *et al.* 2011). In France, one newborn out of 4,200 has cystic fibrosis while 2 million people carry a mutation of the CFTR gene (Farrell 2008).

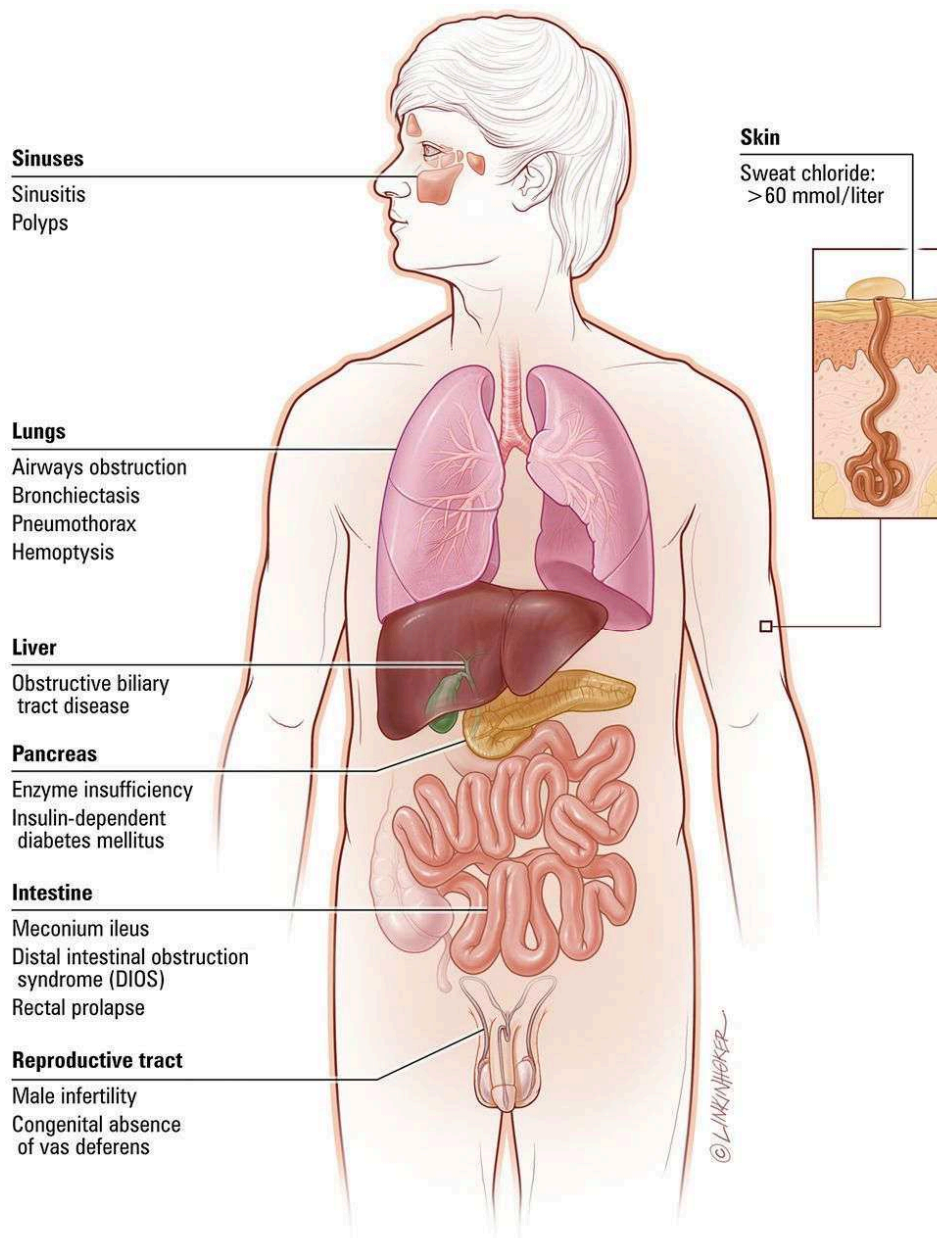


Figure 17: Organs affected by cystic fibrosis. From Paranjape SM *et al.* 2014.

CF is usually symptomatic early in life, sometimes just after birth (Paranjape and Mogayzel 2014). CF is a pleiotropic disease (Paranjape and Mogayzel 2014) (**Figure 17**). Major manifestations impact the respiratory system, pancreas and, more rarely, the intestine or liver. The dysfunction of respiratory system represents the most common cause of mortality in CF patients (Tesse, Cardinale *et al.* 2008).

In CF, the airways are filled with thick, sticky mucus, making breathing difficult for the patient (**Figure 18**). Besides, the thick mucus is also an ideal breeding ground for bacteria and fungi (Paranjape and Mogayzel 2014).

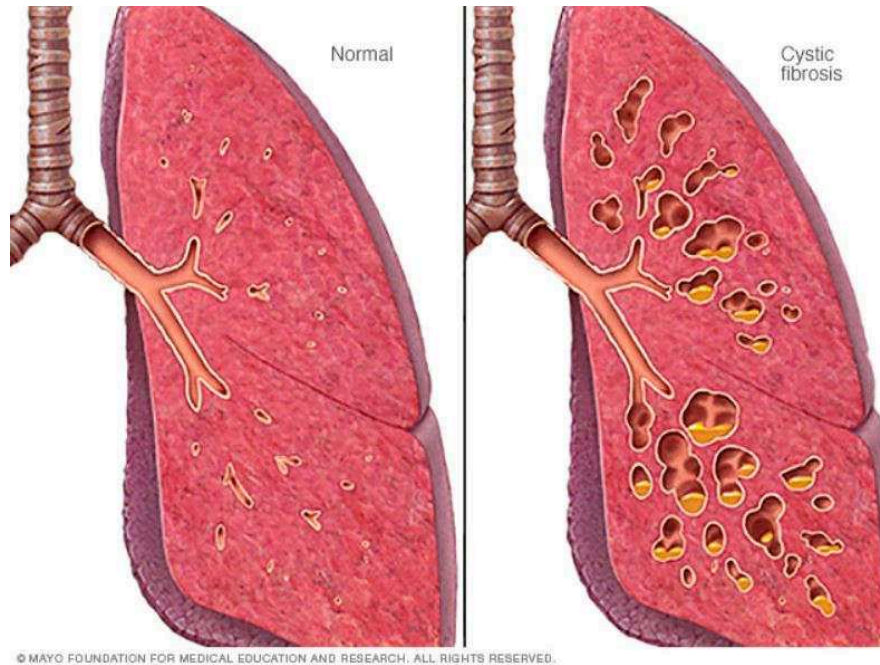


Figure 18: Model of lung from normal people and cystic fibrosis patient. From Mayo Clinic website (<https://www.mayoclinic.org>).

The manifestation of lung disease among CF patients is variable. Most of CF patients do not show symptoms while they are newborns but signs of lung disease, such as wheezing, coughing, and difficulty in breathing, can be seen in infants around six months of age. When the patients get older, coughing becomes a daily occurrence, usually accompanied by sputa. During later stages of the disease, it is common to find blood-streaked sputa.

3.2 Microbiology of cystic fibrosis

As previously mentioned above, the thick mucus in the CF airways provides a perfect home for various bacterial infections. Shortly after birth, initial infection by bacterial pathogens occurs. The impaired function of the mucociliary transport prevents the clearing of the lungs. The antimicrobial peptides located in the airways become inefficient, allowing infections to

take place. Because of these infections, the CF airway displays a prolonged primary inflammatory response.

CF patients followed in cystic fibrosis treatment centers have systematic collections of sputa for microbiological analysis (identification of pathogens and antibiotic susceptibility testing).

A wide range of microorganisms is observed associated with pulmonary infections in CF patients. We will briefly review below the most commonly found bacteria and fungi identified in the sputa of CF patient.

Infants and young children are most likely to be infected with *S. aureus* and *Haemophilus influenzae*. Among older children and adults, *Pseudomonas aeruginosa* is more likely to be found within the airways (Foundation 2017)(**Figure 19**).

H. influenzae is the most common microorganism found in the lower airways at one year of age (Gibson, Burns *et al.* 2003). *H. influenzae* strains infecting CF patients are non-typeable, which means that the vaccine against *H. influenzae* type b has no effect in preventing these infections. Currently, the role of this microorganism in the progressive pulmonary infection and inflammation in CF affected patients has not yet been clearly demonstrated, though it has been proven to be pathogenic in non-CF patients (Gibson, Burns *et al.* 2003).

During childhood, *S. aureus* is the most common pathogen found in CF children. *S. aureus* is often the first organism cultured from the respiratory tract of young children with CF (Armstrong, Grimwood *et al.* 1997). *S. aureus* has gained a lot of attention due to the rise of methicillin resistant *S. aureus* (MRSA). Once MRSA has established itself within the CF airways, it is almost impossible to eradicate through antimicrobial therapy. Studies have investigated how MRSA affects CF patients in comparison to methicillin sensitive *S. aureus* (MSSA). CF patients infected with MRSA as the primary pathogen have lower lung function compared to those infected by MSSA. Meanwhile, patients with MRSA also show a higher rate of hospitalization and use of antibiotics (Ren, Morgan *et al.* 2007).

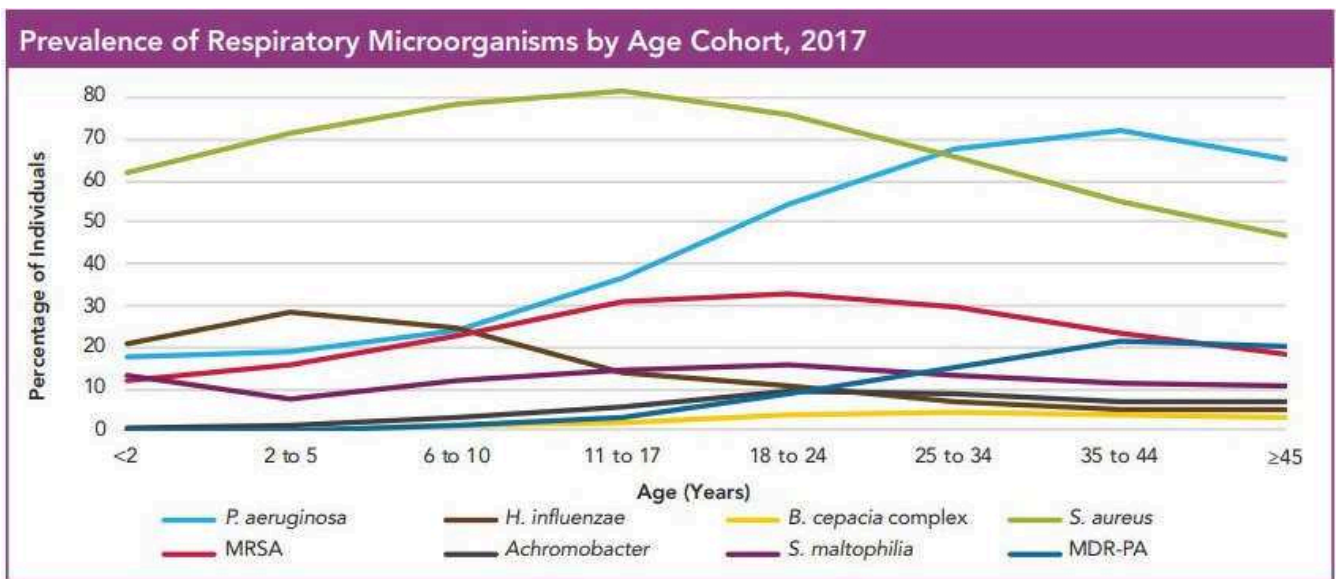


Figure 19: Prevalence of bacterial pathogens in cystic fibrosis. From Cystic Fibrosis Foundation Patient Registry (C) 2017 (USA).

P. aeruginosa is the most significant microorganism associated with CF. About 70% of CF patients will eventually be infected by this pathogen, resulting in poor clinical outcomes (Foundation 2017). The source of *P. aeruginosa* is currently unknown, but the fact that most CF patients harbor different genotypes of the bacteria suggests that the infection comes from the environment (Gibson, Burns *et al.* 2003). Typically, the infection is not initially chronic. Instead, there is a period of recurrent, intermittent colonization by the pathogen until the patient eventually develops a chronic infection. This usually occurs during the late twenties or early thirties in patients. In a chronic infection, there is a continuous growth of *P. aeruginosa* that leads to an increased inflammation. This inflammatory response along with the damages done by the bacteria itself is a major cause of lung function alteration (Folkesson, Jelsbak *et al.* 2012).

Burkholderia cepacia complex (BCC) can also play a role in CF lung function alteration. It can be found in various environments, including agricultural crops and aqueous solutions such as detergent solutions and intravenous fluids commonly found in hospitals. CF patients had a shorter life expectancy when infected by BCC. Now that life expectancy is greater among CF patients, BCC can be found in about 10% of adult CF patients (Gautam, Singhal *et al.* 2011). A pulmonary infection with BCC can persist for months or even years. The infected CF patients may develop bacteremia and die within a few months (Mahenthalingam, Baldwin *et al.* 2008). However, patients with CF may be infected with BCC without a corresponding alteration in

clinical status. Overall, in common with many other opportunistic pathogens, clinical outcomes may vary within CF patients infected with the same BCC strain (Gautam, Singhal *et al.* 2011).

Stenotrophomonas maltophilia and *Achromobacter* spp. are more commonly observed than BCC among CF patients with advanced lung disease but are usually less virulent. There is no significant correlation between these infections and outcomes in CF patients (Goss, Otto *et al.* 2002; Tan, Conway *et al.* 2002).

Finally, fungal colonization and infection of the CF airway in late disease progression is not surprising because these patients receive frequent broad-spectrum antibiotic therapy (Bargon, Dauletbaev *et al.* 1999). *Candida* spp. are the most frequent colonizers, isolated from almost 50 to 75% of patients with CF (Burns, Emerson *et al.* 1998; Bakare, Rickerts *et al.* 2003), but colonization usually does not lead to infection.

3.3 Evolution of *S. aureus* in CF patient

CF airways are an especially suitable model for an *in vivo* study on bacterial evolution and adaptation because within the airways, and especially in the mucus, bacteria are under enormous selective pressure. In this peculiar environment, *S. aureus* has to cope with the presence of polymorphonuclear neutrophils, alveolar macrophages, numerous antibiotic treatments, and competition with other bacteria (Worlitzsch, Tarran *et al.* 2002; Denamur and Matic 2006; Cohen and Prince 2012). Despite these pressures, the same clone of *S. aureus* is able to persist in airway CF patients for months or even years (Kahl, Duebbers *et al.* 2003). The fact that *S. aureus* can persist for a long time supports the notion that *S. aureus* is able to adapt to- and to survive in- the CF airways environment.

There are already several known mechanisms revealing adaptive processes of *S. aureus* in CF airways including: i) phage insertions and excisions (Goerke, Matias y Papenberg *et al.* 2004), ii) changes in the protein A gene (*spa*) repeat region (Kahl, Mellmann *et al.* 2005) as well as, iii) the emergence of SCVs (Kahl, Herrmann *et al.* 1998). In particular, the thymidine-dependent SCVs (TD-SCVs) are observed after prior use of trimethoprim-sulfamethoxazole (SXT) treatment, and genome analyses have shown that random mutations in the highly conserved thymidylate synthase encoding gene *thyA* were involved in the formation of the TD-SCV phenotype (Besier, Zander *et al.* 2008; Chatterjee, Kriegeskorte *et al.* 2008).

McAdam *et al.* investigated the within-host evolution of *S. aureus* during chronic infection of CF patient, via genome analysis of sequential isolates of *S. aureus* obtained from the lung of a patient over a 26-month period and carried out phenotypic analysis of the strains (McAdam, Holmes *et al.* 2011). The majority of the single nucleotide polymorphisms (SNPs) identified in the core genome were specific for each isolate. Furthermore, there was considerable variation in growth rate (leading to small and large colony variants), in hemolytic activity and antibiotic sensitivity. Genetic polymorphisms such as point mutations, insertions and deletions were identified in various loci, including in the genes encoding the global regulators CodY and SigB, which could affect growth and virulence phenotype. In addition, in a late isolate, a mutation in the *fusA* gene (encoding elongation factor G, EF-G) correlated with the resistance to fusidic acid (Nagaev, Bjorkman *et al.* 2001). Finally, consistent with the observations of Goerke *et al.*, phage deletion and/or acquisition was also observed (Goerke, Matias y Papenberg *et al.* 2004). Overall this study, performed on only a small number of isolates, indicated a profound impact on the evolution of *S. aureus* during chronic infection in CF patients.

A longitudinal study of more patients chronically infected with *S. aureus* is needed to further characterize the genomic alterations occurring during chronic infection. Besides genomic studies, proteomic, transcriptomic and metabolomic analyses need to be implemented to comprehensively examine the diversification of infecting populations and the relevance for the pathogenesis of chronic infections in CF.

Objectives

In this thesis work, we addressed the different adaptation strategies of *S. aureus* during chronic infections. Relapse of *S. aureus* infection after a well-conducted antibiotic treatment constitutes a major health issue. One hypothesis is that relapse may result from a lack of access of the antibiotic to the site of infection, especially to the intracellular niche.

1) The time course of *S. aureus* intracellular persistence is still poorly characterized. **The first objective** of my thesis was to better characterize the dynamics of intracellular persistence of the CA-MRSA strain *S. aureus* USA300-LAC in endothelial cells *in vitro*. For this, we quantitatively and qualitatively followed the behavior of intracellular bacteria, by using a combination of confocal and electron microscopy and live-cell imaging. Our results highlighted the heterogeneity of *S. aureus* behavior during cell infection and suggest that intracellular survival is a selective pressure that selects transient slow growing bacteria that are able to persist inside cell cytosol for several days and evade the cells by a mechanism yet to be determined (**Chapter I**).

2) *S. aureus* can dynamically switch phenotypes from a highly aggressive and cytotoxic phenotype to a metabolically inactive phenotype associated with decrease TCA activity. **The second objective** of my thesis was to evaluate the role of pentose phosphate pathway (PPP) during the intracellular persistence of *S. aureus*, by focusing on one of the key enzyme of this pathway, transketolase (TKT) (**Chapter II**). Reprogramming of the TCA cycle notably involves the glycolysis/gluconeogenesis and pentose phosphate pathway (PPP). The contribution of glycolysis/gluconeogenesis to intracellular persistence of *S. aureus* has been already studied. In contrast, the precise role of the PPP is unexplored. The activity of the PPP has been shown to be frequently increased in Gram-positive pathogens in response to environmental stresses and in infection models or in some hospital-acquired isolates.

3) *S. aureus* is the most common pathogen found in children with cystic fibrosis (CF) and *S. aureus* is often the first organism cultured from the respiratory tract of young children with CF. However, very few studies have addressed the adaptations *S. aureus* has undergone in this context. **The third objective** of my thesis was to evaluate the “within host adaptation” of *S. aureus* clinical isolates in this pathogenesis model. By studying serial isolates, we showed that during long-term lung infection, *S. aureus* adaptation occurs through genomic modifications that accumulate over time and lead to major metabolic modifications and protein expression

changes. We also revealed that persistence of *S. aureus* was associated with convergent phenotypic evolution responsible for an increased ability to form biofilm as well as to survive within host cells (**Chapter III**).

Part II Results

**Chapter I: Intracellular survival of
Staphylococcus aureus in
endothelial cells: a matter of growth
or persistence**



Intracellular Survival of *Staphylococcus aureus* in Endothelial Cells: A Matter of Growth or Persistence

Guillaume Rollin^{1,2†}, Xin Tan^{1,2†}, Fabiola Tros^{1,2}, Marion Dupuis^{1,2}, Xavier Nassif^{1,2,3}, Alain Charbit^{1,2*} and Mathieu Coureuil^{1,2*}

¹ Université Paris Descartes, Sorbonne Paris Cité, Bâtiment Leriche, Paris, France, ² Institut National de la Santé et de la Recherche Médicale U1151 - Centre National de la Recherche Scientifique UMR 8253, Institut Necker-Enfants Malades, Equipe 11: Pathogénie des Infections Systémiques, Paris, France, ³ Assistance Publique – Hôpitaux de Paris, Hôpital Necker Enfants Malades, Paris, France

OPEN ACCESS

Edited by:

Catherine Ayn Brissette,
University of North Dakota,
United States

Reviewed by:

Claes Von Wachenfeldt,
Lund University, Sweden
Haike Antelmann,
Freie Universität Berlin, Germany

*Correspondence:

Alain Charbit
alain.charbit@inserm.fr
Mathieu Coureuil
Mathieu.coureuil@inserm.fr

† These authors have contributed
equally to this work.

Specialty section:

This article was submitted to
Microbial Physiology and Metabolism,
a section of the journal
Frontiers in Microbiology

Received: 17 May 2017

Accepted: 04 July 2017

Published: 19 July 2017

Citation:

Rollin G, Tan X, Tros F, Dupuis M,
Nassif X, Charbit A and Coureuil M
(2017) Intracellular Survival of
Staphylococcus aureus in Endothelial
Cells: A Matter of Growth or
Persistence. *Front. Microbiol.* 8:1354.
doi: 10.3389/fmicb.2017.01354

The Gram-positive human pathogen *Staphylococcus aureus* is a leading cause of severe bacterial infections. Recent studies have shown that various cell types could readily internalize *S. aureus* and infected cells have been proposed to serve as vehicle for the systemic dissemination of the pathogen. Here we focused on the intracellular behavior of the Community-Associated Methicillin-Resistant *S. aureus* strain USA300. Supporting earlier observations, we found that wild-type *S. aureus* strain USA300 persisted for longer period within endothelial cells than within macrophages and that a mutant displaying the small colony variant phenotype ($\Delta hemDBL$) had increased intracellular persistence. Time-lapse microscopy revealed that initial persistence of wild-type bacteria in endothelial cells corresponded to distinct single cell events, ranging from active intracellular bacterial proliferation, leading to cell lysis, to non-replicating bacterial persistence even 1 week after infection. In sharp contrast, $\Delta hemDBL$ mutant bacteria were essentially non-replicating up to 10 days after infection. These findings suggest that internalization of *S. aureus* in endothelial cells triggers its persistence and support the notion that endothelial cells might constitute an intracellular persistence niche responsible for reported relapse of infection after antibiotic therapy.

Keywords: *Staphylococcus aureus*, USA300, intracellular persistence, endothelial cells, SCV

INTRODUCTION

The gram-positive bacterial pathogen, *Staphylococcus aureus*, can colonize multiple anatomic sites within its human host, including nares, respiratory tract and skin. Under defined conditions, *S. aureus* is also capable of systemic dissemination and can cause skin and soft tissue infections, pneumonia as well as sepsis, endocarditis, bones and joints infections (Thomer et al., 2016). The rates of infections caused by staphylococci, both community- and hospital-acquired strains, are regularly escalating (Laupland and Church, 2014). However, treatment of these infections is becoming increasingly difficult due to the prevalence of multidrug-resistant strains. In particular, *S. aureus* USA300, an epidemic community-associated methicillin-resistant strain (CA-MRSA), has now emerged as the predominant cause of methicillin-resistant *S. aureus* (MRSA) infections

in the United States and is continuously spreading around the world (DeLeo et al., 2010). Initially classified as strict extracellular pathogen, *S. aureus* is now considered as a non-classical facultative intracellular pathogen (Sendi and Proctor, 2009). Indeed, numerous cell types can ingest *S. aureus* and the bacterium is able to persist within these cells for quite variable periods of time (Fraunholz and Sinha, 2012; Strobel et al., 2016).

Relapse of *S. aureus* infection after a well-conducted antibiotic treatment constitutes a major health issue. One hypothesis is that relapse may result from a lack of access of the antibiotic to the site of infection, especially to the intracellular niche. The molecular mechanisms underlying *S. aureus* virulence have been extensively studied and have been shown to be mediated by a multitude of virulence attributes, including adhesins and toxins that are regulated by complex networks of regulatory systems (Somerville and Proctor, 2009; Felden et al., 2011; Ibarra et al., 2013; Foster et al., 2014). Important contributions have been made over the past 10 years regarding the physiological and metabolic status of intracellular *S. aureus* (Sendi and Proctor, 2009; Tuchscher et al., 2011; Proctor et al., 2014; Thammavongsa et al., 2015). However, the time course of *S. aureus* intracellular persistence is still poorly characterized.

The aim of the present work was to better characterize the dynamic stability of intracellular persistence of the CA-MRSA strain *S. aureus* USA300-LAC. We focused on endothelial cells that have been shown to readily internalize staphylococci *in vitro* (Strobel et al., 2016). We quantitatively and qualitatively followed the behavior of intracellular bacteria, by using a combination of confocal and electron microscopy and live-cell imaging. Our results highlight the heterogeneity of *S. aureus* behavior during cell infection and suggest that intracellular survival is a selective pressure that selects transient slow growing bacteria that are able to persist inside cell cytosol for several days and evade the cells by a mechanism yet to be determined.

MATERIALS AND METHODS

Strains and Culture Conditions

The epidemic clone *S. aureus* USA300-LAC (designated USA300-WT) was provided by the Biodefense and Emerging Infections Research Resources (BEI). The GFP-expressing strain (designated USA300-GFP) was generated by curing the p03 plasmid from USA300-WT and introducing the pCN57-GFP recombinant plasmid (obtained from the BEI) by electroporation (electroporator settings: 2,450 V, 100 Ω , 25 μ F, time constant = 2.3–2.5 ms). Growth curve were performed in BHI broth. Internalization rate and survival inside the EA.hy926 endothelial cell line were similar between the GFP strain and the parental strain.

Construction of the *S. aureus* Δ hemDBL Triple Deletion Mutant

We simultaneously inactivated the three consecutive genes *hemD*, *hemB* and *hemL* of the heme biosynthesis locus in wild-type *S. aureus* USA300 strain and substituted them by the kanamycine resistance gene *nptII* fused with pGro promoter. For this, we used the pMAD-temperature-sensitive

shuttle vector system (Arnaud et al., 2004). Briefly, the recombinant plasmid pMAD- Δ hemDBL was constructed by overlap PCR. First, the two regions (upstream 690 bp, downstream 458 bp) flanking *hemB* and the *nptII* gene (fused with pGro promoter 1,091 bp) were amplified by PCR using the following pairs of primers: i) Primers p1 and p2 amplified the region upstream (hemBUp) of the start codon of the *hemB* coding sequence (p1 5'-CGGAATTCCTGGTTGAGTTAGGCAAAACAGTGAG-3' and p2 5'-TTTAGCTCGACTAATCCATAC AAGGTCCGTGCTGTTTGTCTCC-3'); primers p3 and p4 amplified the region downstream (hemBDown) of the *hemB* stop codon (p3 5'-CCTTCTTGACGAGTTCTTCTGAGCCTGGTG GTGTAATAGTCCAG-3' and p4 5'-CGGGATCCCACCAGGAGAATCCGGCAATCC-3'); iii) primers p5 and p6 amplified the *nptII* gene (p5 5'-TTGTATGGATTAGTCGAGCTAAA-3' and p6 5'-TCAGAAGAAGCTCGTCAAGAAGG-3').

The region hemBUp-npt-hemBDown (2,239 bp) was then amplified by two-step overlap PCR. A first overlap PCR was realized to amplify the region hemBUp-npt, using the hemBUp and npt PCR fragments. The amplified product was cloned in pMiniT 2.0 (using the PCR Cloning Kit New England BioLabs). A second overlap PCR was then realized for the amplification of region hemBUp-npt-hemBDown, using hemBUp-npt and hemBDown PCR fragments. The resulting PCR product was cloned in pMiniT 2.0 (to yield recombinant plasmid pMiniT/hemBUp-npt-hemBDown). This recombinant plasmid was digested with *Bam*HI and *Eco*RI (New England BioLabs) and the *Bam*HI-*Eco*RI hemBUp-npt-hemBDown DNA fragment was finally subcloned into *Bam*HI-*Eco*RI-digested pMAD. All PCR reactions were realized using Phusion High-Fidelity DNA Polymerase (ThermoScientific) and PCR products were purified using QIAquick PCR purification kit (Qiagen).

The pMAD- Δ hemDBL plasmid was first introduced into *E. coli* DH5 α and then transferred to *S. aureus* RN4220 prior to electroporation into *S. aureus* USA300. A standard two-step allelic exchange procedure was used (Arnaud et al., 2004) to create the chromosomal *S. aureus* USA300 Δ hemDBL mutant. The Δ hemDBL mutant strain was finally checked for loss of the corresponding wild-type genes by PCR sequencing (GATC Biotech) using specific primers (p7 5'-CGGAATTCGTCGGAGGCAAAGGCTTATTTG-3' and p8 5'-CCGACTCTGAAACCA GTCATTACTTC-3').

Cell Lines

To study *S. aureus* interaction with endothelial cells we used the EA.hy926 cell line (ATCC[®] CRL-2922[™]), originally derived from human umbilical vein. EA.hy926 cells were grown in Dulbecco's modified Eagle high glucose medium (DMEM, Dominique Dutscher[™]) supplemented with 10% fetal bovine serum (FBS, Eurobio[™]). Culture media contained 1% penicillin/streptomycin and 1% amphotericin B. Antibiotics were removed from culture medium prior to infection.

The human monocytic cell line THP-1 (ATCC[®] TIB-202[™]) was grown in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 5% FBS. Two days before infection, phorbol myristate acetate 200 ng mL⁻¹ (PMA) was added to

the culture medium to induce monocytes differentiation into macrophages.

All cells were incubated in a humidified 5% CO₂ atmosphere at 37°C.

In vitro Infection

Four days prior to infection cells were seeded in plastic 12-well plates in culture medium without antibiotic. At time of infection each well contained a monolayer of $\sim 5 \times 10^5$ cells. Overnight cultures of *S. aureus* in BHI were diluted in BHI broth to an optical density 600 nm (OD₆₀₀) of 0.05. Bacteria were grown at 37°C until the culture reached the initial-middle log phase (OD₆₀₀ = 0.6), and then diluted in 1 mL infection medium. Bacteria were added at a multiplicity of infection (MOI) of 1 and placed in a humidified 5% CO₂ atmosphere at 37°C for 1 h.

One hour after infection, each well was washed three times with 1 mL of phosphate buffer saline (PBS) containing 300 $\mu\text{g}\cdot\text{mL}^{-1}$ gentamicin, to remove extracellular bacteria. Cells were then incubated with 1 mL of cell culture medium containing 50 $\mu\text{g}\cdot\text{mL}^{-1}$ gentamicin. Infected cells were kept in a humidified 5% CO₂ atmosphere at 37°C. Importantly, gentamicin is an antibiotic with a high bactericidal effect on *S. aureus* USA300 (CMI = 2 $\mu\text{g}\cdot\text{mL}^{-1}$) and a very poor penetration inside eukaryotic cells.

At chosen times, the culture medium containing gentamicin was removed from infected cells and wells were washed three times with antibiotic free PBS. Cells were lysed with 1 mL of distilled water during 15 minutes (min) and mechanically detached and serial dilutions were plated on Trypticase Soy Agar plates. Colony forming units were numerated after 24 h (h) at 37°C.

Cytochalasin D Assay

We used Cytochalasin D, a fungal metabolite that binds to actin and reversibly modifies its polymerization, to inhibit *S. aureus* internalization into EA.hy926 endothelial cells. One hour before infection, cytochalasin D (Sigma) 50 $\mu\text{g}\cdot\text{mL}^{-1}$ was added to cell culture medium and maintained during the first hour of infection.

Re-Seeding of *S. aureus*

EA.hy926 were infected as described above. At day 7 and 10 after infection, extracellular and intracellular bacterial load was assessed (**Figure 2A**). Cell monolayers were washed 3 times with PBS containing gentamicin 300 $\mu\text{g}\cdot\text{mL}^{-1}$ and 3 times with antibiotic free PBS. After 8 or 24 h of incubation in antibiotic-free medium the supernatant was collected, centrifugated and the pellet was resuspended in PBS before being plated on TSA for bacterial count. Then, cells were lysed and plated on TSA for intracellular bacteria count. Cells that were infected for 24 h were washed after 8 h and then incubated 16 additional hours in antibiotic-free medium.

Microscopy

Immunofluorescence Microscopy

EA.hy926 cells were grown to confluence on 12-mm diameter glass coverslips coated with 5 μg per cm² of rat tail collagen type

I. Immunofluorescence assays were performed 1 h post infection by USA300-GFP and the day of experiment. Cells were fixed in PFA 4% in PBS for 20 min and permeabilized for 5 min with PBS containing 0.1% Triton X-100. Cells were incubated with DAPI 1 $\mu\text{g}\cdot\text{mL}^{-1}$ and Alexa-633 conjugated phalloidin 1:50 in PBS containing 1% bovine serum albumin (BSA) for 1 h. After several washes, coverslips were mounted in mowiol. Image acquisition was performed on confocal microscope (Leica™ TCS SP5). Images were collected and processed using Image J software. Each image shown corresponds to the Z-projected maximal intensity signal for each fluorochrome.

Time Lapse Microscopy

EA.hy926 cells were grown to confluence on Ibi™ μ slide 8-wells and infected with USA300-GFP at a MOI of 1 as described above. Cells were incubated in cell culture medium containing 2.5 $\mu\text{g}\cdot\text{mL}^{-1}$ propidium iodide and 50 $\mu\text{g}\cdot\text{mL}^{-1}$ gentamicin. Image acquisition of infected cells was performed with an Apotome CO₂ microscope (Zeiss™). One image was acquired every 20 min during 64 h. Images were collected and films were processed using Image J software. Each image corresponds to the Z-projected maximal intensity signal for GFP and to the projected median intensity for bright light.

The mutant strain USA300 Δ hemDBL was imaged using the InCuCyte™ technology. EA.hy926 cells were grown to confluence in ImageLock 96-well plates (Essen BioScience Inc. Ann Arbor, MI, USA) and infected with the mutant strain USA300 Δ hemDBL-GFP (at a MOI of 1). Cytotox Red reagent (Essen Bioscience) was added to the medium (final concentration of 125 nM). Plates were incubated and monitored at 37°C for 6 days in an InCuCyte™ (Essen BioScience), an incubator equipped with a fully automated phase contrast microscope. Images were taken every 20 min. The microscope had a 20 X objective. Time-lapse video were generated by using Image J software.

Transmission Electron Microscopy

EA.hy926 were grown and infected with USA300-WT as previously described. One hour after infection and at day 3 and 7 infected cells were fixed with glutaraldehyde 3% in phosphate buffer during 1 h. Cells were then post-fixed with osmium tetroxide to stabilize lipids and enhance contrast. Sample were then dehydrated with sequential ethanol baths (from 25 to 100%) and embedded in Epon 812 resin with a 48 h polymerization time at 60°C. Embedded samples were sliced in 90 nm thick pieces with an ultramicrotome and laid down on a copper observation grid. Image acquisitions were performed with a JEOL 1011 transmission electron microscope (tungsten filament) at the Institut Cochin (Paris, France).

Statistics

In vitro experiments were at least repeated twice in triplicates. Data were analyzed using GraphPad Prism software. Statistical significance was assessed using Student *t*-test. In figures all results correspond to mean \pm SEM.

RESULTS

Persistence of Intracellular *S. aureus*

In vitro

Whereas several earlier studies have described the capacity of *S. aureus* to persist inside human cells, including non-professional phagocytes such as endothelial cells, other studies clearly showed the extreme propensity of this pathogen to rapidly kill infected cells (Loffler et al., 2014; Jubrail et al., 2016; Strobel et al., 2016). These apparent discrepancies are likely due the heterogeneity of protocols used to infect cells. Moreover, the vast majority of the studies related to *S. aureus* intracellular persistence have focused on early time points (generally up to 24 h post infection).

In this work, our aim was to follow the behavior of *S. aureus* USA300-LAC (referred as to USA300 in this work) when internalized into endothelial cells and to understand how this fast growing extracellular pathogen may survive in the cytosol of these cells. We decided to follow the outcome of intracellular *S. aureus* internalized during the first hours of the infection. Therefore, we adapted a gentamicin protection assay as follows: (i) after 1 h, infected cells were first washed three times with a solution containing $300 \mu\text{g}\cdot\text{mL}^{-1}$ gentamicin (to eliminate all extracellular bacteria); and (ii) the experiments were pursued in the constant presence of $50 \mu\text{g}\cdot\text{mL}^{-1}$ gentamicin in the culture media, to ensure elimination of extracellular staphylococci continuously escaping from lysed infected cells. The antibiotic gentamicin cannot penetrate into eukaryotic cells and has been shown to be poorly effective against intracellular *S. aureus* (Imbuluzqueta et al., 2012; Mohamed et al., 2014).

We first compared the ability of *S. aureus* USA300 to survive inside the Ea.Hy296 endothelial cell line and THP-1 monocytic cell line, for 10 days following infection at a multiplicity of infection (MOI) of 1 (Figure 1A). The number of viable intracellular bacteria (i.e., able to form colonies on solid medium) recovered in human endothelial cell lines remained stable during the first 24 h after the infection. Then, bacterial intracellular persistence started to progressively decrease from day 2 but was still recorded up to 10 days after infection. No viable (i.e., culturable) bacteria were recovered after 14 days. In contrast, THP-1 macrophages had almost completely eliminated bacteria at day 7.

To confirm that surviving *S. aureus* were indeed a consequence of bacterial internalization inside endothelial cells in our assay, we inhibited bacterial uptake at day 0, using cytochalasin D (Figure 1B). A severe 1,000-fold decrease in bacterial uptake was observed in EA.hy926 cells pretreated with cytochalasin D compared to that of untreated cells. As a result, no bacterium was recovered from treated cells 2 days after infection. We next focused on the characterization of *S. aureus* intracellular persistence, using endothelial EA.hy926 cells as model.

Interaction between *S. aureus* and Infected Host Cells

We next performed confocal and transmission electron microscopy (TEM) assays (Figure 2). Confocal microscopy analysis (Figure 2A) indicated that intracellular *S. aureus* actively replicate inside endothelial cells during the first 3 days of infection. After day 3, a progressive reduction in the number of GFP-expressing intracellular bacteria was observed concomitant with an increase in the number of bacteria only stained by DAPI that are likely corresponding to dead bacteria. To ascertain the fate of bacteria and that of their intracellular location inside endothelial cell we used TEM (Figure 2B). Image analysis confirmed the intracellular location of *S. aureus*. At day 0, a majority of intracellular bacteria were alive, according to their high cytoplasmic density and envelope integrity. After 3 days of infection, most of the intracellular bacteria appeared to be seriously damaged; only 23% of intracellular bacteria were still morphologically intact. As previously described, less than 5% of intracellular bacteria were surrounded by a visible vacuolar membrane (Grosz et al., 2014). After 7 days, only few intracellular bacteria were morphologically living. Confocal microscopy is consistent with this observation since dead bacteria were stained with DAPI but no longer express GFP.

S. aureus USA300 Uses Multiple Intracellular Survival Strategies

To follow the fate of single *S. aureus* USA300 after entry into endothelial cells, we next used time lapse-microscopy on EA.hy926 cells infected by USA300-GFP. As above, gentamicin

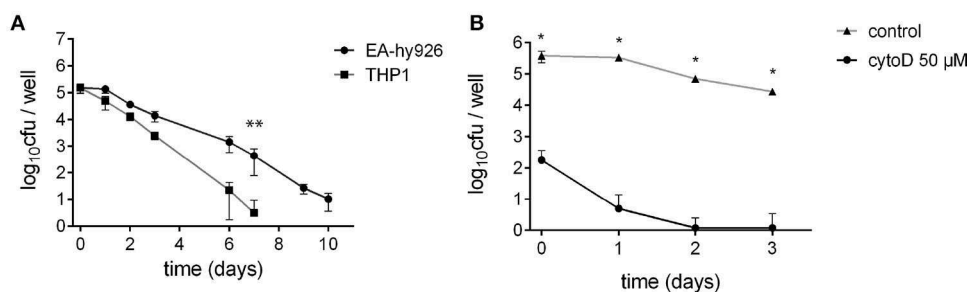


FIGURE 1 | *S. aureus* can invade and survive inside various cell types. **(A)** *S. aureus* survival inside non-professional-phagocyte cells: EA.hy926 endothelial cells derived from human umbilical vein and THP-1 monocyte-macrophages. **Wilcoxon-Mann-Whitney *U*-test ($p < 0.05$). **(B)** Endothelial cells EA.hy926 treatment with $50 \mu\text{M}$ cytochalasin D significantly reduced *S. aureus* uptake. *Student *t*-test ($p < 0.01$).

was present throughout the experiment to prevent extracellular bacterial growth and new infection. One image was captured every 20 min during 64 h, starting 1 h after infection (Videos S1; Supplementary Materials). At the single cell level, three distinct types of behaviors were observed: (i) in the majority of cases, *S. aureus* actively proliferated during the first 48 h, leading to host cell death and to bacterial release in the extracellular medium (as illustrated in the screen captures of **Figure 3A**, Videos S2, S3); (ii) in some cases, however, *S. aureus* remained inside the host cell without replicating throughout the observation period **Figure 3B**; or (iii) after a transient phase of bacterial multiplication, host cell could control and partly eliminate intracellular *S. aureus* (**Figure 3C**, Video S4). Interestingly, at day 7 we did not detect intracellular replication anymore (Video S6).

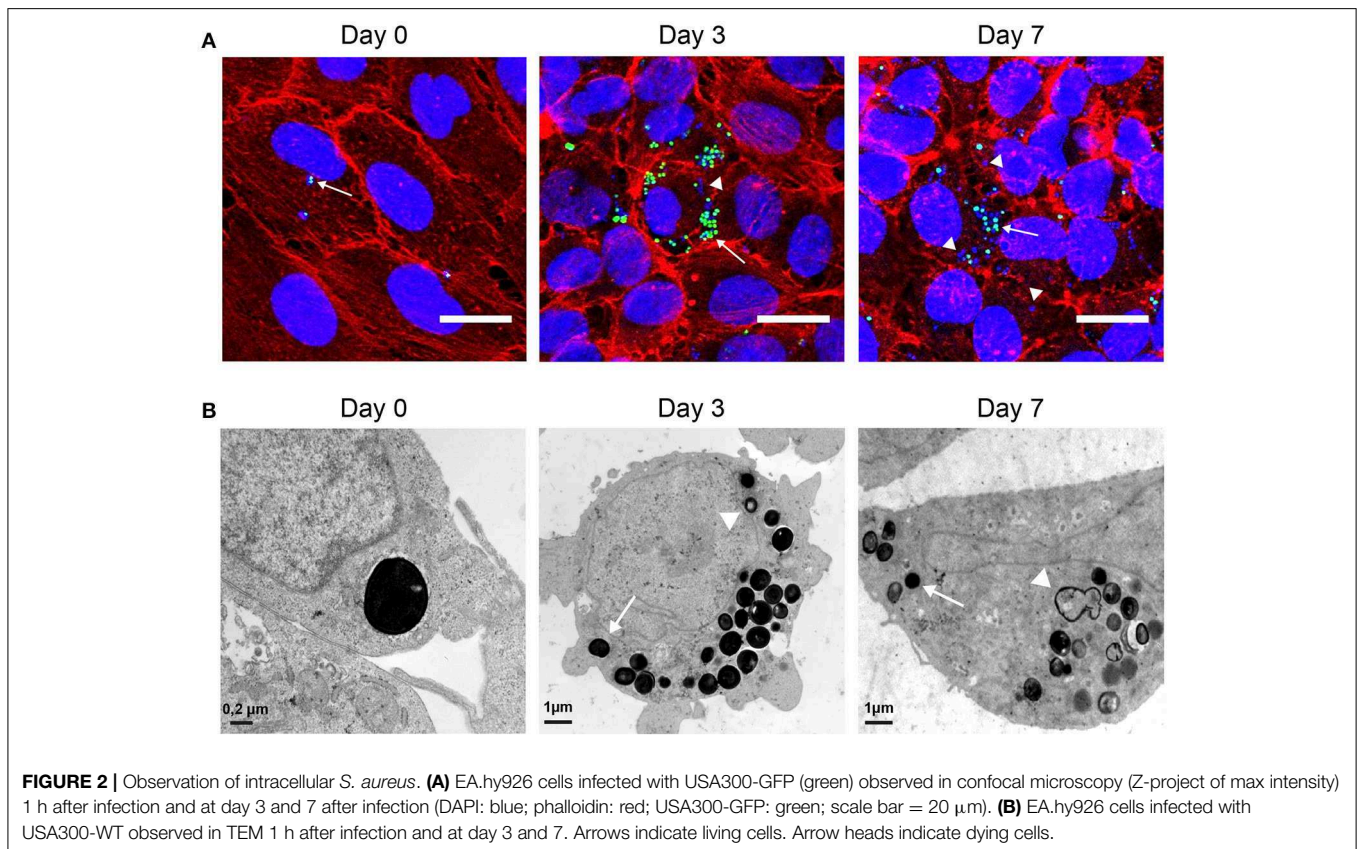
Altogether these results indicate that the infection of endothelial cells by *S. aureus* is not as straightforward as suggested by colony forming units counts (CFU). In some circumstances *S. aureus* can proliferate in the cytosol of endothelial cells, leading to cell lysis, while endothelial cells may also limit bacteria proliferation leading to the emergence of persistent bacteria.

Inactivation of the Heme Biosynthetic Pathway Improves Intracellular Persistence

S. aureus small colony variants (SCVs) constitute a slow growing subpopulation which can be recovered from patients who present

persisting or relapsing infections, as it may be observed in cystic fibrosis (Goerke and Wolz, 2010). SCV phenotype (colony that exhibits significantly reduced size) can be either transient (i.e., reversible), mostly due to transcription regulation mechanisms, or permanent due to a variety of genetic defects, such as those leading to an impaired electron transport chain (due to mutations inhibiting heme or menadione biosynthesis) and/or resulting in thymidine auxotrophy (Löffler et al., 2014). Laboratory engineered mutants that mimic the SCV phenotype have been largely used to study *S. aureus* persistence and notably Δ *hemB* mutants (inactivated heme biosynthetic pathway). For example, early work of von Eiff et al. (1997) have shown that a Δ *hemB* mutant of *S. aureus* (NCTC 8325 strain) was able to persist longer than wild-type strain (over a 48 h period) within cultured bovine aortic endothelial cells, likely due to reduced cytotoxicity. More recently, one of the rare studies that addressed long term *S. aureus* persistence (Tuchscherer et al., 2011), reported that staphylococcal strains could survive within cultured host cells for up to 4 weeks and suggested that SCV formation was directly linked to chronic infection.

These observations prompted us to count the proportion of SCVs in wild type *S. aureus* that infected endothelial cells (**Figure 4A**). Ten days after infection, only 30–40% of CFUs obtained after cell lysis showed a SCV phenotype suggesting that surviving internalized *S. aureus* only transiently slowdown their metabolism and growth. Since SCVs are known to better survive in human cells we decided to follow the fate of internalized SCVs. We thus engineered a SCV mutant by inactivating the heme



utilization locus ($\Delta hemDBL$) in wild-type *S. aureus* USA300 and USA300-GFP. Then we infected endothelial cells and recovered viable CFUs in infected cells (**Figure 4B**). While the CFUs count in **Figure 4B** were similar between wild type and $\Delta hemDBL$ mutant strains up to day 3, the $\Delta hemDBL$ mutant persisted more than the wild type parental strain at days 7 and 10. We then imaged the growth of our SCV mutant over a 7 day-course, using time lapse-microscopy (**Figure 4C**, Video S5). Neither proliferation inside endothelial cells nor cell detachment was noticed during the time of infection. This is consistent with previous reports, which indicate that SCVs induce less cell death after invasion of human cells (Tuchscher et al., 2010, 2011). Interestingly this result recapitulated what was observed with wild type strain USA300-GFP between day 5 and day 10 of endothelial infection, suggesting that surviving wild type USA300 between day 5 and day 10 may be potent persister SCVs.

S. aureus Can Escape from Infected Cells

We next tested whether *S. aureus* USA300 could escape from infected EA.hy926 cells and re-seed the extracellular medium after prolonged intracellular persistence, upon removal of antibiotic treatment. For this, we set up the infectious scheme depicted in **Figure 5A**. Briefly, 7 and 10 days after

infection, we removed gentamicin from the culture media (see Materials and Methods). Cells were then washed several times and incubated 8 h in antibiotic-free medium and supernatant were tested for the presence of *S. aureus*. No bacteria were detected in the cell culture media 8 h after removal of antibiotics, indicated that no detectable *S. aureus* survived in the extracellular media nor were released by cells early after removal of gentamicin. Cells were then incubated for 16 additional hours before counting extracellular and intracellular bacteria. At days 7 and 10, the number of *S. aureus* was dramatically increased in the supernatants of 5 out of 9 wells, and 7 out of 12 wells, respectively (**Figures 5B,C**). Bacterial multiplication in cell culture supernatant was associated with an increase in the number of intracellular CFUs of the corresponding well. Considering that we never observed an increase in the intracellular CFU count in the presence of gentamicin at day 7 and 10, these data tend to suggest that *S. aureus* USA300 that have been internalized for 7–10 days in endothelial cells are still able to evade the cells. However, as mentioned above we did not detect proliferation of intracellular wild type bacteria by time-lapse microscopy at day 7 (Video S6).

To confirm that single bacteria did not escape from the intracellular compartment by cell lysis (due to overwhelming

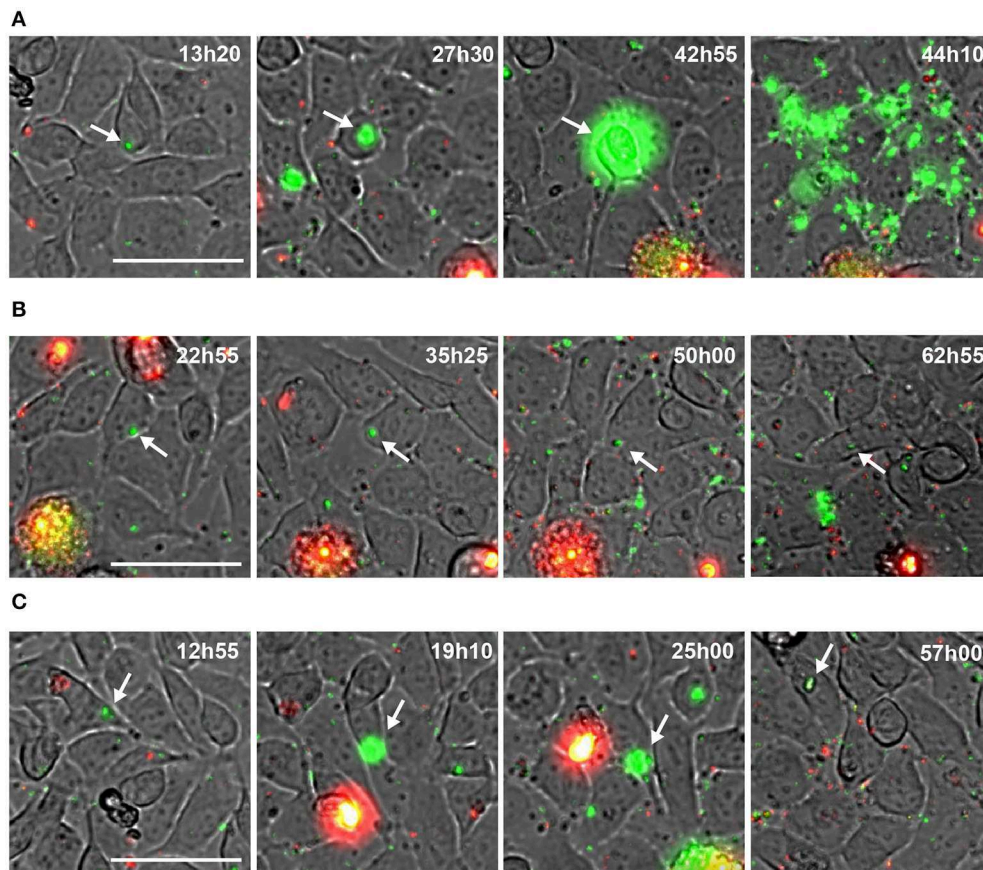


FIGURE 3 | Behavior of intracellular wild type *S. aureus*. (**A–C**) Screenshots of time lapse microscopy (see Video S1–S4). EA-hy926 cells were infected by USA300-GFP and time lapse microscopy was processed for 64 h. Images were extracted at different time point. USA300-GFP: green; DNA of dying cells (propidium iodide): red. Time is in hours and minutes. Bar = 50 μ m. White arrow heads point to multiplying USA300-GFP.

intracellular proliferation as observed at day 1–3), we also tested the relapse of the $\Delta hemDBL$ mutant 10 days after infection (Figures 5D,E). As for the wild type parental strain, *S. aureus* $\Delta hemDBL$ mutant bacteria proliferated in the supernatant to reach $\approx 10^6$ CFU in 5 wells out of 6. This proliferation was also associated with an increase in the intracellular CFU count.

Altogether, these data are compatible with the idea that single *S. aureus* USA300 bacteria are: (i) able evade endothelial cells after 10 days of intracellular persistence (either actively or passively, after cell extrusion or cell death), (ii) proliferate in the extracellular medium, and (iii) eventually infect neighboring cells, in the absence of antibiotics.

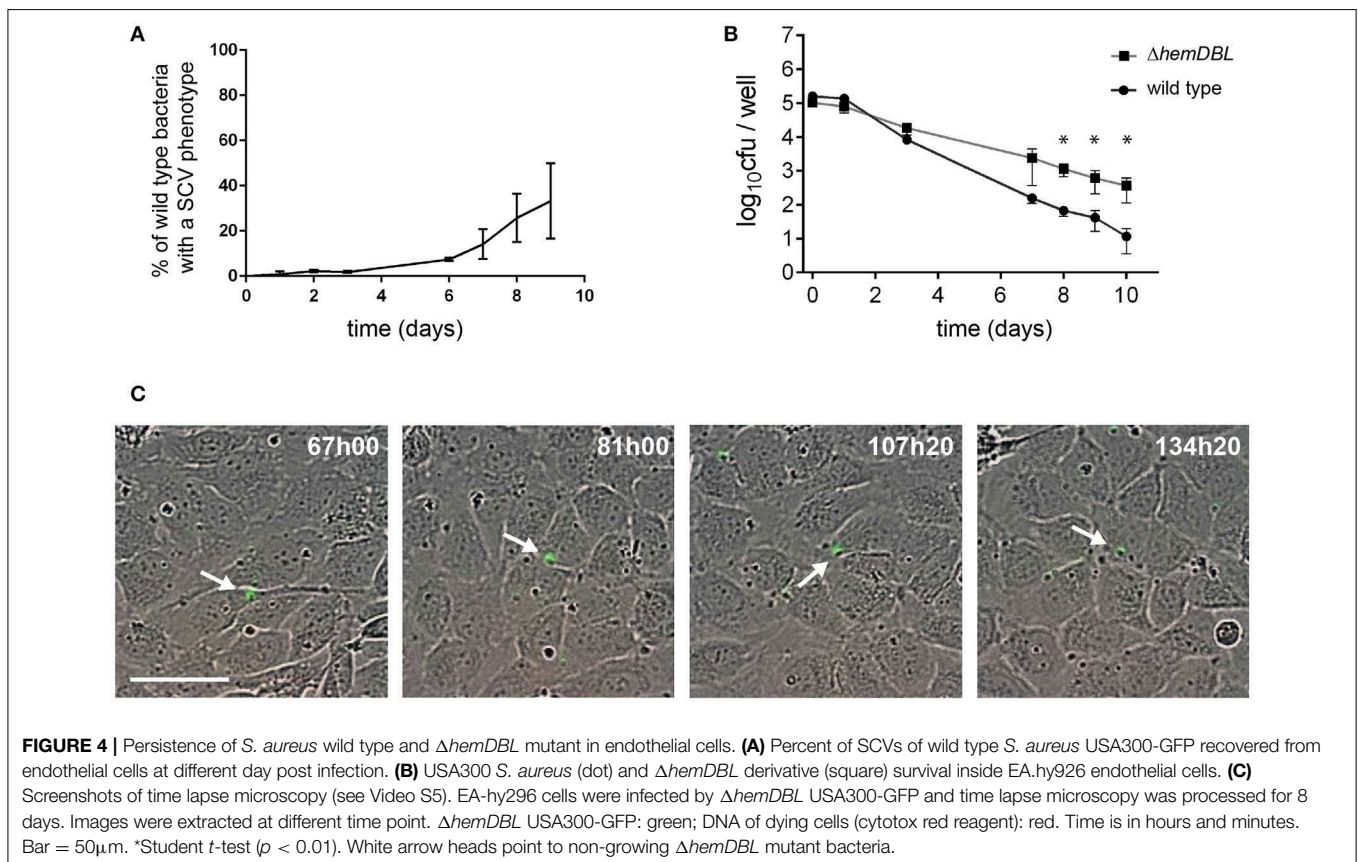
DISCUSSION

Having confirmed the increased intracellular persistence of *S. aureus* USA300 in endothelial cells as compared to macrophages, we show here that the initial persistence of wild-type *S. aureus* in endothelial cells corresponds to distinct simultaneous situations at the single cell level. Indeed, time-lapse microscopy revealed that, although in most cases, rapid bacterial proliferation leading to cell lysis was observed; in some cells, a transient bacterial multiplication ultimately controlled by the host, as well as a prolonged bacterial persistence without apparent morphological cellular damage, could also be observed. This dynamic view of *S. aureus* intracellular persistence provides

a complementary and necessary assessment of the intracellular lifestyle of the bacterium that should contribute to better understand the *in vivo* situation than any classical snapshot analysis.

Persistence in Endothelial Cells Corresponds to Different Single Cell Events

Two main outcomes of host cell invasion by *S. aureus* are generally described: (i) rapid host cell lysis, triggered by the secretion of toxins and other pro-inflammatory factors, which induce strong inflammatory and cytotoxic effects; (ii) persistence within morphologically-intact host cells for extended periods of time, where secretion of virulence factors is either down-regulated or not expressed (Proctor et al., 2014). Of note, these persisting bacteria generally correspond to so-called “small colony variants” (SCV). The association between *S. aureus* SCVs and persistent infection was first reported by Proctor and co-workers in 1995 (Proctor et al., 1995) and recent studies have shown that the impaired growth of *S. aureus* SCVs was due to decreased metabolic activity (Amato et al., 2014). Since a number of other pathogenic bacteria species have been shown to persist within their host as SCVs (Kahl et al., 2016), it may be considered as a major survival strategy in chronic bacterial infections. Summarizing the prolific literature addressing *S. aureus* intracellular persistence is quite complicated and may even be misleading. Indeed the comparison of results obtained



in one experimental setup with those obtained in another one is quite often impossible. Of interest, recent studies have revealed that the general stress transcription Sigma B (SigB) was a major player of the dynamic switch between full virulence and long-term persistent phenotypes in wild-type strains. SigB by down-regulating the quorum sensing *agr* system, silences toxins production and improves persistence within the host cell (Tuchscher et al., 2015).

Here, we thought to use video microscopy to follow the behavior of individual *S. aureus* USA300-GFP infected endothelial cells over a seven day-period. This technique has been very recently used by Jubrail et al. (2016) to visualize the fate of GFP-labeled *S. aureus* strain Newman in infected

THP-1 macrophages, over a 72 h-period. This experiment showed that intracellular bacteria re-emerged from macrophage from 24 to 72 h. This leads to a constant reseeding of the extracellular compartment. Our experiment in endothelial cells revealed that, whereas most infected cells were unable to restrict bacterial growth and were lysed within the first 48 h, some cells were able to resolve the infection. In these cells, morphologically intact bacteria were observed at least for 7 days.

Thus, upon entry of *S. aureus* into endothelial cells, simultaneous single cellular fates coexist which could not have been anticipated from classical confocal microscopy analyses or quantification of viable intracellular bacteria at fixed time points.

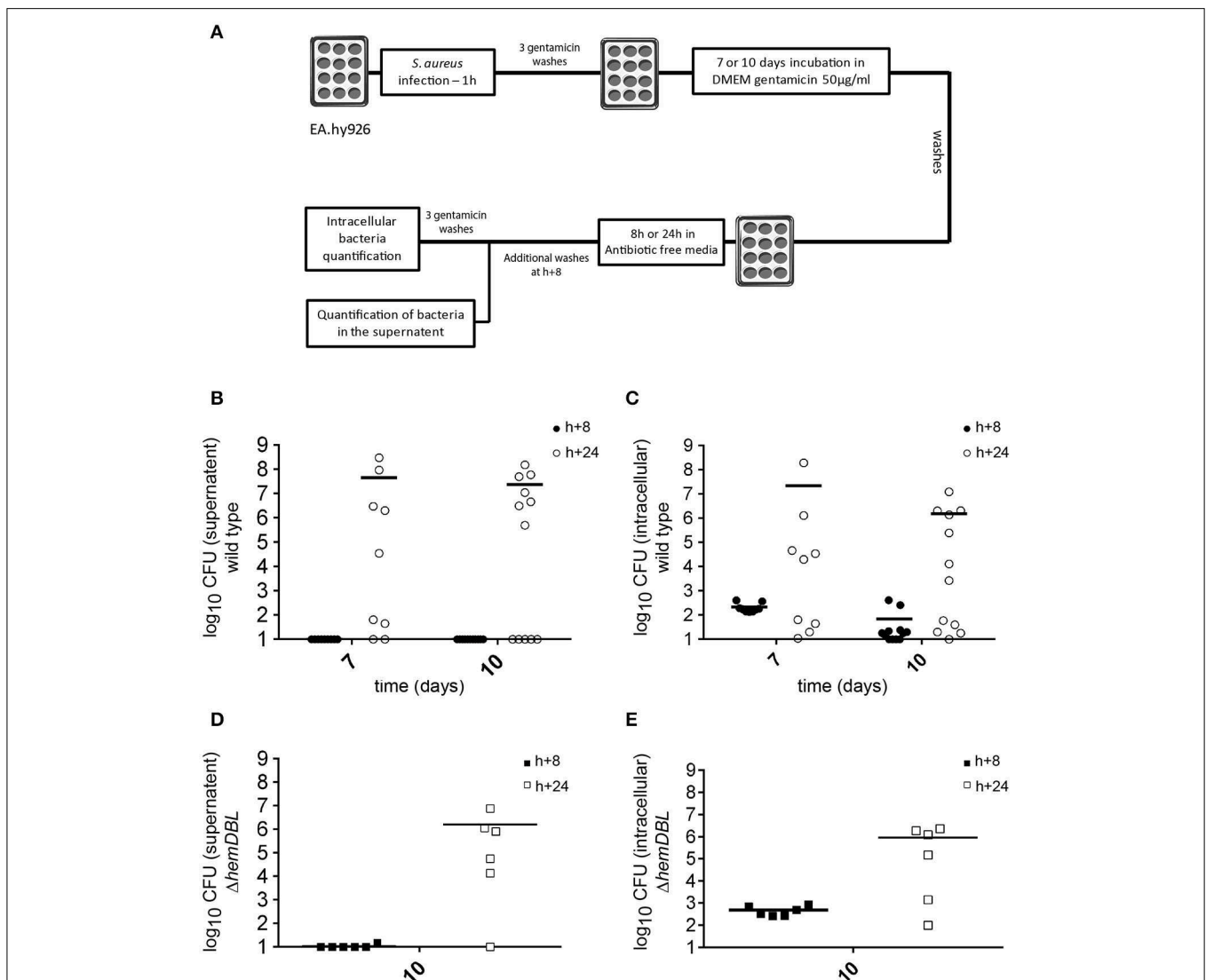


FIGURE 5 | Intracellular *S. aureus* may escape from infected host cells up to 10 days after infection. **(A)** Schematic representation of the experimental set-up (see Materials and Methods). Briefly, EA.hy926 cells were infected as described. At day 7 and 10 after infection cell monolayer was washed 3 times with PBS containing gentamicin 300 µg/mL and 3 times with antibiotic free PBS. After 8 h of incubation in antibiotic free medium the number of bacteria in the supernatant was assessed. The cells are then incubated 16 h more hours in antibiotic free medium and bacteria load in the supernatant and inside cells was assessed. **(B–E)** Mean of viable bacterial load 8 h (black) and 24 h (white) after incubation with antibiotic free medium, 7 or 10 days after infection. **(B,D)** Bacterial load in the supernatant. **(C,E)** Bacterial load inside cells. **(B,C)** USA300-GFP *S. aureus*. **(D,E)** Δ*hemD*BL USA300-GFP. Ten CFU were considered as a detection threshold.

Persistence and Exit of *S. aureus* from Human Cells

Our results and those of others indicate that, within the first hours after internalization, bacteria tend to actively multiply inside human cells (and ultimately lyse them) whereas infected cells attempt to restrict their proliferation. This selective pressure on *S. aureus* may favor the emergence of transient persisters which are able to survive into host cells for several days, without multiplication, before re-emerging in the extracellular compartment where they can rapidly proliferate and infect neighboring cells.

Of note, after several days of intracellular persistence, *S. aureus* is no longer capable of proliferation. Thus, the evasion of SCVs in the supernatant of infected cells (**Figure 5D**) in the absence of antibiotic, suggests that single non-proliferative bacteria are able to evade cells by an unknown, possibly active, process yet to be determined.

AUTHOR CONTRIBUTIONS

GR, XT, FT, and MD performed the experiments; GR, AC, and MC designed the research; GR, XN, AC, and MC discussed the data and the manuscript; GR, AC, and MC co-wrote the manuscript.

FUNDING

These studies were supported by INSERM, CNRS and Université Paris Descartes Paris Cité Sorbonne. GR was funded by a

fellowship from the “Fondation pour la Recherche Médicale” (FRM: DEA20150633005). XT was funded by a scholarship from the China Scholarship Council (n° CSC NO. 201508500097). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We thank Elise Borezee-Durant for the gift of *S. aureus* strain RN4220 and helpful technical advises. We greatly acknowledge Corinne Cordier and Jerome Megret (Necker Institute Cytometry Facility), Nicolas Ugolin (Necker Institute Imaging Facility) and Alain Schmitt (Cochin Institute Electron Microscopy Facility) for their technical support. We also thank Diana Mihaela Dincă for kindly providing support with the live cell image analysis system InCuCyte™. The following reagent was provided by the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) for distribution by BEI Resources, NIAID, NIH: *Staphylococcus aureus*, Strain USA300-0114, NR-46070; *Escherichia coli*—*Staphylococcus aureus* Shuttle Vector pNR-46158, Recombinant in *Staphylococcus aureus*, NR-46158.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.01354/full#supplementary-material>

REFERENCES

- Amato, S. M., Fazen, C. H., Henry, T. C., Mok, W. W., Orman, M. A., Sandvik, E. L., et al. (2014). The role of metabolism in bacterial persistence. *Front. Microbiol.* 5:70. doi: 10.3389/fmicb.2014.00070
- Arnaud, M., Chastanet, A., and Debarbouille, M. (2004). New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Appl. Environ. Microbiol.* 70, 6887–6891. doi: 10.1128/AEM.70.11.6887-6891.2004
- DeLeo, F. R., Otto, M., Kreiswirth, B. N., and Chambers, H. F. (2010). Community-associated methicillin-resistant *Staphylococcus aureus*. *Lancet* 375, 1557–1568. doi: 10.1016/S0140-6736(09)61999-1
- Felden, B., Vandenesch, F., Boulloc, P., and Romby, P. (2011). The *Staphylococcus aureus* RNome and its commitment to virulence. *PLoS Pathog.* 7:e1002006. doi: 10.1371/journal.ppat.1002006
- Foster, T. J., Geoghegan, J. A., Ganesh, V. K., and Hook, M. (2014). Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. *Nat. Rev. Microbiol.* 12, 49–62. doi: 10.1038/nrmicro3161
- Fraunholz, M., and Sinha, B. (2012). Intracellular *Staphylococcus aureus*: live-in and let die. *Front. Cell. Infect. Microbiol.* 2:43. doi: 10.3389/fcimb.2012.00043
- Goerke, C., and Wolz, C. (2010). Adaptation of *Staphylococcus aureus* to the cystic fibrosis lung. *Int. J. Med. Microbiol.* 300, 520–525. doi: 10.1016/j.ijmm.2010.08.003
- Grosz, M., Kolter, J., Paprotka, K., Winkler, A. C., Schafer, D., Chatterjee, S. S., et al. (2014). Cytoplasmic replication of *Staphylococcus aureus* upon phagosomal escape triggered by phenol-soluble modulins. *Cell. Microbiol.* 16, 451–465. doi: 10.1111/cmi.12233
- Ibarra, J. A., Perez-Rueda, E., Carroll, R. K., and Shaw, L. N. (2013). Global analysis of transcriptional regulators in *Staphylococcus aureus*. *BMC Genomics* 14:126. doi: 10.1186/1471-2164-14-126
- Imbuluzqueta, E., Lemaire, S., Gamazo, C., Elizondo, E., Ventosa, N., Veciana, J., et al. (2012). Cellular pharmacokinetics and intracellular activity against *Listeria monocytogenes* and *Staphylococcus aureus* of chemically modified and nanoencapsulated gentamicin. *J. Antimicrob. Chemother.* 67, 2158–2164. doi: 10.1093/jac/dks172
- Jubrail, J., Morris, P., Bewley, M. A., Stoneham, S., Johnston, S. A., Foster, S. J., et al. (2016). Inability to sustain intraphagolysosomal killing of *Staphylococcus aureus* predisposes to bacterial persistence in macrophages. *Cell. Microbiol.* 18, 80–96. doi: 10.1111/cmi.12485
- Kahl, B. C., Becker, K., and Löffler, B. (2016). Clinical significance and pathogenesis of staphylococcal small colony variants in persistent infections. *Clin. Microbiol. Rev.* 29, 401–427. doi: 10.1128/CMR.00069-15
- Laupland, K. B., and Church, D. L. (2014). Population-based epidemiology and microbiology of community-onset bloodstream infections. *Clin. Microbiol. Rev.* 27, 647–664. doi: 10.1128/CMR.00002-14
- Löffler, B., Tuchscher, L., Niemann, S., and Peters, G. (2014). *Staphylococcus aureus* persistence in non-professional phagocytes. *Int. J. Med. Microbiol.* 304, 170–176. doi: 10.1016/j.ijmm.2013.11.011
- Mohamed, W., Sommer, U., Sethi, S., Domann, E., Thormann, U., Schutz, I., et al. (2014). Intracellular proliferation of *S. aureus* in osteoblasts and effects of rifampicin and gentamicin on *S. aureus* intracellular proliferation and survival. *Eur. Cell. Mater.* 28, 258–268. doi: 10.22203/eCM.v028a18
- Proctor, R. A., Kriegeskorte, A., Kahl, B. C., Becker, K., Löffler, B., and Peters, G. (2014). *Staphylococcus aureus* Small Colony Variants (SCVs): a road map for the metabolic pathways involved in persistent infections. *Front. Cell. Infect. Microbiol.* 4:99. doi: 10.3389/fcimb.2014.00099

- Proctor, R. A., van Langevelde, P., Kristjansson, M., Maslow, J. N., and Arbeit, R. D. (1995). Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clin. Infect. Dis.* 20, 95–102. doi: 10.1093/clinids/20.1.95
- Sendi, P., and Proctor, R. A. (2009). *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. *Trends Microbiol.* 17, 54–58. doi: 10.1016/j.tim.2008.11.004
- Somerville, G. A., and Proctor, R. A. (2009). At the crossroads of bacterial metabolism and virulence factor synthesis in *Staphylococci*. *Microbiol. Mol. Biol. Rev.* 73, 233–248. doi: 10.1128/MMBR.00005-09
- Strobel, M., Pfortner, H., Tuscherr, L., Volker, U., Schmidt, F., Kramko, N., et al. (2016). Post-invasion events after infection with *Staphylococcus aureus* are strongly dependent on both the host cell type and the infecting *S. aureus* strain. *Clin. Microbiol. Infect.* 22, 799–809. doi: 10.1016/j.cmi.2016.06.020
- Thammavongsa, V., Kim, H. K., Missiakas, D., and Schneewind, O. (2015). Staphylococcal manipulation of host immune responses. *Nat. Rev. Microbiol.* 13, 529–543. doi: 10.1038/nrmicro3521
- Thomer, L., Schneewind, O., and Missiakas, D. (2016). Pathogenesis of *Staphylococcus aureus* bloodstream infections. *Annu. Rev. Pathol.* 11, 343–364. doi: 10.1146/annurev-pathol-012615-044351
- Tuscherr, L., Bischoff, M., Lattar, S. M., Noto Llana, M., Pfortner, H., Niemann, S., et al. (2015). Sigma Factor SigB Is crucial to mediate *Staphylococcus aureus* adaptation during chronic infections. *PLoS Pathog.* 11:e1004870. doi: 10.1371/journal.ppat.1004870
- Tuscherr, L., Heitmann, V., Hussain, M., Viemann, D., Roth, J., von Eiff, C., et al. (2010). *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. *J. Infect. Dis.* 202, 1031–1040. doi: 10.1086/656047
- Tuscherr, L., Medina, E., Hussain, M., Volker, W., Heitmann, V., Niemann, S., et al. (2011). *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. *EMBO Mol. Med.* 3, 129–141. doi: 10.1002/emmm.201000115
- von Eiff, C., Heilmann, C., Proctor, R. A., Woltz, C., Peters, G., and Gotz, F. (1997). A site-directed *Staphylococcus aureus* hemB mutant is a small-colony variant which persists intracellularly. *J. Bacteriol.* 179, 4706–4712. doi: 10.1128/jb.179.15.4706-4712.1997

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2017 Rollin, Tan, Tros, Dupuis, Nassif, Charbit and Coureuil. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Paper 1 Intracellular survival of Staphylococcus aureus in endothelial cells: a matter of growth or persistence

Contributions by Xin TAN

Methods

- Generation of Erythromycin-sensitive USA300 wild-type strain and the GFP-expressing USA300 wild type strain.
- Construction of USA300 Δ hemDBL strain and the GFP-expressing USA300 Δ hemDBL strain.
- In vitro infection assay for USA300 Δ hemDBL strain and wild-type strain in endothelial cells.
- Time lapse microscopy for USA300 Δ hemDBL strain.

Results

Figures 3 and Figure 4

**Chapter II: Transketolase of
Staphylococcus aureus is involved in
the control of master regulators of
stress response during infection**

1 **Transketolase of Staphylococcus aureus is involved in the control**
2 **of master regulators of stress response during infection**

3

4 Xin Tan^{1,2}, Elodie Ramond^{1,2}, Anne Jamet^{1,2}, Jean-Philippe Barnier^{1,2}, Baptiste
5 Decaux-Tramoni^{1,2}, Marion Dupuis^{1,2}, Daniel Euphrasie^{1,2}, Fabiola Tros^{1,2}, Ivan
6 Nemazanyy^{1,2,3}, Jason Ziveri^{1,2}, Xavier Nassif^{1,2}, Alain Charbit^{1,2} * and Mathieu
7 Coureuil^{1,2} *

8

9 ¹ Université de Paris, Paris, France

10 ² INSERM U1151 - CNRS UMR 8253, Institut Necker-Enfants Malades, Paris, France

11 ³ Plateforme Métabolomique Institut Necker, Structure Fédérative de Recherche SFR Necker,
12 Université Paris Descartes, Paris 75015 France

13

14 Running title: Transketolase in S. aureus infection

15 Keywords: Transketolase, Staphylococcus aureus, metabolic adaptation, Pentose Phosphate
16 Pathway, sigma B, RpiRc.

17 Abstract: 142 words; Text: 3315 words.

18

19 * Corresponding authors: Alain Charbit, Mathieu Coureuil

20 e-mail: alain.charbit@inserm.fr ; mathieu.coureuil@inserm.fr

21 Tel: 33 1 – 72 60 65 11 — Fax: 33 1 - 72 60 65 13

22

23 Conflict of interest: No potential conflict of interest was reported by the authors.

24 Funding: These studies were supported by INSERM, CNRS and Université Paris Descartes
25 Paris Cité Sorbonne. This work was supported by Vaincre La Mucoviscidose (grant
26 RF20180502254). Xin Tan was funded by a scholarship from the China Scholarship Council
27 (n° CSC NO. 201508500097). The funders had no role in study design, data collection and
28 analysis, decision to publish, or preparation of the manuscript.

29 **Brief Abstract**

30 Inactivation of the *Staphylococcus aureus* transketolase activity leads to impaired early
31 intracellular proliferation and kidneys colonization, associated with reduced Sigma B and
32 RpiRc transcription. The data suggest that TKT -or a functional PPP- strongly influences *S.*
33 *aureus* intracellular proliferation.

34

35

36 **Abstract**

37

38 *Staphylococcus aureus* is a leading cause of both acute and chronic infections in humans.
39 The importance of the pentose phosphate pathway (PPP) during *S. aureus* infection is
40 currently largely unexplored. Here, we focused on one key PPP enzyme, transketolase. We
41 showed that inactivation of the unique gene encoding transketolase activity in *S. aureus*
42 USA300 (Δ tkt) led to drastic metabolomic changes. Using time-lapse video imaging and mice
43 infection, we observed a major defect of the Δ tkt strain compared to wild-type strain in early
44 intracellular proliferation and in the ability to colonise kidneys. Transcriptional activity of the
45 two master regulators Sigma B and RpiRc was drastically reduced in the Δ tkt mutant during
46 host cells invasion. The concomitant increased RNAlII transcription, suggests that TKT -or a
47 functional PPP- strongly influences the ability of *S. aureus* to proliferate within host cells by
48 modulating key transcriptional regulators.

49

50

51 **Background**

52

53 Staphylococcus aureus is a leading cause of severe skin and soft tissue infections,
54 pneumonia, endocarditis and bone and joint infections [1, 2]. *S. aureus* invades and survives
55 within eukaryotic host cells [3-9]. This is likely to play an important role in chronicity and
56 treatment failures [10-12]. To evade host defences and silently persist within human cells [13,
57 14], *S. aureus* can dynamically switch phenotypes from a highly aggressive and cytotoxic
58 phenotype to a metabolically inactive phenotype associated with decrease TCA activity [15-
59 18]. Reprogramming of the TCA cycle notably involves the glycolysis/gluconeogenesis and
60 pentose phosphate pathway (PPP) [19].

61

62 The contribution of glycolysis/gluconeogenesis to intracellular persistence of *S.*
63 *aureus* has been recently studied [20-23]. In contrast, the precise role of the PPP is
64 unexplored. The PPP is important for producing nucleotide precursors for DNA synthesis and
65 repair as well as precursors for aromatic amino acids or peptidoglycan synthesis. In addition,
66 the oxidative branch of the PPP contributes to bacterial tolerance to oxidative stress by
67 generating the redox molecule NADPH/H⁺, which is the substrate for other reducing agents
68 such as glutathione. The activity of the PPP has been shown to be frequently increased in
69 Gram-positive pathogens in response to environmental stresses and in infection models [24-
70 26] or in some hospital-acquired isolates [27]. While it has been suggested that the
71 transcriptional regulator RpiRc might control the PPP [28-30], no gene regulation has been
72 yet associated with any change in its activity in *S. aureus*.

73

74 Here, we study the role of PPP during the intracellular persistence of *S. aureus* by focusing
75 on one of the key enzyme of this pathway, transketolase (TKT). *tkt* genes are found in a wide
76 range of organisms including bacteria, plants and mammals, underlining the ubiquitous role
77 of this enzyme in biology. We show here that *tkt* inactivation in *S. aureus* USA300 leads to
78 deregulation of whole cell metabolism. This correlates with impaired proliferation of
79 intracellular staphylococci in vitro and kidney colonisation defect in infected mice.

80 Unexpectedly, we found that the tkt defective mutant was unable to regulate the two master
81 regulators of virulence RpirC and SigB, leading to a dramatic increase of RNAIII during
82 intracellular invasion of human cells. Altogether our data suggest a major role of the PPP in
83 the control of *S. aureus* virulence.

84

85

86

87

88

89

90

91

92

93

94

95

96

97

98

99

100

101

102

103

104

105

106

107

108 **Methods**

109

110 **Strains, Cells, Culture conditions and Infection.**

111 *S. aureus* USA300-LAC (designated USA300-WT) was provided by the Biodefense and
112 Emerging Infections Research Resources (BEI). The GFP-expressing strain, the Δ tkt mutant
113 strain and the complemented strain were constructed and cultured as described in
114 **supplementary methods**. Strains and plasmids used are summarized in **supplementary**
115 **methods**.

116 *S. aureus* were grown in brain heart infusion (BHI) or chemically defined medium (CDM)
117 supplemented with ribose or fructose or glycerol when it was required [31].

118 EA.hy926 endothelial cells (ATCC CRL-2922) were grown in DMEM 10 % FBS (5 % CO₂ at
119 37 °C). When indicated, cells were incubated with no glucose DMEM supplemented with
120 glucose, ribose or fructose at a concentration of 25mM. EA.hy926 expressing nuclear
121 mKate2 were transduced with the IncuCyte® NucLight Red Lentivirus.

122 Cells were infected at a multiplicity of infection (MOI) of 1 for 1 h and washed three times
123 with 100 μ L of phosphate buffer saline (PBS) containing 300 μ g/ml gentamicin to remove
124 extracellular bacteria. Cells were then incubated in PBS-gentamicin (50 μ g/ml). Gentamicin is
125 an antibiotic with a high bactericidal effect on *S. aureus* USA300 (CMI=2 μ g/ml) and a very
126 poor penetration inside eukaryotic cells. Its use was necessary to abolish extracellular
127 proliferation.

128 LAMP-1 colocalization assay in EA.hy926 infected cells is described in the **supplementary**
129 **methods**.

130

131 **Time lapse microscopy**

132 EA.hy926 cells expressing nuclear mKate2 were seeded in ImageLock 96-well plates (Essen
133 BioScience Inc). Cells were infected with WT and Δ tkt mutant of the GFP-expressing
134 USA300 strain. Plates were incubated and monitored at 5% CO₂ and 37°C for 24 hours in
135 the fully automated microscope Incucyte® S3 (Essen BioScience). The detailed analysis is
136 further described in the **supplementary methods**.

137

138 **Transcriptional analyses**

139 The bacteria were recovered from lysed cells or planktonic overnight culture. Nucleic acids
140 were released by resuspending bacteria in TE buffer containing lysostaphin. RNAs recovery,
141 reverse transcription and PCR were performed as described in the **supplementary methods**.
142 We used the “The Relative Standard Curve Method” for analyzing the qRT-PCR data.

143

144 **Metabolomic analyses**

145 Metabolite profiling of *S. aureus* isolates grown to stationary phase in BHI Broth was
146 performed by liquid chromatography–mass spectrometry (LC-MS) as described in the
147 **supplementary methods** [9, 32, 33].

148

149 **Transketolase activity assay**

150 TKT activity was analysed on bacteria grown to stationary phase in BHI as described in the
151 **supplementary methods** [34]. TKT activity was expressed as units per microgram of total
152 protein. One unit of enzyme was defined as the amount of enzyme that oxidized 1 μmol of
153 NADH per minute.

154

155 **In vivo infection**

156 Mice were infected intravenously (iv) in the tail vein as described in the **supplementary**
157 **methods**. For bacterial burden determination, mice were euthanized after 4, 24, 48 and 72
158 hours. Sequential dilutions of homogenized preparations from spleen and kidney were
159 spread onto BHI agar plates.

160

161 **Statistics**

162 Statistical significances were assessed using one-way analysis of variance (ANOVA) with
163 Dunnett’s correction, unpaired two-tail Student’s t-test, or Kruskal Wallis test. P values of
164 $p < 0.05$ were considered to indicate statistical significance.

165

166 **Ethics statement**

167 All experimental procedures involving animals were conducted in accordance with guidelines
168 established by the French and European regulations for the care and use of laboratory
169 animals (Decree 87–848, 2001–464, 2001–486 and 2001–131 and European Directive
170 2010/63/UE) and approved by the INSERM Ethics Committee (Authorization Number: 75-
171 906).

172

173

174

175

176

177

178

179

180

181

182

183

184

185

186

187

188

189

190

191

192

193

194

195 Results

196

197 Transketolase inactivation in *S. aureus* USA300

198 *S. aureus* genomes possess a unique transketolase-encoding gene, designated tkt,
199 which is highly conserved within species. Transketolase (TKT) is an enzyme of the non-
200 oxidative branch of the PPP involved in two main reversible enzymatic reactions: i) Fructose-
201 6-P + Glyceraldehyde-3-P \leftrightarrow Erythrose-4-P + Xylulose-5-P; and ii) Sedoheptulose-7-P +
202 Glyceraldehyde-3-P \leftrightarrow Ribose-5-P + Xylulose-5-P (**Supplementary Figure S2A**). Several
203 TKT structures have been solved [35] and allow the modelisation of the *S. aureus* TKT
204 (**Supplementary Figure S1B**). The protein of *S. aureus* is 662 amino acid long and harbours
205 three domains (**Supplementary Figure S1A**). Of note, although ubiquitously expressed in
206 Eukaryotes and Bacteria, *S. aureus* and *Homo sapiens* TKT proteins share only 22.4%
207 amino acid identity (**Supplementary Figure S1C**). We constructed a chromosomal deletion
208 of the tkt gene in *S. aureus* strain USA300 (Δ tkt strain), and generated a complemented
209 strain (designated Cp-tkt) by expressing the wild-type tkt allele preceded by its own promoter
210 in the Δ tkt strain. Gene inactivation was confirmed by quantification of the transketolase
211 activity (**Supplementary Figure S2**).

212 The Δ tkt mutant showed only a slight growth decrease compared to the WT strain in
213 complete BHI Broth or in Dulbecco's Modified Eagle Medium (DMEM) (**Figure 1A**). In
214 contrast, the Δ tkt mutant strain was unable to grow in chemically defined medium [31]
215 supplemented with ribose (which can be converted to Ribose-5-phosphate by ribokinase)
216 (**Supplementary Figure S2C**). In all assays, functional complementation was observed in
217 the Cp-tkt strain.

218 We next performed a whole cell metabolomic analysis on WT and Δ tkt strains grown
219 for 24h in BHI Broth (i.e., when identical numbers of cells were recorded) to evaluate the
220 global impact of tkt deletion on bacterial metabolism (**Figure 1B and Supplementary Figure**
221 **S3 and Table S1**). Remarkably, we observed a massive accumulation of ribose-5-phosphate
222 (R5P; 56-fold increase) concomitant with a decrease in ribose content (4-fold decrease) in
223 the Δ tkt mutant compared to WT. This accumulation was associated with an increased

224 amount of metabolites derived from Ribose-5P, such as inosine-5-monophosphate (IMP),
225 xanthosine-5-monophosphate (XMP) and hypoxanthine. We also observed a 2.5-fold
226 decrease in D-sedo-heptulose-7P amount in the Δtkt mutant compared to WT accompanied
227 by a significant decrease in tyrosine, histidine and tryptophan amounts (5, 10 and 12 fold
228 respectively), three aromatic amino-acids produced from the PPP. The glutamate pathway
229 seemed to be significantly more active in the Δtkt strain compared to WT as the amounts of
230 glutamate, proline and ornithine were all increased. The higher amounts of glutamate,
231 possibly fuelling the TCA cycle at the level of α -ketoglutarate, could be responsible for the
232 increased expression of some TCA cycle metabolites (i.e., fumarate, malate, succinate and
233 α -ketoglutarate) in the Δtkt strain. Of note, ATP content was not altered in Δtkt strain.

234 These results demonstrate that deregulation of the PPP by *tkt* inactivation has direct
235 effects on the whole cell metabolism, including aromatic amino acid production, TCA activity
236 and pathways involved in adenine, guanine or peptidoglycan synthesis.

237

238 **Tkt is essential for intracellular proliferation of *S. aureus***

239 Intracellular staphylococci exist as two different populations: bacteria that actively
240 proliferate during the first 24 hrs after invasion, leading to their release in extracellular milieu
241 as a consequence of host cell death, and bacteria that do not replicate. These latter
242 staphylococci can persist within host-cell cytoplasm for several days [3-9, 36]. Here we
243 addressed the role of TKT in these two phenotypes in EA.hy926 human endothelial cells by
244 using: i) time lapse-microscopy, to evaluate early intracellular proliferation of *S. aureus* and ii)
245 colony forming units counting, to monitor the survival kinetics of internalized bacteria over a
246 ten days period.

247 Time-lapse video microscopy. EA.hy926 cells expressing mKate2 nuclear fluorescent
248 protein were infected by GFP-expressing bacteria. Gentamicin was used throughout the
249 experiment to eliminate extracellular bacteria and prevent re-infection (see Methods),
250 allowing us to solely focus on the fate of intracellular persistent bacteria. Cell infection was
251 monitored using an IncuCyte[®] S3 imaging system and images were captured every 30
252 minutes. Bacterial entry into endothelial cells was similar for WT and Δtkt strains, showing

253 0.3~0.5 GFP expressing particles recorded per cell (**Figure 2A-B**). Active proliferation of WT
254 *S. aureus* inside EA.hy926 cells was confirmed after 24 hours of infection (**Figure 2A-B and**
255 **Movie 1**) with an average number of GFP expressing particles rising from 0.5 to 2 per cells.
256 At the single cell level, WT *S. aureus* showed active intracellular multiplication, ultimately
257 leading to cell death and bacterial release in the extracellular medium (**Figure 2A-B and**
258 **Movie 1**). Thanks to the permanent presence of gentamicin in the medium bacterial release
259 did not lead to proliferation in the extracellular medium. In contrast, the Δ tkk mutant strain
260 showed limited proliferation during the first 24 hours (**Figure 2A-B and Movie 2**). Finally,
261 using LAMP-1 colocalization assay (Lysosome-associated membrane protein 1), we
262 confirmed that the intracellular Δ tkk strain growth defect was not due to a decreased
263 phagosomal escape (**Supplementary Figure S4**).

264 Long-term intracellular survival. Using the same infection conditions as for time-lapse
265 experiment, we monitored survival of internalised Δ tkk mutant compared to that of the WT
266 strain at three, seven or ten days after infection by CFU counting. The Δ tkk strain behaved
267 like the WT strain throughout the infection period, ultimately leading to its complete
268 elimination after 10 days (**Figure 2C**).

269 Thus, the role of TKT during *S. aureus* host cell infection seems to be essential for
270 bacteria proliferation and restricted to early time points.

271

272 **Tkk inactivation is associated with an altered rpiRc and sigB expression.**

273 *S. aureus* is known to sense its environment and to regulate virulence factors
274 depending on the availability of carbon sources. For example, recent studies have shown
275 that the transcriptional regulator RpiRc could sense metabolic shifts, especially in the PPP,
276 and control expression of RNAlII [29, 30]. RNAlII is a key effector in the quorum-sensing
277 system that regulates the expression of a large number of virulence factors [37]. RpiRc is
278 also linked to the alternative Sigma B factor (SigB) that has pleiotropic roles in gene
279 regulation and is a master regulator of intracellular survival [29, 38, 39].

280 These data prompted us to follow the expression profile of the master regulator SigB
281 and RpiRc in the WT and the Δ tkk mutant strains during endothelial cells invasion (**Figure 3**).

282 EA.hy296 cells were infected as described above to solely focus on intracellular bacteria.
283 Between day 1 and day 7, sigB and rpiRc transcription increased in the WT strain (by 4-fold
284 and 3-fold, respectively) (**Figure 3**). In sharp contrast, as early as one day after infection,
285 both genes were significantly down-regulated in the Δ tkr mutant strain compared to WT (8-
286 fold and 7-fold decrease for sigB and rpiRc expression, respectively). Consistent with reports
287 demonstrating inhibition of RNAlII expression by RpiRc and SigB [18], we observed a
288 dramatic increase in the expression of RNAlII in the Δ tkr mutant strain (**Figure 3**), suggesting
289 that the Δ tkr mutant strain is unable to control rpiRc and sigB expression during intracellular
290 survival. Functional complementation always restored the wild type phenotype
291 (**Supplementary Figure S5**). To further confirm these data, we monitored the expression of
292 two major regulators of SigB activity, RsbU and RsbW [40]. RsbW, which is co-expressed
293 with sigB, is known to sequester and inhibit SigB. RsbU, whose expression is under the
294 control of SigA, is a phosphatase involved in the dephosphorylation of RsbV. In turn,
295 dephosphorylated RsbV (dRsbV) is able to sequester RsbW, allowing the release of active
296 SigB. RsbU is thus considered as an activator of SigB, allowing a fine-tuning of SigB activity.

297 We found that the transcription of both rsbW and rsbU genes was down-regulated in
298 the Δ tkr mutant during infection of endothelial cells. The down-regulation of rsbW is
299 consistent with the down-regulation of its co-expressed gene sigB (**Figure 3**). The
300 concomitant down-regulation of rsbU suggests a global inhibition of the sigB pathway in the
301 Δ tkr mutant strain. Finally, we followed expression of asp23, a sigB-dependent locus [41].
302 The recorded down-regulation of asp23 transcription in the Δ tkr mutant strain confirmed the
303 inhibition of SigB-dependent transcription. All together these transcriptional analyses suggest
304 that the metabolic deregulation observed in Δ tkr strain is sufficient to promote a dramatic
305 reprogramming of the RpiRc and SigB pathways when bacteria invade human cells.

306

307 **Tkr inactivation impairs proliferation of *S. aureus* in the kidney of infected mice.**

308 The ability of internalized *S. aureus* to readily proliferate inside host cell cytoplasm
309 may be seen as an effective mechanism to avoid host innate immunity, ultimately allowing an
310 increased colonization of the infected site. In this respect, it has been previously shown in the

311 mouse model that after intravenous (IV) injection, although rapidly cleared from the
312 bloodstream, bacteria were able to proliferate in the kidney, leading to abscesses formation
313 within the first days of infection [42].

314 Here, we monitored the kinetics of in vivo proliferation of WT, Δ tkk and Cp-tkk strains
315 in kidneys and spleens of BalB/c mice (**Figure 4**) after IV infection with 10^7 bacteria per
316 mouse. As previously reported, *S. aureus* poorly proliferates in the spleen of infected mice.
317 We therefore used the spleen as a non proliferative control. The bacterial burden in kidneys
318 and spleens was quantified by plating CFUs at 4 h, 24 h, 48 h and 72 h after infection. We
319 observed no significant differences between the bacterial counts in the spleens of mice
320 infected either with WT, the Δ tkk mutant or the Cp-tkk strain. In each case, a progressive
321 reduction of the bacterial burden was recorded (ranging from 2.5×10^5 , 1.1×10^5 and 1.5×10^5 at
322 4 h; down to 3×10^3 , 7.3×10^2 and 1.2×10^2 at 72 h, for WT, Δ tkk and Cp-tkk respectively). In
323 contrast, in kidneys, whereas the WT and the Cp-tkk strains readily proliferated (from 3.9×10^4
324 $\pm 8.6 \times 10^3$ WT and $2.1 \times 10^4 \pm 4.6 \times 10^2$ Cp-tkk *S. aureus* per organ at 4 hrs to $7.7 \times 10^7 \pm 3.3 \times 10^7$
325 WT and $3.6 \times 10^7 \pm 1.3 \times 10^7$ Cp-tkk *S. aureus* per organ at 72 hrs), multiplication of the Δ tkk
326 mutant was severely impaired at all time-points tested, with a 10^4 -fold reduction of Δ tkk
327 counts at 24h compared to WT counts (**Figure 4**). This result confirms the importance of TKT
328 -and most likely of the PPP- in bacterial proliferation in vivo and especially during the first 24
329 hours after infection. This result is consistent with the previously described role of SigB in
330 intracellular growth in vivo [43].

331

332 **The SigB pathway may be controlled by modulating carbon source availability.**

333 Inside the cytoplasm of host cells, the Δ tkk mutant is likely to deal simultaneously with
334 PPP blocking and altered levels of carbon-based nutrients compared to the outside
335 compartment. The above results suggested that the PPP might take control of rpiRc and sigB
336 expression when bacteria invade endothelial cells. We thus hypothesized that PPP-
337 associated carbon sources may possibly influence rpiRc and/or sigB expression in vitro. We
338 therefore grew WT USA300 in CDM supplemented either with glucose (CDM-glucose),
339 ribose (CDM-ribose) or fructose (CDM-fructose) a carbon source possibly fuelling the PPP)

340 and quantified by qRT-PCR the transcription of sigB, rpiRc, rsbU/W and asp23 genes.
341 Remarkably, we found that the regulation of the whole sigB operon was impacted by the
342 carbon source used in CDM (**Figure 5**). Indeed, expression of sigB, rsbU/W and rpiRc
343 decreased in CDM-fructose or CDM-ribose, compared to CDM-glucose. In contrast, asp23
344 expression was only decreased by 2-fold in CDM-fructose and increased by 3-fold in CDM-
345 ribose, suggesting that in this growth conditions, asp23 expression might be regulated by
346 other factor than sigB (**Figure 5**). Hence, the availability of carbon sources, and especially
347 those related to PPP, may be sensed by the bacteria and affect the regulation of rpiRc and
348 sigB expression.

349

350

351

352

353

354

355

356

357

358

359

360

361

362

363

364

365

366

367

368

369 Discussion

370 Inactivation of the *tkt* gene, encoding the unique transketolase of *S. aureus* USA300,
371 led to a major decrease in early intracellular proliferation and impaired in vivo the
372 multiplication in a kidney colonization model. Our results suggest that the intracellular
373 proliferation defect of the Δ *tkt* mutant occurs through the inhibition of *sigB* and *rpiRc*
374 expression and the concomitant increased RNAIII transcription, thus highlighting a novel
375 connection between the PPP and these master regulators during cell invasion.

376 Our whole cell metabolomic analysis of planktonic-grown bacteria (**Figure 1**,
377 **Supplementary Figure S2 and Table S1**) showed a huge increase in R5P intermediates in
378 the Δ *tkt* mutant compared to WT that could be explained by the impaired entry of R5P into
379 glycolysis and a concomitant significant decrease in SH7P relative concentration. In *S.*
380 *aureus*, ribose is imported by the RbsU transporter and converted to R5P by the RbsD/RbsK
381 pathway. RbsR, the repressor of *rbsUDK* operon, is itself highly regulated by SigB [44].
382 Interestingly, *S. aureus* grown in CDM-ribose showed a decreased expression of *sigB*.
383 Hence, stress signals that modulate SigB activity are likely to be important effectors for
384 controlling the quantity of RbsR, thereby affecting ribose uptake.

385 Significant changes in the relative concentrations of TCA cycle intermediates were also
386 recorded in the Δ *tkt* mutant compared to WT (**Supplementary Figure S3**). The TCA cycle
387 plays a central role in maintaining the bacterial metabolic status and has been repeatedly
388 implicated in the regulation of staphylococcal virulence [45, 46]. Hence, changes in TCA
389 cycle activity are likely to induce alterations of the overall metabolome of the bacterium that,
390 in turn, may modulate the activity of metabolite-responsive global regulators such as SigB or
391 RpiR.

392 RpiRc, a RpiR family-member controlling enzymes involved in sugar catabolism in *S.*
393 *aureus*, has been shown to repress RNAIII [28], supporting the notion of a direct connection
394 between the PPP and the regulation of *S. aureus* virulence. The expression of *sigB* has also
395 been shown to play a crucial role during the intracellular life of bacteria possibly by down-
396 regulating pro-inflammatory virulence factors and increasing the expression of factors
397 promoting persistence [18, 39].

398 We propose (**Figure 6**) that in wild-type *S. aureus*, transketolase activity results in a
399 positive regulation of *rpiRc* and *sigB* transcription and allows the repression of *RNAIII*.
400 Consequently, repression of toxins production favours intracellular growth. In contrast, in a
401 Δ *tkt* mutant, *rpiRc* and *sigB* transcriptions are not activated, possibly due to metabolic
402 changes (such as R5P accumulation) and *RNAIII* is not repressed. At this stage, it cannot be
403 excluded that the enzyme itself might also directly affect transcription of these regulators.

404 TKT inhibitors are currently actively tested in cancer therapy [47] and TKT could
405 constitute an efficient target against tuberculosis [35] and malaria [48]. The present work,
406 highlighting an unprecedentedly reported role of transketolase in *S. aureus* intracellular
407 survival, suggests that modulation of the pentose phosphate pathway activity may also
408 represent an interesting mean to fight *S. aureus* infections.

409

410 Legends

411

412 **Figure 1. Phenotypic characterization of the Δ tkk mutant**

413 **A. Growth of USA300 wild type (WT), Δ tkk mutant (Δ tkk) and its complemented**
414 **derivative (Cp-tkk).** USA300 WT, Δ tkk and Cp-tkk strains were grown in BHI Broth or DMEM
415 for 21 hours at 37°C. Optic densities were measured every hour from 0 to 8 hours and then
416 at 21 hours. Each panel is a representative experiment.

417 **B. Metabolomic analysis of the Δ tkk mutant strain.** Quantitative metabolomic analysis was
418 performed by ion chromatography and tandem mass spectrometry (IC-MS/MS) after
419 overnight cultures, in BHI Broth, from WT USA300 and Δ tkk mutant strains (see **Table S1**).
420 The heatmap, obtained using MetaboAnalyst (<https://www.metaboanalyst.ca>), illustrates the
421 metabolic shift between the two strains.

422

423 **Figure 2. Intracellular proliferation and persistence of *S. aureus*.**

424 **A, B. Intracellular proliferation of USA300 wild type (WT) and Δ tkk mutant (Δ tkk) strains.**

425 Endothelial EA.hy926 cells expressing mKate2 nuclear restricted red fluorescent protein
426 were infected with GFP expressing WT and Δ tkk strains. One hour after infection, cells were
427 washed several times with gentamicin containing medium to eliminate extracellular bacteria.
428 Gentamicin concentration of 50 μ g/ml was maintained throughout the experiment. Images
429 were acquired every 30 minutes using Incucyte[®] S3 live cell imaging system. A.
430 Representative images (see also Movie 1 and 2). Red: cell nuclei. Green: GFP expressing
431 bacteria. For each line, a white circle was centred on a unique infected cell. Bar = 100 μ m. B.
432 Quantitative analysis representing the number of green particles divided by the number of
433 red nuclei during the first day after infection. Blue line: WT strain. Red line: Δ tkk strain. Black
434 line: non infected cells.

435 **C. Persistence of intracellular WT and Δ tkk strains.** Endothelial EA.hy926 cells were
436 infected with WT and Δ tkk strains as described above. Gentamicin was maintained
437 throughout the experiment. Survival of *S. aureus* was assessed by plating and counting

438 CFUs three, seven and ten days after infection. Results are shown as mean \pm SD of triplicate
439 measurements.

440

441 **Figure 3. Transcription profile of intracellular *S. aureus*.**

442 Endothelial EA.hy926 cells were infected with USA300 WT and Δ tkt strain as described in
443 figure 2. One, three or seven days after infection, cells were washed and collected. RNA
444 from intracellular *S. aureus* were prepared and gene expression of RNAIII, rpiRc, sigB, rsbU,
445 rsbW and asp23 was analysed by quantitative RT-PCR. Gene expression level was
446 normalized by that of gyrA at days 1, 3 and 7 after infection of EA-hy296 cells. Data are
447 shown as mean normalized expression \pm SD of triplicate measurements from two
448 independent experiments. (ANOVA, ns: not statistically different; * $p < 0.01$, ** $p < 0.001$).
449 Functional complementation was assessed on two other independent experiments and
450 presented in **Supplementary Figure S5**.

451

452 **Figure 4. Proliferation of *S. aureus* in the spleen and the kidney of infected mice.**

453 Bacterial loads in the spleen and the kidney of BalB/c mice infected intravenously with 10^7
454 bacteria of the WT, Δ tkt and Cp-tkt strain. Bacterial counts are expressed in CFU/organ (WT
455 and Δ tkt $n=10$; Cp-tkt $n=6$). Data are shown as mean \pm SD. Statistical significance was
456 determined using the Kruskal Wallis test with Dunn's correction (ns: not statistically different,
457 *** $p < 0.001$, **** $p < 0.0001$). No statistical significance between strains was observed in the
458 spleen of infected mice.

459

460 **Figure 5. Carbon sources influence the transcription profile of the SigB pathway.**

461 *S. aureus* USA300 WT strain was grown to stationary phase in CDM supplemented with
462 glucose or ribose or fructose and gene expression profile of rpiRc, sigB, rsbU, rsbW and
463 asp23 was analysed by quantitative RT-PCR. Gene expression level was normalized by that
464 of gyrA. Data are shown as mean normalized expression \pm SD of triplicate measurements
465 from two independent experiments. Statistical significance was assessed in comparison to
466 bacteria grown in CDM glucose (ANOVA, * $p < 0.01$, ** $p < 0.001$).

467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495

Figure 6. Schematic depiction of the impact of TKT inactivation on sigB and rpiRc regulation.

SigB dependent regulation of RNAlII in Δ tkk context.

Movie 1: Intracellular proliferation of USA300 wild type strain. Endothelial EA.hy926 cells expressing a nuclear restricted Red Fluorescent Protein were infected with GFP expressing wild type strains. One hour after infection cells were washed several times with gentamicin containing medium to eliminate extracellular bacteria (see materials and methods section). Gentamicin concentration of 50 μ g/ml was maintained throughout the experiment. Images were acquired every 30 minutes using Incucyte[®] S3 live cell imaging system.

Movie 2: Intracellular proliferation of USA300 Δ tkk mutant strain. Same protocol as above in movie 1.

Acknowledgements

We are grateful to the Cell imaging core facility of the “Structure Fédérative de Recherche” Necker INSERM US24/CNRS UMS3633 for his technical support.

The following reagents were provided by the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA) for distribution by BEI Resources, NIAID, NIH: Staphylococcus aureus, Strain USA300-0114, NR-46070; Escherichia coli – Staphylococcus aureus Shuttle Vector pNR-46158, Recombinant in Staphylococcus aureus, NR-46158.

496 **References**

497

498 1. Otto M. Community-associated MRSA: what makes them special? *Int J Med Microbiol*
499 **2013**; 303:324-30.

500 2. Thomer L, Schneewind O, Missiakas D. Pathogenesis of *Staphylococcus aureus*
501 Bloodstream Infections. *Annual review of pathology* **2016**; 11:343-64.

502 3. Proctor RA, von Eiff C, Kahl BC, et al. Small colony variants: a pathogenic form of bacteria
503 that facilitates persistent and recurrent infections. *Nature reviews Microbiology* **2006**; 4:295-
504 305.

505 4. Tuchscher L, Heitmann V, Hussain M, et al. *Staphylococcus aureus* small-colony variants
506 are adapted phenotypes for intracellular persistence. *The Journal of infectious diseases*
507 **2010**; 202:1031-40.

508 5. Strobel M, Pfortner H, Tuchscher L, et al. Post-invasion events after infection with
509 *Staphylococcus aureus* are strongly dependent on both the host cell type and the infecting *S.*
510 *aureus* strain. *Clin Microbiol Infect* **2016**; 22:799-809.

511 6. Hayes SM, Howlin R, Johnston DA, et al. Intracellular residency of *Staphylococcus aureus*
512 within mast cells in nasal polyps: A novel observation. *The Journal of allergy and clinical*
513 *immunology* **2015**; 135:1648-51.

514 7. Hanssen AM, Kindlund B, Stenklev NC, et al. Localization of *Staphylococcus aureus* in
515 tissue from the nasal vestibule in healthy carriers. *BMC microbiology* **2017**; 17:89.

516 8. Moldovan A, Fraunholz MJ. In or out: phagosomal escape of *Staphylococcus aureus*.
517 *Cellular microbiology* **2018**:e12997.

518 9. Tan X, Coureuil M, Ramond E, et al. Chronic *Staphylococcus aureus* lung infection
519 correlates with proteogenomic and metabolic adaptations leading to an increased
520 intracellular persistence. *Clin Infect Dis* **2019**.

521 10. Branger C, Gardye C, Lambert-Zechovsky N. Persistence of *Staphylococcus aureus*
522 strains among cystic fibrosis patients over extended periods of time. *J Med Microbiol* **1996**;
523 45:294-301.

- 524 11. Kahl BC, Duebbers A, Lubritz G, et al. Population dynamics of persistent *Staphylococcus*
525 *aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study.
526 *Journal of clinical microbiology* **2003**; 41:4424-7.
- 527 12. Fisher RA, Gollan B, Helaine S. Persistent bacterial infections and persister cells. *Nature*
528 *reviews Microbiology* **2017**; 15:453-64.
- 529 13. Grosz M, Kolter J, Paprotka K, et al. Cytoplasmic replication of *Staphylococcus aureus*
530 upon phagosomal escape triggered by phenol-soluble modulins. *Cellular microbiology*
531 **2014**; 16:451-65.
- 532 14. Korea CG, Balsamo G, Pezzicoli A, et al. Staphylococcal Esx proteins modulate
533 apoptosis and release of intracellular *Staphylococcus aureus* during infection in epithelial
534 cells. *Infection and immunity* **2014**; 82:4144-53.
- 535 15. Kohler C, von Eiff C, Liebeke M, et al. A defect in menadione biosynthesis induces global
536 changes in gene expression in *Staphylococcus aureus*. *Journal of bacteriology* **2008**;
537 190:6351-64.
- 538 16. Kriegeskorte A, König S, Sander G, et al. Small colony variants of *Staphylococcus*
539 *aureus* reveal distinct protein profiles. *Proteomics* **2011**; 11:2476-90.
- 540 17. Kriegeskorte A, Grubmüller S, Huber C, et al. *Staphylococcus aureus* small colony
541 variants show common metabolic features in central metabolism irrespective of the
542 underlying auxotrophism. *Frontiers in cellular and infection microbiology* **2014**; 4:141.
- 543 18. Tuchscherer L, Löffler B. *Staphylococcus aureus* dynamically adapts global regulators and
544 virulence factor expression in the course from acute to chronic infection. *Current genetics*
545 **2016**; 62:15-7.
- 546 19. Stincone A, Prigione A, Cramer T, et al. The return of metabolism: biochemistry and
547 physiology of the pentose phosphate pathway. *Biological reviews of the Cambridge*
548 *Philosophical Society* **2015**; 90:927-63.
- 549 20. Harper L, Balasubramanian D, Ohneck EA, et al. *Staphylococcus aureus* Responds to
550 the Central Metabolite Pyruvate To Regulate Virulence. *mBio* **2018**; 9.
- 551 21. Purves J, Cockayne A, Moody PC, Morrissey JA. Comparison of the regulation,
552 metabolic functions, and roles in virulence of the glyceraldehyde-3-phosphate

553 dehydrogenase homologues gapA and gapB in *Staphylococcus aureus*. *Infection and*
554 *immunity* **2010**; 78:5223-32.

555 22. Vitko NP, Spahich NA, Richardson AR. Glycolytic dependency of high-level nitric oxide
556 resistance and virulence in *Staphylococcus aureus*. *mBio* **2015**; 6.

557 23. Vitko NP, Grosser MR, Khatri D, Lance TR, Richardson AR. Expanded Glucose Import
558 Capability Affords *Staphylococcus aureus* Optimized Glycolytic Flux during Infection. *mBio*
559 **2016**; 7.

560 24. Gardner SG, Marshall DD, Daum RS, Powers R, Somerville GA. Metabolic Mitigation of
561 *Staphylococcus aureus* Vancomycin Intermediate-Level Susceptibility. *Antimicrobial agents*
562 *and chemotherapy* **2018**; 62.

563 25. Fleury B, Kelley WL, Lew D, Gotz F, Proctor RA, Vaudaux P. Transcriptomic and
564 metabolic responses of *Staphylococcus aureus* exposed to supra-physiological temperatures.
565 *BMC microbiology* **2009**; 9:76.

566 26. Joseph B, Schneiker-Bekel S, Schramm-Gluck A, et al. Comparative genome biology of
567 a serogroup B carriage and disease strain supports a polygenic nature of meningococcal
568 virulence. *Journal of bacteriology* **2010**; 192:5363-77.

569 27. Mekonnen SA, Palma Medina LM, Michalik S, et al. Metabolic niche adaptation of
570 community- and hospital-associated methicillin-resistant *Staphylococcus aureus*. *Journal of*
571 *proteomics* **2018**.

572 28. Zhu Y, Nandakumar R, Sadykov MR, et al. RpiR homologues may link *Staphylococcus*
573 *aureus* RNAlII synthesis and pentose phosphate pathway regulation. *Journal of bacteriology*
574 **2011**; 193:6187-96.

575 29. Gaupp R, Wirf J, Wollenberg B, et al. RpiRc Is a Pleiotropic Effector of Virulence
576 Determinant Synthesis and Attenuates Pathogenicity in *Staphylococcus aureus*. *Infection*
577 *and immunity* **2016**; 84:2031-41.

578 30. Balasubramanian D, Ohneck EA, Chapman J, et al. *Staphylococcus aureus* Coordinates
579 Leukocidin Expression and Pathogenesis by Sensing Metabolic Fluxes via RpiRc. *mBio*
580 **2016**; 7.

- 581 31. Hussain M, Hastings JG, White PJ. A chemically defined medium for slime production by
582 coagulase-negative staphylococci. *J Med Microbiol* **1991**; 34:143-7.
- 583 32. Mackay GM, Zheng L, van den Broek NJ, Gottlieb E. Analysis of Cell Metabolism Using
584 LC-MS and Isotope Tracers. *Methods in enzymology* **2015**; 561:171-96.
- 585 33. Chong J, Soufan O, Li C, et al. MetaboAnalyst 4.0: towards more transparent and
586 integrative metabolomics analysis. *Nucleic acids research* **2018**; 46:W486-W94.
- 587 34. Shaw JA, Henard CA, Liu L, Dieckman LM, Vazquez-Torres A, Bourret TJ. *Salmonella*
588 *enterica* serovar Typhimurium has three transketolase enzymes contributing to the pentose
589 phosphate pathway. *The Journal of biological chemistry* **2018**; 293:11271-82.
- 590 35. Fullam E, Pojer F, Bergfors T, Jones TA, Cole ST. Structure and function of the
591 transketolase from *Mycobacterium tuberculosis* and comparison with the human enzyme.
592 *Open biology* **2012**; 2:110026.
- 593 36. Rollin G, Tan X, Tros F, et al. Intracellular Survival of *Staphylococcus aureus* in
594 Endothelial Cells: A Matter of Growth or Persistence. *Frontiers in microbiology* **2017**; 8:1354.
- 595 37. Boisset S, Geissmann T, Huntzinger E, et al. *Staphylococcus aureus* RNAIII coordinately
596 represses the synthesis of virulence factors and the transcription regulator Rot by an
597 antisense mechanism. *Genes & development* **2007**; 21:1353-66.
- 598 38. Mader U, Nicolas P, Depke M, et al. *Staphylococcus aureus* Transcriptome Architecture:
599 From Laboratory to Infection-Mimicking Conditions. *PLoS genetics* **2016**; 12:e1005962.
- 600 39. Tuchscher L, Bischoff M, Lattar SM, et al. Sigma Factor SigB Is Crucial to Mediate
601 *Staphylococcus aureus* Adaptation during Chronic Infections. *PLoS pathogens* **2015**;
602 11:e1004870.
- 603 40. Pane-Farre J, Jonas B, Hardwick SW, et al. Role of RsbU in controlling SigB activity in
604 *Staphylococcus aureus* following alkaline stress. *Journal of bacteriology* **2009**; 191:2561-73.
- 605 41. Gertz S, Engelmann S, Schmid R, Ohlsen K, Hacker J, Hecker M. Regulation of sigmaB-
606 dependent transcription of sigB and asp23 in two different *Staphylococcus aureus* strains.
607 *Molecular & general genetics : MGG* **1999**; 261:558-66.

608 42. Cheng AG, Kim HK, Burts ML, Krausz T, Schneewind O, Missiakas DM. Genetic
609 requirements for *Staphylococcus aureus* abscess formation and persistence in host tissues.
610 *Faseb J* **2009**; 23:3393-404.

611 43. Tuchscher L, Geraci J, Loffler B. *Staphylococcus aureus* Regulator Sigma B is Important
612 to Develop Chronic Infections in Hematogenous Murine Osteomyelitis Model. *Pathogens*
613 **2017**; 6.

614 44. Lei MG, Lee CY. RbsR Activates Capsule but Represses the rbsUDK Operon in
615 *Staphylococcus aureus*. *Journal of bacteriology* **2015**; 197:3666-75.

616 45. Somerville GA, Cockayne A, Durr M, Peschel A, Otto M, Musser JM. Synthesis and
617 deformylation of *Staphylococcus aureus* delta-toxin are linked to tricarboxylic acid cycle
618 activity. *Journal of bacteriology* **2003**; 185:6686-94.

619 46. Ding Y, Liu X, Chen F, et al. Metabolic sensor governing bacterial virulence in
620 *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences of the United*
621 *States of America* **2014**; 111:E4981-90.

622 47. Xu IM, Lai RK, Lin SH, et al. Transketolase counteracts oxidative stress to drive cancer
623 development. *Proceedings of the National Academy of Sciences of the United States of*
624 *America* **2016**; 113:E725-34.

625 48. Thota S, Yerra R. Drug Discovery and Development of Antimalarial Agents: Recent
626 Advances. *Current protein & peptide science* **2016**; 17:275-9.

627

628

Supplementary Methods

Strains, Cells, Culture conditions and Infection.

S. aureus. The epidemic clone *S. aureus* USA300-LAC (designated USA300-WT) was provided by the Biodefense and Emerging Infections Research Resources (BEI). The GFP-expressing strain was generated by curing the p03 plasmid from USA300-WT and introducing the pCN57-GFP recombinant plasmid (obtained from the BEI) by electroporation as described before [1]. The Δ tkl mutant strain and the complemented strain were constructed as described below. Strains and plasmids used are summarized at the end of these supplementary methods.

Before experiments bacteria were grown 24 hours at 37°C on BHI agar plates supplemented with antibiotics when required and then overnight in BHI Broth from an optical density 600 nm (OD_{600}) of 0.05. The day of experiment, bacteria were resuspended in BHI broth to an OD_{600} of 0.05 and grown at 37°C until the culture reached the initial-middle log phase (OD_{600} of 0.6).

Growth curve were performed in brain heart infusion, DMEM and chemically defined medium (CDM) supplemented with ribose or fructose or glycerol when it was required [2].

Internalization rate and survival inside the EA.hy926 endothelial cell line were similar between the GFP strain and the parental strain.

Cell line. EA.hy926 cell line (ATCC CRL-2922), originally derived from human umbilical vein, were grown in Dulbecco's modified Eagle high glucose medium (DMEM, Dominique Dutscher) supplemented with 10 % fetal bovine serum (FBS, Thermofisher scientific) in a humidified 5 % CO₂ atmosphere at 37 °C. When indicated cells were incubated with no glucose DMEM supplemented with glucose, ribose or fructose at a concentration of 25mM. To facilitate cell identification and counting during time lapse experiments, EA.hy926 cells were transduced with the IncuCyte[®] NuLight Red Lentivirus, following the manufacturer's

recommendations, to obtain red nuclear labelling of living cells due to the expression of the red fluorescent protein mKate2 containing a nuclear localization signal.

Infection.

When fresh cultures reached OD₆₀₀ of 0.6, bacteria were added to endothelial cells at a multiplicity of infection (MOI) of 1 and placed in a humidified 5 % CO₂ atmosphere at 37 °C for 1 h. One hour after infection, each well was washed three times with 100 µL of phosphate buffer saline (PBS) containing 300 µg/mL gentamicin, to remove extracellular bacteria. Cells were then incubated with 100 µL of cell culture medium containing 50 µg.mL⁻¹ gentamicin. Infected cells were kept in a humidified 5 % CO₂ atmosphere at 37 °C. Importantly, gentamicin is an antibiotic with a high bactericidal effect on *S. aureus* USA300 (CMI=2 µg.mL⁻¹) and a very poor penetration inside eukaryotic cells. In our model the use of gentamicin in the extracellular medium was necessary to abolish extracellular proliferation.

Construction of the *S. aureus* Δ*tk*t chromosomal deletion mutant

We inactivated the *tk*t gene in wild-type *S. aureus* USA300 strain and substituted it by the kanamycin resistance gene *nptII* fused with pGro promoter. For this, we used the pMAD-temperature-sensitive shuttle vector system [3]. Briefly, the recombinant plasmid pMAD-Δ*tk*t was constructed by overlap PCR. First, the two regions (upstream 694bp, downstream 567bp) flanking *tk*t and the *nptII* gene (fused with pGro promoter 1,091bp) were amplified by PCR. The region (2,352 bp) was then amplified by two-step overlap PCR. The resulting PCR product was cloned in pMiniT 2.0 (to yield recombinant plasmid pMiniT/*tk*tUp-*npt*-*tk*tDown). This recombinant plasmid was digested with BamHI and EcoRI (New England BioLabs) and the BamHI-EcoRI *tk*tUp-*npt*-*tk*tDown DNA fragment was finally subcloned into BamHI-EcoRI-digested pMAD. All PCR reactions were realized using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific) and PCR products were purified using QIAquick PCR purification kit (Qiagen). The pMAD-Δ*tk*t plasmid was first introduced into *S. aureus* RN4220 prior to electroporation into *S. aureus* USA300. We used a standard two-step allelic

exchange procedure [3] to create the chromosomal *S. aureus* USA300 Δ tkk strain. The Δ tkk strain was finally checked for loss of the corresponding wild-type genes by PCR sequencing (Eurofins Genomics) using specific primers (described at the end of these supplementary methods).

Construction of the complementation of *S. aureus* Cp-tkk strain

The plasmid pCN57-CPtkk was constructed by homologous recombination [4]. The pCN57 linear vector and tkk gene fragments were amplified by PCR. To allow recombination, overlapping region of 20pb was added at each ends. PCN57 was linearized using SphI (New England BioLabs). *E. coli* DH5 α were chemically transfected with a mix of both PCR product (insert: vector stoichiometry 2:1, 100ng of vector) in 10 μ l MilliQ water. The colonies containing plasmid pCN57-CP-tkk were checked by PCR and the construct was sequenced. The pCN57-CP-tkk vector was then transfected into *S. aureus* RN4220 prior to electroporation into *S. aureus* USA300 Δ tkk. The Δ tkk-Cp-tkk (Cp-tkk) strain was finally checked for complementation of wild-type genes by PCR sequencing (Eurofins Genomics) using specific primers.

Time lapse microscopy

Four days prior to infection, NuLight Red labelled EA.hy926 cells were seeded in ImageLock 96-well plates (Essen BioScience Inc) in culture medium without antibiotic. Cells were infected with wild-type and mutant of the GFP-expressing USA300 strain as described above. Plates were incubated and monitored at 5% CO₂ and 37°C for 24 hours in the fully automated microscope Incucyte[®] S3 (Essen BioScience). Images were taken every 30 minutes with the 20X objective. The medium was changed once during the observation period. Analysis and time-lapse videos (from which images were extracted) were generated by using Incucyte[®] S3 software.

Detailed analysis: Green items were analysed with a threshold of 1 GCU and using Top-hat segmentation mode. Red item were analysed with a threshold of 3 RCU and using Top-hat

segmentation mode and edge sensitivity of -25. A spectral unmixing of 10% was applied to remove red signal from green signal. Green item counts were considered as an approximation of the number of intracellular bacteria, and red item counts considered as the number of EA-hy296 cells. (Green items/Red items) represents the number of intracellular bacteria per cell during the time of infection.

Transcriptional analyses

Intracellular bacteria RNA extraction: EA.hy926 cells were grown to confluence in 75 cm² flasks and infected with *S. aureus* as described above. At selected time points, the culture medium was removed and the flasks were washed twice with PBS without antibiotics. Cells were lysed with 5 mL of distilled water for 10 min and the surface of the flask was scraped to detach the cells. The contents were recovered and centrifuged for 5 min at 3000 g. The supernatant was carefully removed and the pellet (which contained cell debris and bacteria) was resuspended in 100 µL of TE buffer containing 20 µg of lysostaphin and incubated for 5 minutes at 37 °C [5] to allow cell wall peptidoglycan degradation.

Planktonic bacteria RNA extraction: *S. aureus* was resuspended in CDM supplemented with glucose, ribose or fructose to an OD₆₀₀ of 0.05 and grown overnight at 37°C . Bacteria were centrifuged at 12 000 g for 2 min and incubated in 50 µL of TE buffer containing 10 µg of lysostaphin for 5 minutes at 37 °C. The samples were resuspended in Trizol (ThermoFisher Scientific) and treated with chloroform. RNAs were recovered using RNeasy Clean-up column (Qiagen) and DNA was digested using Turbo Dnase (ThermoFisher Scientific). RNA concentration was measured using NanoDrop 1000 (ThermoFisher Scientific). Reverse transcription was carried out with 500 ng of RNA and 0.5 µg of random primers (Promega). After denaturation at 70 °C for 5 min, 15 µL of the mix containing 4 µL M-MLV 5 X reaction buffer, 1 µL of 0.5 mM PCR Nucleotide Mix, 0.5 µL of RNasin Ribonuclease Inhibitor, and 1 µL M-MLV Reverse Transcriptase (Promega) was added. Samples were incubated 5 min at 25 °C, 1 h at 42 °C, then 15 min at 70 °C and chilled on ice. Quantitative RT-PCR was performed using FastStart SYBR Green qPCR Master Mix (ThermoFischer Scientific) or

Luna Universal qPCR Master Mix (New England Biolabs) according to the manufacturer's recommendations. Transcript levels were analyzed using a 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the standard settings of the system software. The thermal cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene specific primer sequences are presented at the end of these supplementary methods. Expression of the gene *gyrA* was chosen as a reference. We used the "The Relative Standard Curve Method" for analyzing the qRT-PCR data.

Metabolomic analyses

Bacteria: Metabolite profiling of *S. aureus* isolates was performed by liquid chromatography–mass spectrometry (LC-MS) as described [6, 7]. Two independent experiments with three biological replicates were performed for each isolate. Briefly, *S. aureus* strains were grown in BHI Broth overnight to stationary phase and pelleted by centrifugation. Metabolic activity was blocked by immersion in liquid nitrogen for 10 sec. Metabolites were extracted using a solvent mixture of Methanol/ACN/H₂O (50:30:20) at –20 °C (Extraction solution). Samples were vortexed for 5 min at 4°C, and then centrifuged at 16,000 g for 15 minutes at 4°C.

Endothelial cells: EA.hy926 endothelial cells were grown to confluence on a 12-well plate in no glucose DMEM supplemented with glucose or fructose or ribose (25 mM). One independent experiment with three biological replicates was performed for each condition. At each time points the cells were washed with cold PBS. Metabolism was blocked by plunging the plate into liquid nitrogen for 10 seconds. The cell metabolites were extracted from the samples by placing the cell culture plate on a rocking shaker at 4°C for 10 minutes. The extraction solution from each well was then collected and centrifuged at 16,000 g for 15 minutes at 4°C.

The supernatants were collected and analysed by LC-MS using SeQuant ZIC-pHilic column (Millipore) for the liquid chromatography separation [6]. The aqueous mobile-phase solvent was 20 mM ammonium carbonate plus 0.1% ammonium hydroxide solution and the organic mobile phase was acetonitrile. The metabolites were separated over a linear gradient from

80% organic to 80% aqueous phase for 15 min. The column temperature was 48 °C and the flow rate was 200 µl/min. The metabolites were detected across a mass range of 75–1,000 m/z using the Q-Exactive Plus mass spectrometer (Thermo) at a resolution of 35,000 (at 200 m/z) with electrospray ionization and polarity switching mode. Lock masses were used to insure mass accuracy below 5 ppm. The peak areas of different metabolites were determined using TraceFinder software (Thermo) using the exact mass of the singly charged ion and known retention time on the HPLC column. By this approach we were able to identify and measure: Glucose, Glucose-6P (G6P), UDP-glucose (UDP-Glc), Sedoheptulose-7-P (SH-7P), Ribose 5-phosphate, Glyceraldehyde 3-phosphate, Lactate, Pyruvate, Phospho-Enol-Pyruvate (PEP), Citrate (Cit), cis-aconitate (cis-aco), Succinate (Suc), Fumarate (Fum), Malate (Mal), alpha ketoglutarate (aKG), dihydroxyacetone phosphate (DHAP), N-carbamoyl-L-aspartate, Nicotinamide adenine dinucleotide (NAD), NADH, Nicotinamide adenine dinucleotide phosphate (NADP), NADPH, Adenine, Adenosine, Adenosine Mono, Di and Tri-phosphate (AMP, ADP, ATP), Cytidine, Cytidine Mono, Di and Tri-phosphate (CMP, CDP, CTP), Uridine Mono, Di and Tri-phosphate (UMP, UDP, UTP), Hypoxanthine, Guanine, Guanosine, Guanidine Mono and Di-phosphate (GMP, GDP), Inosine monophosphate (IMP), Pantothenate, Dephospho-Coenzyme A, Coenzyme A (CoA), Flavin adenine dinucleotide (FAD), Niacin, Nicotinamide, 3-hydroxybutyrate, Urate, 5-oxo-L-Proline, Acetyl-aspartate, Acetyl-glutamine, Alanine, Arginine, Argininosuccinate, Asparagine, Aspartate, Betaine, Citrulline, Cystathionine, Cystine, Glutamine, Glutamate, Glycine, Histidine, IsoLeucine, Leucine, Sarcosine, Lysine, Methionine, Ornithine, Phenylalanine, Proline, Serine, Taurine, S-Adenosyl-L-methionine, Threonine, Tryptophan, Tyrosine, Valine, Butyric acid, Carnitine, Decanoic acid, Lauric acid, Hexanoic acid, Linoleic acid, Myristic acid, Octanoic acid, Oleic acid, Palmitic acid.

Statistical analyses were performed using MetaboAnalyst 4.0 software [8, 9]. The algorithm for heatmap clustering was based on the Pearson distance measure for similarity and the Ward linkage method for biotype clustering. Metabolites with similar abundance patterns were positioned closer together.

Transketolase activity assay

Bacteria were grown to stationary phase in BHI Broth and 1 ml of culture was harvested. Bacteria were washed in PBS, pelleted by centrifugation, and resuspended in 300 μ l of 50 mM Tris with 60 μ g lysostaphin, pH 7.5. Samples were incubated at 37 $^{\circ}$ C for 5 min and then sonicated at 4 $^{\circ}$ C (30s on, 30s off, repeated 30 times) (Diagenode). Samples were centrifuged at 12,000 g and 4 $^{\circ}$ C for 10 min to remove cell debris and protein concentration was determined using BCA assay with BSA as a standard (Thermo Fisher Scientific). TKT activity was analysed as described previously [10]. Briefly, each enzymatic reactions were performed at a final volume of 100 μ l containing 20 μ l of supernatant, 50 mM Tris (pH 7.5), 0.24 mM $MgCl_2$, 10 μ M thiamine pyrophosphate, 0.5 mM NADH, 3 units of glycerol-3-phosphate dehydrogenase, 10 units of triosephosphate isomerase, 0.5mM D-xylulose-5-phosphate, and 0.5mM D-erythrose-4-phosphate (all reagents obtained from Sigma-Aldrich). The reaction proceeded at 37 $^{\circ}$ C for 5 min, and the decrease in $A_{340\text{ nm}}$ was monitored with a microplate reader (Molecular Devices). Transketolase enzyme activity in bacteria lysates was expressed as units per microgram of total protein. One unit of enzyme was defined as the amount of enzyme that oxidized 1 μ mol of NADH per minute.

LAMP-1 colocalization assay

EA.hy926 endothelial cells were grown to confluence on 8-well Slides (Lab Tek II) and infected with *S. aureus* as described above. For each point, cells were washed 3 times with PBS and fixed 20 min in PBS-4% paraformaldehyde at 4 $^{\circ}$ C. Fixation was stopped in PBS- NH_4Cl 50 mM. Cells were permeabilized with PBS containing 0.1% saponin and 5% goat serum for 10 min on ice. Cells were incubated for 30 min with anti-LAMP-1 (Lysosomal-associated membrane protein 1) rabbit polyclonal antibody (1/200e, AbCam) diluted in PBS containing 5% goat serum (staining buffer). After washing, cells were incubated for 30 min with Alexa-568 conjugated donkey anti-rabbit secondary antibodies (1/400e, AbCam) diluted in staining buffer. After washing, 4,6-diamidino-2-phenylindole (DAPI) was added (1/10,000e)

for 20 min and glass coverslips were mounted in Mowiol (Cityfluor Ltd.). Cells were examined using an X63 oil-immersion objective on an Apotome microscope (Zeiss™). Colocalization of GFP expressing bacteria with LAMP-1 staining was quantified by using Image J software (plug in: Coloc based Sur-vol _ Beta v6). Data were obtained from more than 200 infected cells for each condition and expressed as % of bacteria associated with LAMP-1 \pm SD.

In vivo infection

For all experiments we used 6 to 10 weeks-old female BALB/c (Janvier Labs) with access to food and water ad libidum.

Infection. Overnight cultures of *S. aureus* in BHI Broth were diluted in BHI Broth to an OD₆₀₀ of 0.05. Bacteria were grown at 37°C until the culture reached the initial-middle log phase (OD₆₀₀=0.6) and resuspended in a sterile isotonic saline solution to an OD₆₀₀ of 0.4.

Mice were infected with 10⁷ bacteria intravenously (iv) in the tail vein. Inoculum titers were checked by spreading dilutions of the preparations on BHI agar plates. For bacterial burden determination, mice were euthanized after 4, 24, 48 and 72 hours. Sequential dilutions of homogenized preparations from spleen and kidney were spread onto BHI agar plates.

Mice infected with the complemented strain were treated with erythromycin to avoid loss of the complementing plasmid. For this, mice received one dose of erythromycin 16 hours before infection and 2 doses of erythromycin per day until they were sacrificed (500 μ g of erythromycin per dose).

Statistics

Data were analyzed using GraphPad Prism software. Results are presented either with one representative experiment for clarity or as means \pm standard deviation (SD). The number of biological and technical replicates is indicated in the legend of each figure. For two-sample comparisons statistical significance was measured using unpaired two-tail Student's t-test. For comparisons between more than two groups, statistical significance was measured using

one-way analysis of variance (ANOVA) with Dunnett's correction. P values of $p < 0.05$ were considered to indicate statistical significance.

Ethics statement

All experimental procedures involving animals were conducted in accordance with guidelines established by the French and European regulations for the care and use of laboratory animals (Decree 87–848, 2001–464, 2001–486 and 2001–131 and European Directive 2010/63/UE) and approved by the INSERM Ethics Committee (Authorization Number: 75-906).

Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)	Source of reference
Strains		
E.coli		
DH5 α	$\Phi 80'$ lacZ Δ M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	Invitrogen
S.aureus		
USA300 LAC	MRSA containing phage Φ SA2usa (PVL), Ery ^R	BEI Resources
USA300-Ery ^S	Erythromycin-sensitive derivative of USA300	Laboratory collection
RN4220	Restriction-deficient transformation recipient S. aureus	Laboratory collection
USA300 Δ tkt	tkt defective mutant, Kan ^R	This study
USA300 Δ tkt-CPtk	Complemented strain, USA300 Δ tkt containing pCN57-CPtk, Ery ^R Kan ^R	This study
USA300 LAC-gfp	Strain containing pCN57-GFP, Ery ^R	Laboratory collection
USA300 Δ tkt-gfp	USA300 Δ tkt containing pCN57, Ery ^R Kan ^R	This study
Plasmids		
pMAD	Plasmid with a thermosensitive origin, used for gene deletion; Amp ^R Ery ^R	Laboratory collection
pMAD- Δ tkt	Derivative of pMAD with upstream and downstream sequences from tkt gene and nptII ^a , Amp ^R Ery ^R Kan ^R	This study
pCN57	Plasmid containing gfp expression cassette (constitutive expression), Ery ^R	Laboratory collection
pXT02	Derivative of pCN57. The gfp expression cassette was replaced by the sequence of the tkt gene with its own promoter.	This study

pMiniT 2.0

Plasmid used to clone upstream and downstream regions from tkt gene and nptII, Amp^R New England Biolabs

^a. nptII: neomycin phosphotransferase gene, determining kanamycin resistance.

Primers used for Δ tkt mutant construction and complementation

Primer name	Primer sequence (From 5' to 3')
tk tup	F: CGGAATTCGAAGTAGGATTA ACTCAAGAAGAAGC
	R: TTTAGCTCGACTAATCCATACA ACTGCTAAAGCTAATCCTACTGAC
tk tdn	F: CCTTCTTGACGAGTTCTTCTGAGGATGGCATAAATATGTAGGTACTG
	R: CGGGATCCATTTGCATCATCATACGAAGC
nptII	F: TTGTATGGATTAGTCGAGCTAAA
	R: TCAGAAGA ACTCGTCAAGAAGG
s_tkt	F: GAATATCGAAAGAATTAACGAGTTAGC
	R: CGCACTCTTCATATTTTGATCCAT
s_pMAD	F: TCTGGCCATTGCTCTGGGTTA
	R: GCTGTCCCTGATGGTCGTCAT
HRtkt	F: CTTTGAGTGAGCTGGCGGCCGGATCCGGGTTTAAACAACAAATTGAAAATAC
	R: CATAGTAAGCTGCATGCAGCCCCGGGTTATAAGCTCATAACTTGTTTAAAGATATTTTC
HRpCN57	F: ACCAAGTTATGAGCTTATAACCCGGGGCTGCATGCAGCTTACTATG
	R: TCAATTTGTTGTTTAAACCCGGATCCGGCCGCCAGCTCACTCAAAG

Primers used for RT-qPCR.

Gene name	Primer sequence (From 5' to 3')
RNAIII	F: GCCATCCCAACTTAATAACCATGTAAAATTAGC
	R: GCGATGTTGTTTACGATAGCTTACATGCTAG
rpirC	F: CATAACGTA CTACACGCGCACTTAATC
	R: GTCGGTAGCAACTACAAAAGATGCG
tkt	F: GGTCATAATGGTGCATCTCGTGC
	R: CATTGGATTTGTTTGAACCAGCAAGG
gyrA	F: GGTGGATTTGAAA ACTTAGAGGACGAAGAC
	R: CATACTTGAACACCACGACCACC
sigB	F: GGTGCCATAAATAGATTTCGATATGTCCTT
	R: CTTTTGATTTCA CCGATTACAGTAGGTACT
rsbU	F: CGCGTGAAGATGTGTTCAAGAC
	R: CTATCTCTTTATCGTGA ACTTGAAG
rsbW	F: GAAGATTTTATCGAAATGCGC
	R: CATCATATGTAGCACCAGCTCTC

asp23	F: TCGCTGCACGTGAAGTTAAA
	R: CACCAACTTCAACAGATACACC

References

1. Rollin G, Tan X, Tros F, et al. Intracellular Survival of *Staphylococcus aureus* in Endothelial Cells: A Matter of Growth or Persistence. *Frontiers in microbiology* **2017**; 8:1354.
2. Hussain M, Hastings JG, White PJ. A chemically defined medium for slime production by coagulase-negative staphylococci. *J Med Microbiol* **1991**; 34:143-7.
3. Arnaud M, Chastanet A, Debarbouille M. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. *Applied and environmental microbiology* **2004**; 70:6887-91.
4. Jacobus AP, Gross J. Optimal cloning of PCR fragments by homologous recombination in *Escherichia coli*. *PloS one* **2015**; 10:e0119221.
5. Garzoni C, Francois P, Huyghe A, et al. A global view of *Staphylococcus aureus* whole genome expression upon internalization in human epithelial cells. *BMC genomics* **2007**; 8:171.
6. Mackay GM, Zheng L, van den Broek NJ, Gottlieb E. Analysis of Cell Metabolism Using LC-MS and Isotope Tracers. *Methods in enzymology* **2015**; 561:171-96.
7. TAN X, Coureuil M, Ramond E, et al. Chronic *Staphylococcus aureus* lung infection correlates with proteogenomic and metabolic adaptations leading to an increased intracellular persistence. *bioRxiv* **2018**.
8. Chong J, Soufan O, Li C, et al. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic acids research* **2018**; 46:W486-W94.
9. Xia J, Wishart DS. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. *Current protocols in bioinformatics* **2016**; 55:14 0 1- 0 91.
10. Shaw JA, Henard CA, Liu L, Dieckman LM, Vazquez-Torres A, Bourret TJ. *Salmonella enterica* serovar Typhimurium has three transketolase enzymes contributing to the pentose phosphate pathway. *The Journal of biological chemistry* **2018**; 293:11271-82.

Figure 1

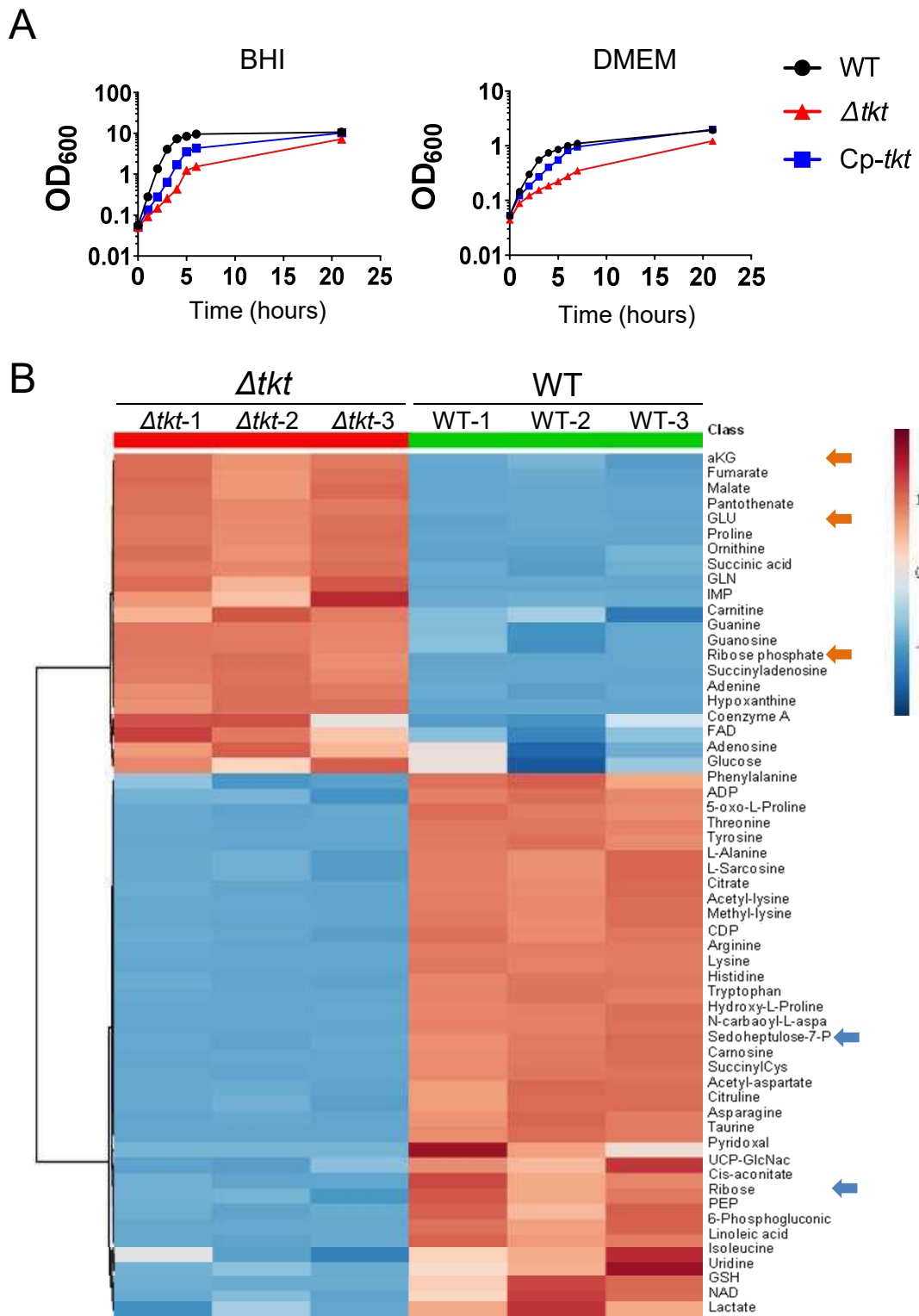


Figure 2

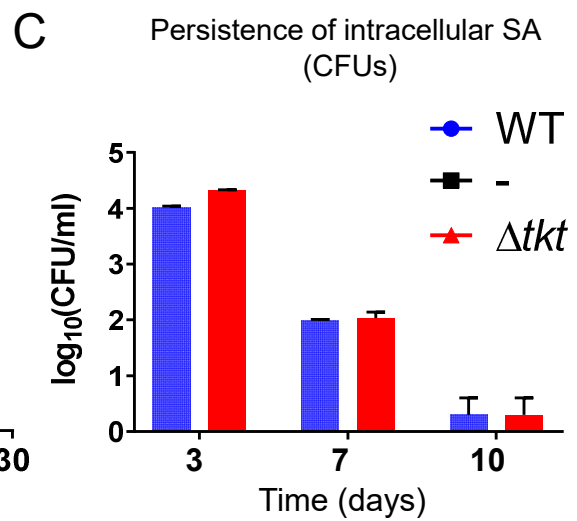
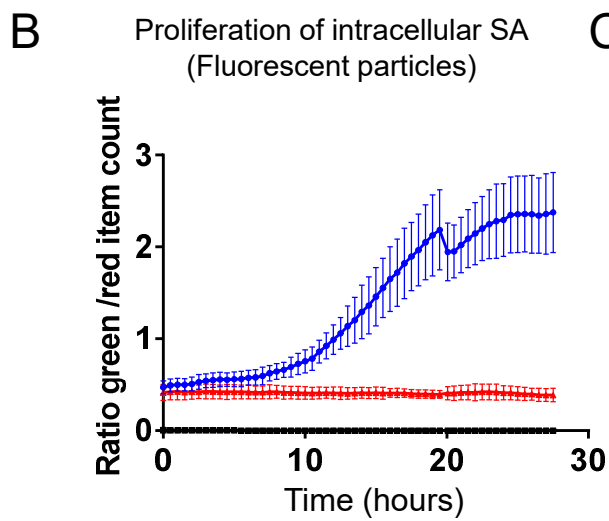
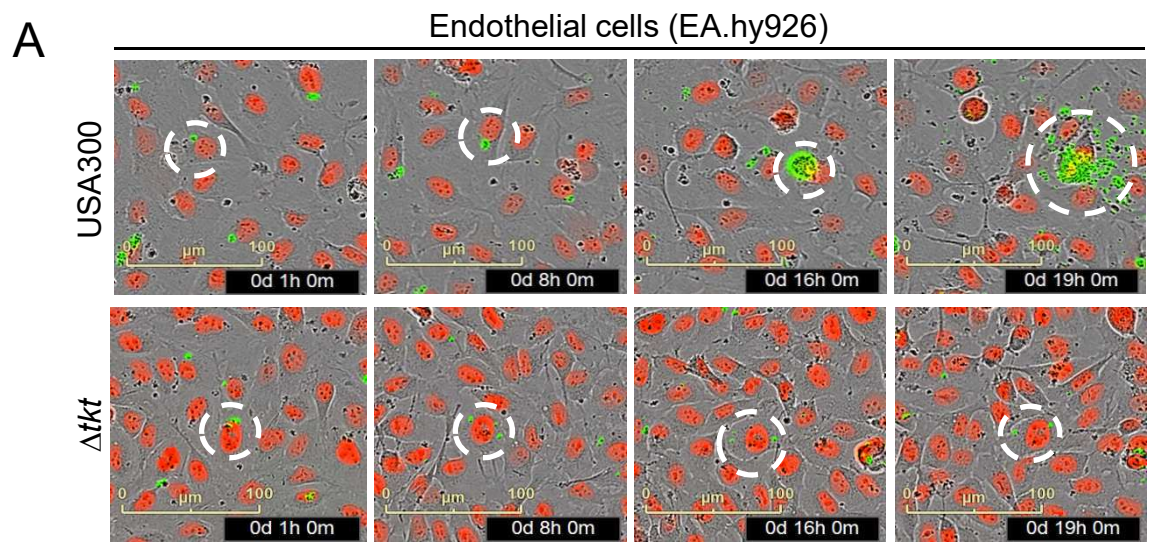


Figure 3

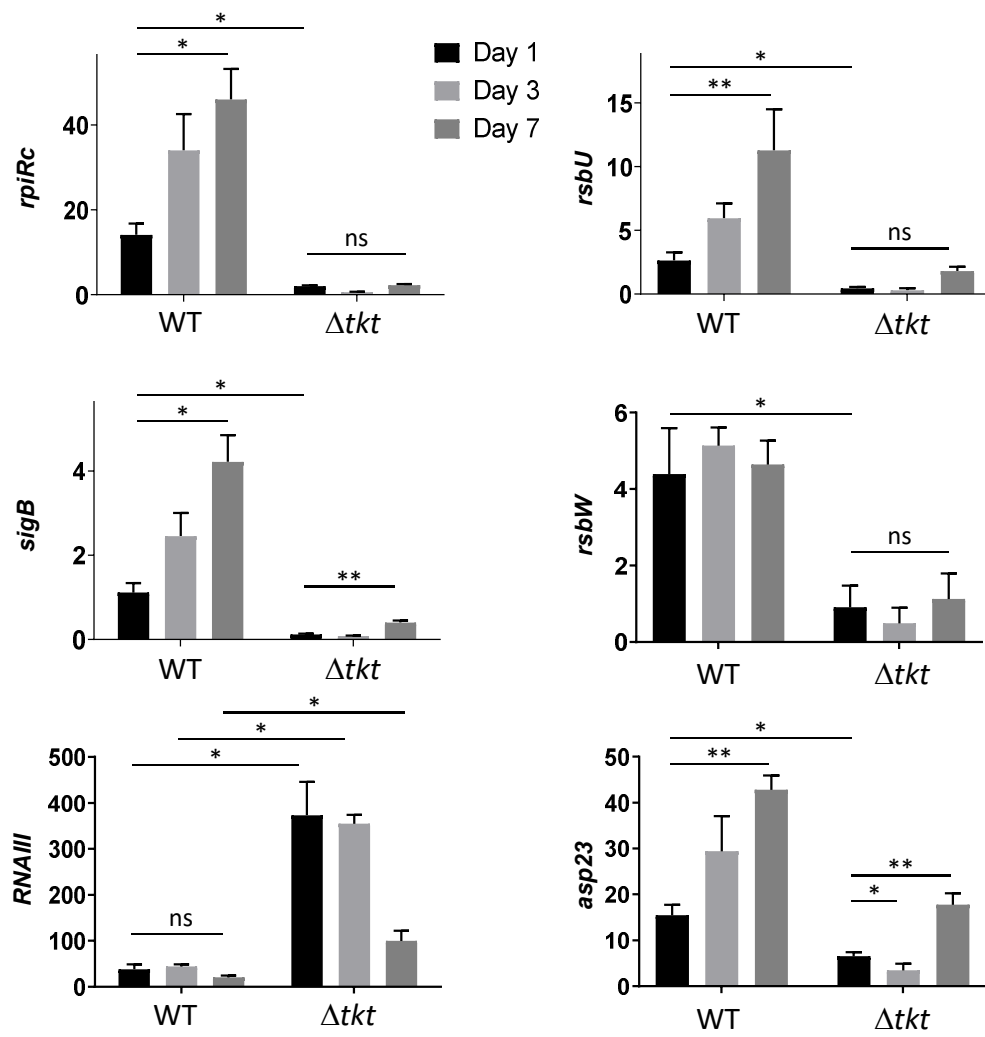


Figure 4

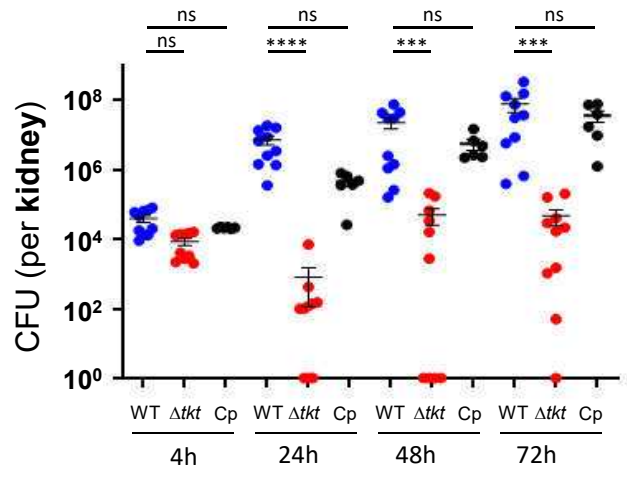
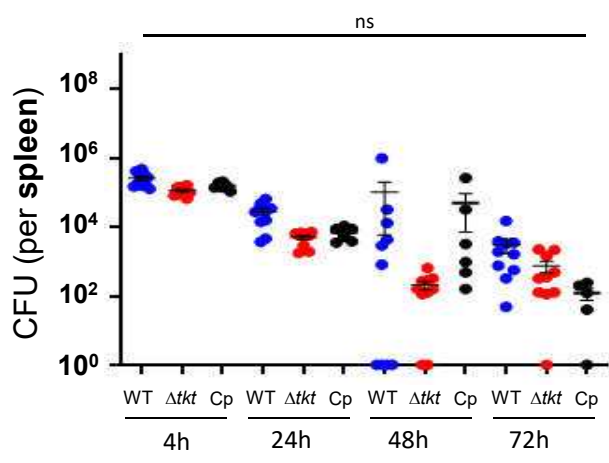


Figure 5

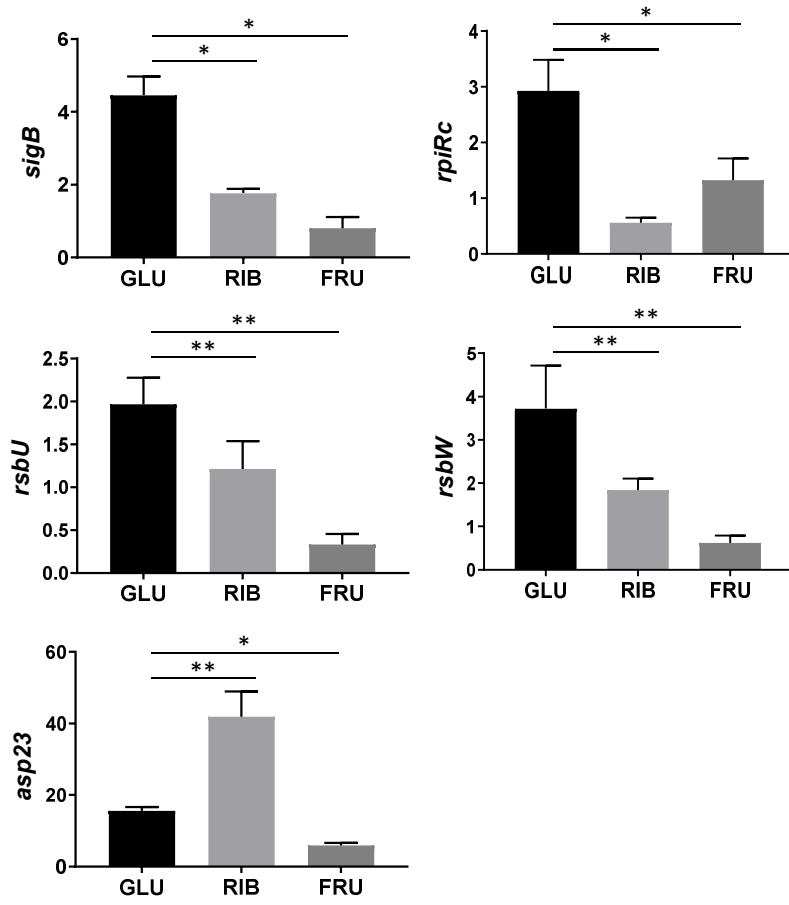


Figure 6

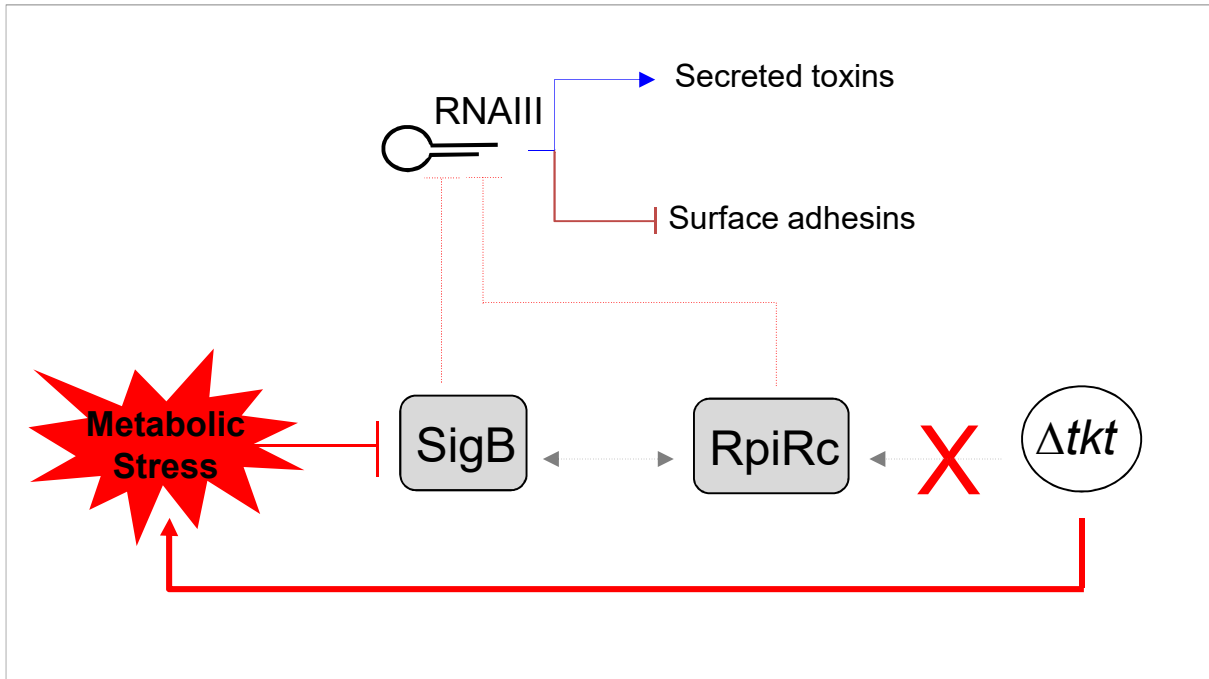


Figure S1

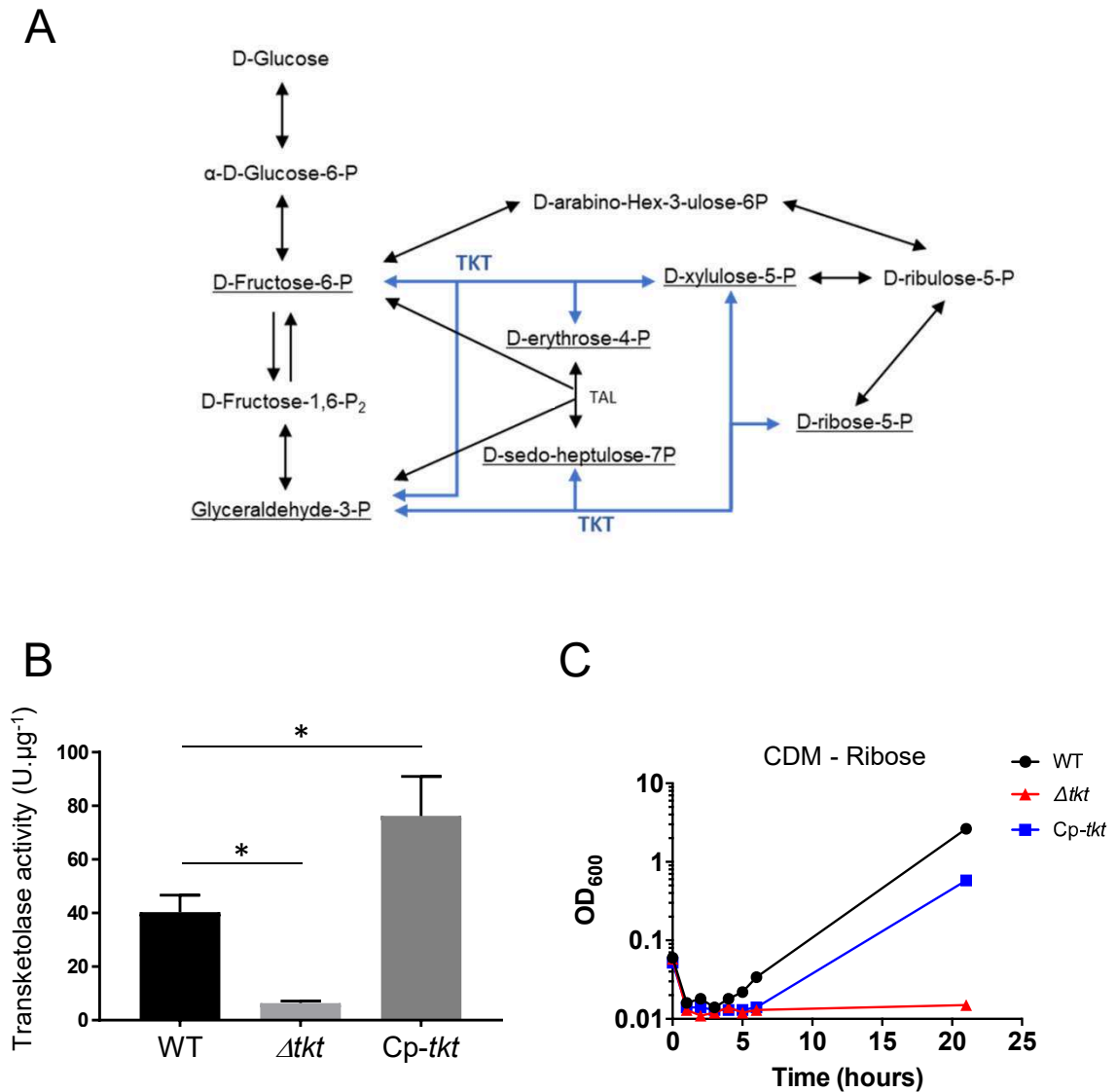


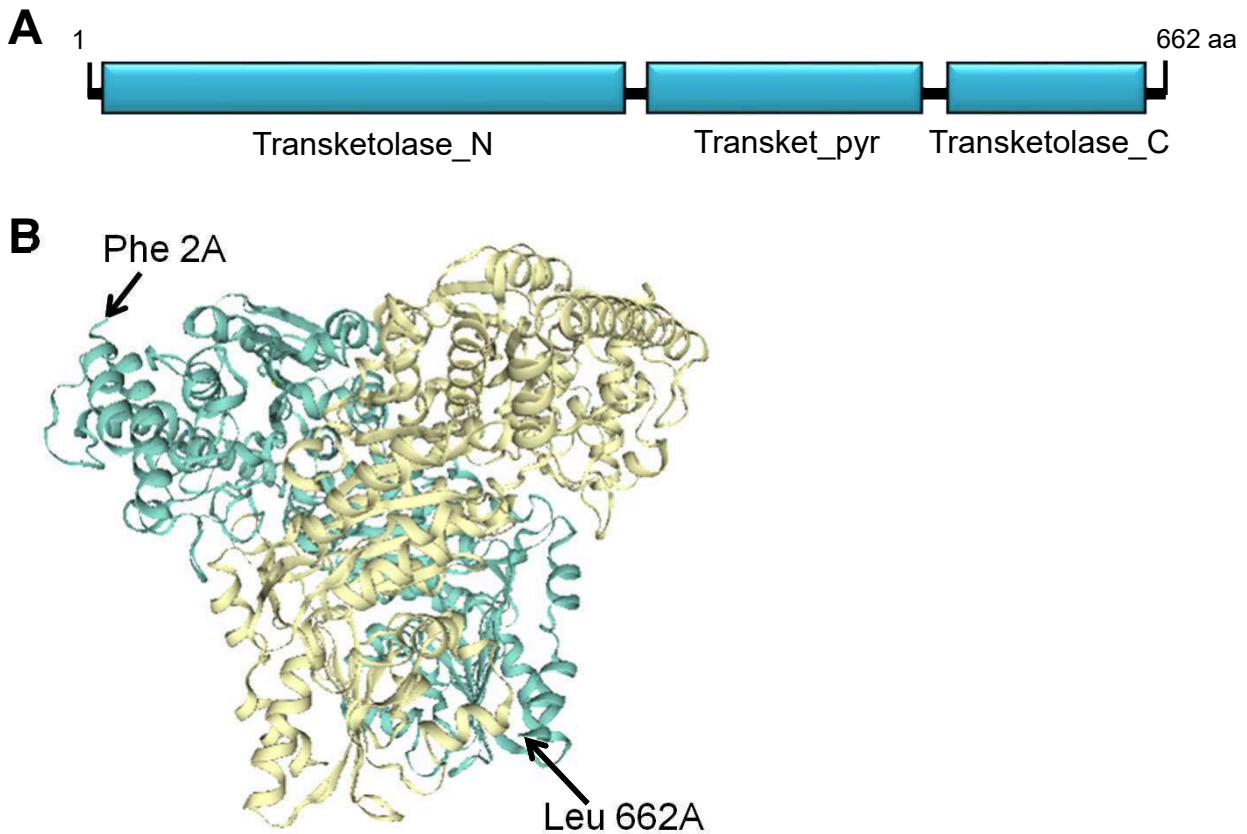
Figure S1. Characterization of the Δtkt mutant strain.

A. Schematic depiction of the Pentose Phosphate Pathway. Light blue: TKT enzymatic reaction.

B. Transketolase activity. Transketolase activity was monitored in USA300 WT, Δtkt and Cp-*tkt*. Inactivation of *tkt* gene led to a significant decrease in TKT enzyme activity (ANOVA: * $p < 0.001$).

C. Growth of USA300 wild type (WT), Δtkt mutant (Δtkt) and its complemented derivative (Cp-*tkt*). USA300 WT, Δtkt and Cp-*tkt* strains were grown in CDM supplemented with ribose as sole carbon source for 21 hours at 37°C. Optic densities were measured every-hour from 0 to 8 hours and then at 21 hours. Each panel is a representative experiment.

Figure S2



C

Protein ID	% coverage	% identity	Species
TKT_HUMAN	100.0%	100.0%	<i>Homo sapiens</i>
TKT_MOUSE	100.0%	94.7%	<i>Mus musculus</i>
TKT2_ECOLI	94.5%	24.1%	<i>Escherichia coli</i>
TKT_HAEIN	94.2%	22.9%	<i>Haemophilus influenzae</i>
TKT1_VIBCH	94.4%	24.0%	<i>Vibrio cholerae</i>
TKT_DICDI	94.2%	22.6%	<i>Dictyostelium discoideum</i>
TKT1_YEAST	94.5%	23.8%	<i>Saccharomyces cerevisiae</i>
TKT_TREPA	93.9%	21.9%	<i>Treponema pallidum</i>
TKT_MYCBO	96.6%	20.6%	<i>Mycobacterium bovis</i>
TKT_STRPN	94.2%	23.0%	<i>Streptococcus pneumoniae</i>
TKT_STAAS	94.4%	22.4%	<i>Staphylococcus aureus</i>
TKT_BACSU	94.9%	22.7%	<i>Bacillus subtilis</i>

Figure S2. Transketolase proteins domain organization and structure.

A. Conserved domains found in *S. aureus* transketolase. Transketolase_N (PF00456 domain): Transketolase, thiamine diphosphate binding domain; Transket_pyr (PF02779 domain): Transketolase, pyrimidine binding domain; Transketolase_C (PF02780): Transketolase, C-terminal domain (putative regulatory molecule binding site).

B. Protein model of *S. aureus* transketolase. Swiss-Model was used to predict the structure of *S. aureus* TKT (SAUSA300_1239) as a homodimer based on the template 3M49.1.A (Crystal Structure of Transketolase from *Bacillus anthracis*). The QMQE score and QMEAN were 0.83 and -0.59 respectively.

C. Table showing coverage percentage between transketolase amino acid sequences and percentage of identical amino acids between aligned transketolase amino acid sequences. Protein sequences were retrieved from UniProt database and aligned using Clustal Omega.

Figure S3

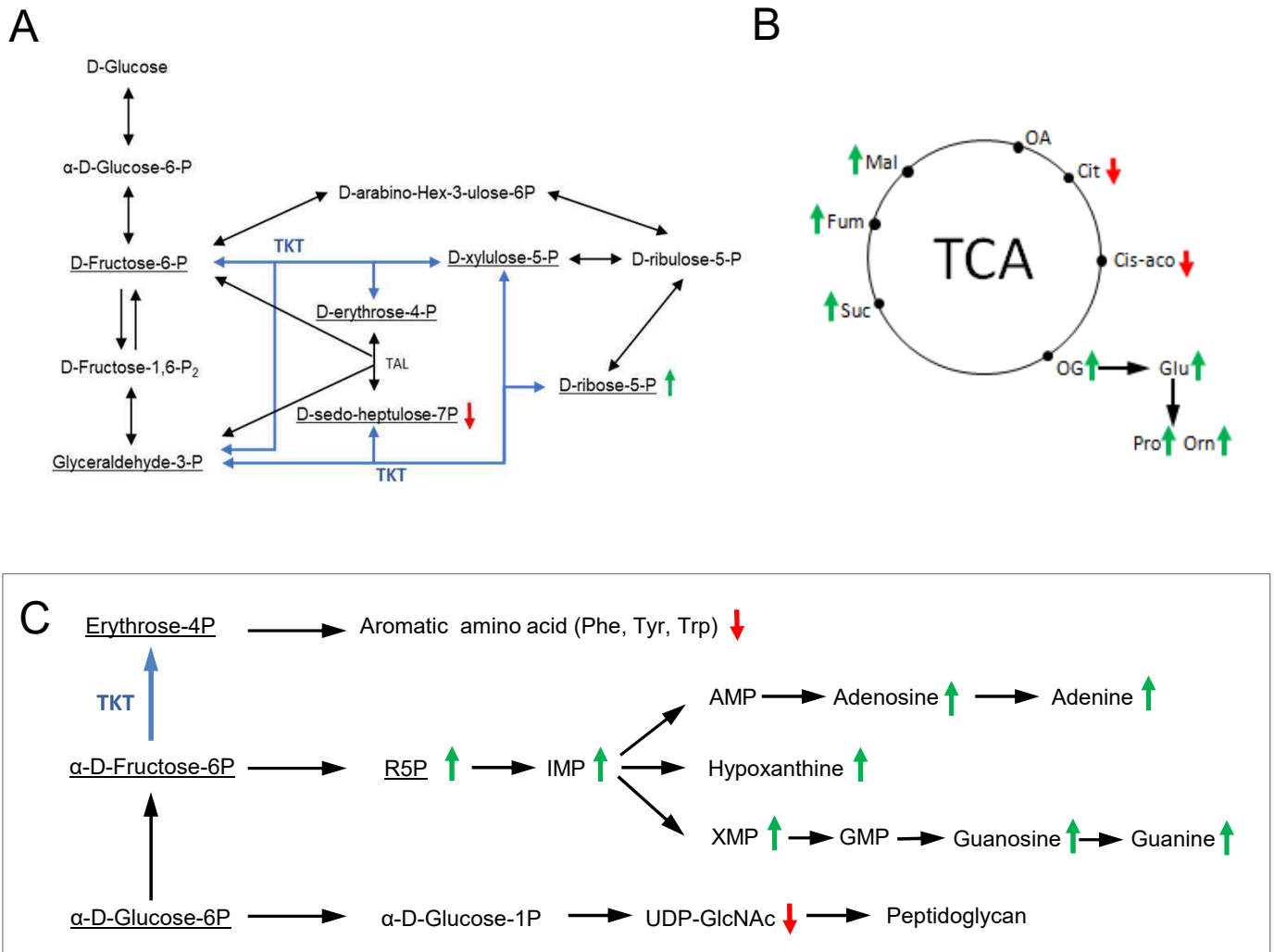


Figure S3. Metabolomic analysis of the Δtkt mutant strain.

Quantitative metabolomic analysis was performed by ion chromatography and tandem mass spectrometry (IC-MS/MS) after overnight cultures, in BHI Broth, from WT USA300 and Δtkt mutant strains (see **Table S1**). A, B and C. Highlight on metabolic pathways. Light blue: TKT enzymatic reaction. Green arrow: increased concentration in Δtkt mutant compared to WT strain. Red arrow: decreased concentration in Δtkt mutant compared to WT strain.

Figure S4

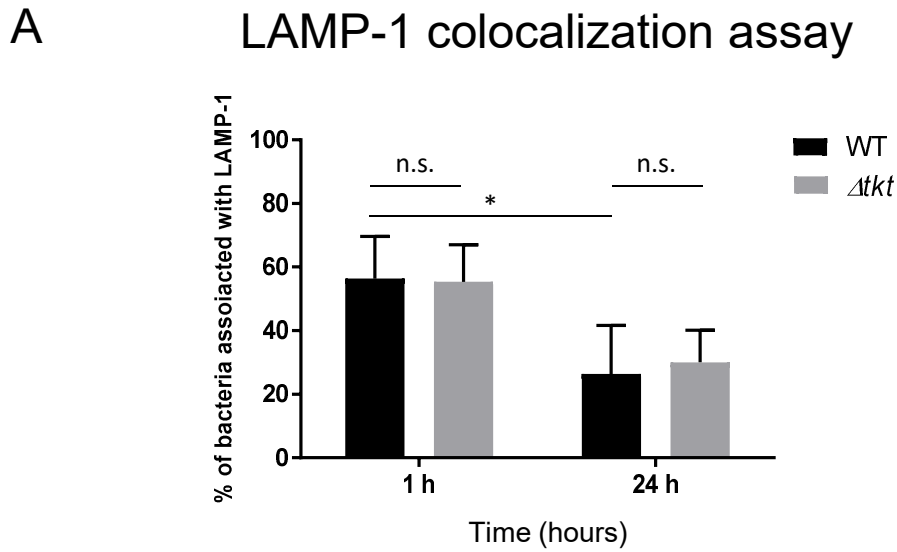


Figure S4. LAMP-1 colocalization assay

LAMP-1 colocalization assay. Endothelial EA.hy926 cells were infected with GFP-expressing WT and *Dtkl* mutant strains. One hour after infection, cells were washed several times with gentamicin containing medium to eliminate extracellular bacteria. A gentamicin concentration of 50 μ g/ml was maintained throughout the experiment. At 1 hour and 24 hours after infection cells were fixed using 4% PFA and processed for immunohistochemistry. Lysosomes were stained using anti-LAMP1 antibodies and images were acquired by confocal microscopy. Quantification of colocalization between GFP expressing USA300 WT or Δtkl bacteria and LAMP-1 staining was obtained using ImageJ software. Data were obtained from more than 200 infected cells for each condition and expressed as % of bacteria associated with LAMP-1 \pm SD. (ANOVA; n.s.: not statistically different; * $p < 0.05$).

Figure S5

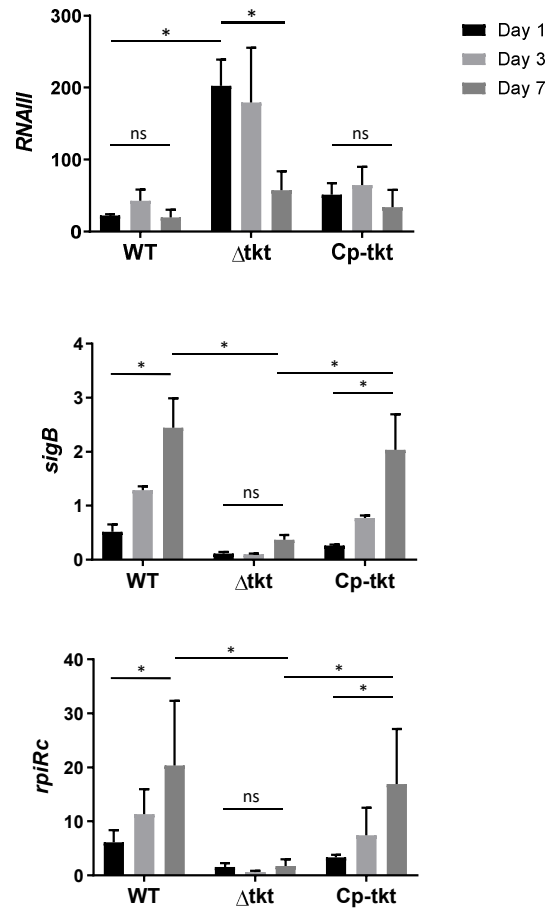


Figure S5. Transcription profile of intracellular *S. aureus*.

Endothelial EA.hy926 cells were infected with USA300 WT, Δ tkk and Cp-tkk strains as described in figure 2. One, three or seven days after infection cells were washed and collected. RNA from intracellular *S. aureus* were prepared and gene expression of *RNAIII*, *rpiRc* and *sigB* was analysed by quantitative RT-PCR. Gene expression level was normalized by that of *gyrA* at days 1, 3 and 7 after infection of EA-hy296 cells. Data are shown as mean normalized expression \pm SD of triplicate measurements from two independent experiments. (ANOVA, ns : not statistically different; * $p < 0.01$, ** $p < 0.001$).

Experiments presented in Figure S5 were independent of those presented in Figure 3.

Paper 2 Transketolase of *Staphylococcus aureus* is involved in the control of master regulators of stress response during infection

Contributions by Xin TAN

Methods

- Construction of USA300 Δtkt strain and the GFP-expressing USA300 Δtkt strain and construction of USA300 Δtkt Cp-*tkt* strain
- Growth curve of all the strains.
- Time lapse microscopy and immunofluorescence microscopy for USA300 Δtkt strain and wild-type strain.
- Preparation of cell samples for metabolomic studies.
- In vivo* infection assay.
- RT-qPCR.
- Transketolase activity assays.

Results

Figure 1, 2, 3, 4, 5 and Figure S2, S3 and S4

Chapter III: Chronic *Staphylococcus aureus* lung infection correlates with proteogenomic and metabolic adaptations leading to an increased intracellular persistence

Chronic *Staphylococcus aureus* Lung Infection Correlates With Proteogenomic and Metabolic Adaptations Leading to an Increased Intracellular Persistence

Xin Tan,¹ Mathieu Coureuil,¹ Elodie Ramond,¹ Daniel Euphrasie,¹ Marion Dupuis,¹ Fabiola Tros,¹ Julie Meyer,¹ Ivan Nemazanyy,² Cerina Chhuon,³ Ida Chiara Guerrero,⁴ Agnes Ferroni,⁵ Isabelle Sermet-Gaudelus,⁶ Xavier Nassif,¹ Alain Charbit,¹ and Anne Jamet^{1,6}

¹Université Paris Descartes, INSERM U1151 - CNRS UMR 8253, Institut Necker-Enfants Malades. Team: Pathogenesis of Systemic Infections, ²Plateforme d'étude du métabolisme, Structure Fédérative de Recherche INSERM US24/CNRS UMS3633, ³Plateforme Protéome Institut Necker-Enfants Malades, PPN, Structure Fédérative de Recherche SFR Necker, University Paris Descartes, ⁴Proteomics platform 3P5-Necker, Université Paris Descartes - Structure Fédérative de Recherche Necker, INSERM US24/CNRS UMS3633, ⁵Laboratoire de Microbiologie de l'hôpital Necker, University Paris Descartes, and ⁶Université Paris Descartes, INSERM U1151 - CNRS UMR 8253, Institut Necker-Enfants Malades. Team: Canalopathies épithéliales: la mucoviscidose et autres maladies, Paris, France

Background. Chronic lung infection in cystic fibrosis (CF) patients by *Staphylococcus aureus* is a well-established epidemiological fact. Indeed, *S. aureus* is the most commonly identified pathogen in the lungs of CF patients. Improving our understanding of the mechanisms associated with the persistence of *S. aureus* is therefore an important issue.

Methods. We selected pairs of sequential *S. aureus* isolates from 3 patients with CF and from 1 patient with non-CF chronic lung disease. We used a combination of genomic, proteomic, and metabolomic approaches with functional assays for in-depth characterization of *S. aureus* long-term persistence.

Results. In this study, we show that late *S. aureus* isolates from CF patients have an increased ability for intracellular survival in CF bronchial epithelial-F508del cells compared to ancestral early isolates. Importantly, the increased ability to persist intracellularly was confirmed for *S. aureus* isolates within the own-patient F508del epithelial cells. An increased ability to form biofilm was also demonstrated. Furthermore, we identified the underlying genetic modifications that induce altered protein expression profiles and notable metabolic changes. These modifications affect several metabolic pathways and virulence regulators that could constitute therapeutic targets.

Conclusions. Our results strongly suggest that the intracellular environment might constitute an important niche of persistence and relapse necessitating adapted antibiotic treatments.

Keywords. cystic fibrosis; *Staphylococcus aureus*; intracellular persistence; biofilm; proteogenomics.

Staphylococcus aureus and *Pseudomonas aeruginosa* are the most common pathogens that infect the lungs of patients with a chronic lung disease (CLD) including cystic fibrosis (CF) [1, 2]. Furthermore, *S. aureus* is one of the earliest bacteria detected in infants with CF. However, very few studies have addressed the adaptations *S. aureus* has undergone in this context [3–6].

Staphylococcus aureus has the ability to form biofilm [7–9] and to survive within a wide range of eukaryotic host cells [10–19]. These abilities are likely to contribute to the persistence of *S. aureus* in airways of patients with CLDs despite appropriate antimicrobial treatments [20, 21]. *Staphylococcus aureus* persistence is associated with a drastic decrease in metabolism [22], a decrease in the expression of virulence factors, and an increase

in the expression of bacterial adhesins [23]. Such a profile is typical of small-colony variants (SCVs) that are defined by small-sized colonies [17, 24, 25]. In addition to SCVs, strains with normal colony morphology can exhibit similar patterns of “low toxicity” that allow them to persist intracellularly without being cleared by host cell defense mechanisms [23]. A low-toxicity pattern can be achieved either transiently, following changes in the expression of genes encoding toxins and/or regulators, or permanently by mutations in global regulators [26–28].

The alternative sigma B factor (SigB) has pleiotropic roles in gene regulation and has been shown to be a master regulator of intracellular persistence [29, 30]. SigB was shown to downregulate the *agr* system, which is responsible for enhanced inflammatory activity. Hence, the expression of SigB during chronic persistence is linked to its capacity to downregulate proinflammatory virulence factors and to increase the expression of factors that promote persistence [28, 30]. SigB enables the bacteria to downregulate its aggressive phenotype and to switch toward a silent SCV phenotype that allows for long-term intracellular persistence.

Received 11 July 2018; editorial decision 28 January 2019; accepted 31 January 2019; published online February 7, 2019.

Correspondence: A. Jamet, Bâtiment Leriche, 14 Rue Maria Helena Vieira Da Silva CS 61431 - 75993 Paris, France (anne.jamet@inserm.fr).

Clinical Infectious Diseases® 2019;XX(XX):1–9

© The Author(s) 2019. Published by Oxford University Press for the Infectious Diseases Society of America. All rights reserved. For permissions, e-mail: journals.permissions@oup.com. DOI: 10.1093/cid/ciz106

By studying serial isolates, we show that during long-term lung infection, *S. aureus* adaptation occurs through genomic modifications that accumulate over time and lead to major metabolic modifications and protein expression changes. We also reveal that persistence of *S. aureus* is associated with convergent phenotypic evolution responsible for an increased ability to form biofilm as well as to survive within host cells. These observations should be taken into account in therapeutic decisions aimed at eradicating *S. aureus* chronic infections by choosing drugs that specifically target biofilm-embedded and intracellular bacteria.

METHODS

Whole-genome sequencing was performed on an Illumina MiSeq instrument (2 × 150 bp), and the sequences were processed using the Nullarbor bioinformatic pipeline software v1.20 and RAST server. The sequences reported here are available at the National Center for Biotechnology Information's BioProject database under accession PRJNA446073.

Quantification of biofilm formation was assessed with crystal violet staining in polystyrene 96-well plates. CF bronchial epithelial cell line CFBE41o- and primary nasal epithelial cell were infected with a multiplicity of infection of 100 using an inoculum taken from cultures of *S. aureus* grown in brain-heart infusion (BHI) until the exponential growth phase. Infected cells were kept for 6 days in a medium that contained 50 µg/mL gentamicin to kill extracellular bacteria. E-tests were used to determine minimal inhibitory concentrations (MICs) to gentamicin. Gentamicin has a high bactericidal effect on *S. aureus* USA300-LAC (MIC = 2 µg/mL) and a very poor penetration inside eukaryotic cells. In addition, the measured MICs to gentamicin of all tested clinical strains were similar to that of USA300 reference strain (≤2 µg/mL). Hence, an accumulation of gentamicin within epithelial cells should impact equally all tested strains and is unlikely to explain differences between isolates.

For proteomics, proteins were digested and analyzed using liquid chromatography (LC) coupled with tandem mass spectrometry (nanoLC-MS/MS). For metabolomics, metabolite profiling of *S. aureus* isolates was performed using LC-MS. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [31] partner repository with the dataset identifier PXD011281. The metabolomics raw data are provided in [Supplementary Table S1](#). A full description of methods is available in the [Supplementary Methods](#).

Statistical Analyses

Data were analyzed using R or GraphPad Prism software. Results are presented either with 1 representative experiment for clarity or as means ± standard deviation. The number of biological and technical replicates is indicated per figure.

For 2-sample comparisons, statistical significance was measured using unpaired 2-tail Student *t* test or Wilcoxon rank sum test as indicated in the figure legends. For comparisons between more than 2 groups, statistical significance was measured using 1-way analysis of variance with multiple comparisons (Dunnnett's correction) performed, with each value compared to that of the reference strain. *P* values < .05 were considered to be statistically significance.

Ethics Statement

All experiments were performed in accordance with the guidelines and regulations described by the Declaration of Helsinki and the low Huriet-Serusclet on human research ethics, and informed consent was obtained for all participating patients. Serial isolates of *S. aureus* were obtained from airway secretions from 4 patients with chronic lung infection at the Necker-Enfants Malades University Hospital, Paris, France. Sputum sampling is part of routine standard care. The research procedure is validated by Ile de France 2 IRB (ID-RCB/Eudract: 2016 A00309-42).

RESULTS

Selection of *S. aureus* Sequential Isolates From Patients With Chronic Lung Infection

Three patients with CF (CF1, CF2, and CF3) and, for comparison purposes, 1 patient with non-CF CLD were chosen. For each patient, we selected 1 early and 1 late isolate separated by 3- to 9-year intervals. By definition, in a pair of isolates that belong to the same clone, the "early isolate" corresponds to the isolate collected earlier than the second isolate that had been collected later and that corresponds to the "late isolate." Whole-genome sequencing confirmed that each pair of isolates belonged to 4 distinct clones ([Figure 1](#)). Patient diseases and treatments are detailed in the [Supplementary Methods](#).

S. aureus Clinical Isolates From CF Patients Evolved an Increased Persistence Ability Within CFBE-F508del Epithelial Cell Line

Numerous studies have shown that *S. aureus* has the ability to survive within human cells [10–19]. We subsequently aimed at determining if during the course of within-lung adaptation, *S. aureus* isolates have evolved a greater ability to persist within epithelial cells. We infected the bronchial CFBE epithelial cell line (F508del +/+ cystic fibrosis transmembrane conductance regulator [CFTR] mutation) with clinical isolates, the control strain USA300-LAC, and a stable SCV mutant altered in the haemin biosynthetic pathway (hereafter, named Δhem). As expected, wild-type bacteria were not able to persist, whereas the Δhem mutant was able to persist intracellularly during the whole course of the experiment ([Supplementary Figure S1](#)) [15]. All early and late clinical isolates were able to persist at least 2.6-fold, and up to 900-fold, more than the USA300-LAC reference strain at day 3 and day 6 post-infection ([Figure 2A, 2B](#)). Furthermore, at day 3 and day 6 post-infection, all the late

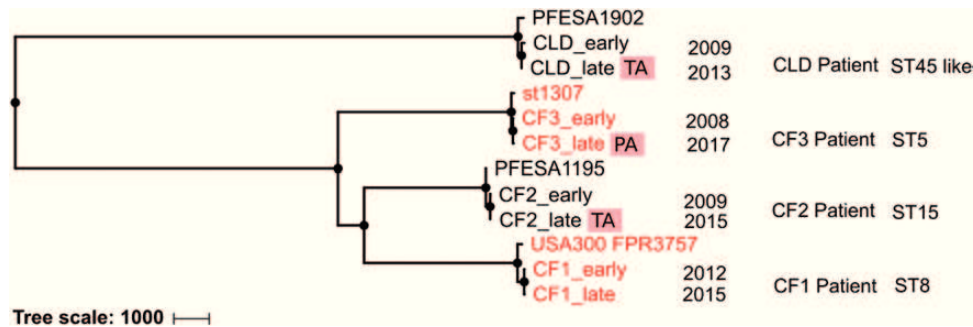


Figure 1. Selection of 4 pairs of *Staphylococcus aureus* isolates belonging to 4 sequence types (STs) in 4 patients. Dendrogram generated by wgsa.net from the genomes of the 8 clinical isolates retrieved from the respiratory samples of 3 patients with cystic fibrosis (CF), 1 patient with non-CF chronic lung infection, and 4 reference genomes from public databases. Branch length is proportional to the number of variant nucleotide sites within the core genes. For each patient, the isolate taken first is termed “early” and the isolate taken later is termed “late.” The dates of sampling and the ST of the isolates are indicated. “TA” and “PA” mean that the isolate is auxotrophic for thymidine or pantothenate, respectively. The name of the isolate is indicated in red when it is methicillin-resistant *S. aureus*. Reference strains included are PFESA1902 (ERR54197), st1307 (ERR158691), and PFESA1195 (ERR554722). Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease.

isolates recovered from CF patients exhibited an improved ability to persist intracellularly within CFBE-F508del epithelial cells compared to cognate early isolates (Figure 2A). Interestingly, the CLD_late isolate recovered from the non-CF patient did not exhibit an improved ability to persist within CFBE epithelial cells compared to CLD_early isolate (Figure 2B). These data suggest that *S. aureus* adaptation within CF lungs correlates with an improved ability to persist intracellularly in cells with a CFTR dysfunction.

Late *S. aureus* Isolate of CF3 Patient Exhibits an Increased Persistence Within Primary F508del Epithelial Own-patient Cells

To confirm the relevance of the results obtained with the bronchial CFBE epithelial cell line, we first assessed the persistence of CF3 isolates within primary epithelial cells isolated from the nose of a healthy donor (Figure 2C). In addition, we performed an infection assay with the CF3 primary epithelial own-patient cells (F508del +/- CFTR mutation) to verify the specific within-patient adaptation of *S. aureus* recovered from long-term infection (Figure 2D). These experiments confirmed that the late isolate persistence ability is improved compared to the early isolate at day 3 and day 6 within both primary nasal epithelial cells retrieved from a healthy donor and from the CF3 patient.

S. aureus Clinical Isolates From Chronically Infected Patients Evolved High Biofilm Formation Ability

Assuming that isolates retrieved from chronic infections might have a high biofilm-forming capacity, we studied the biofilm formation ability of the pairs of isolates. Remarkably, all isolates displayed a greater capacity to form biofilms compared to that of the weak biofilm-producer USA300-LAC reference strain ($P < .001$; Supplementary Figure S2). Furthermore, for 3 chronically infected patients, the late isolates formed more biofilm than the early isolates, revealing that long-term adaptation within lungs had improved their biofilm formation capacity ($P < .001$; Figure 3).

Late *S. aureus* Clinical Isolates From Chronically Infected Patients Acquired Auxotrophies

Compared to that of USA300-LAC, all patients’ early isolates and the CF1 late isolate displayed similar colony morphology on BHI agar plates and a wild-type growth in a liquid broth that mimics sputum (CF sputum medium [CFSM]; Supplementary Figures S3 and 4A). In contrast, the late isolate of CF2 and CLD patients displayed a typical stable SCV phenotype with very small colonies on BHI agar (Supplementary Figure S4) and CF2_late, CF3_late, and CLD_late isolates exhibited a growth defect in CFSM broth (Figure 4B–4D). Thymidine-dependent SCVs are frequently isolated from patients treated with sulfamethoxazole-trimethoprim (SXT) [32]. Indeed, supplementation with thymidine restored most wild-type growth for CF2_late and CLD_late isolates (Figure 4B, 4D). We used genomic data to determine the auxotrophy of CF3_late isolate and identified a frameshift in *panB* gene, which is involved in de novo biosynthesis of pantothenic acid (Table 1). Accordingly, growth of CF3_late isolate in the presence of pantothenate restored wild-type growth (Figure 4C). Thus, isolates from 3 of 4 patients with *S. aureus* chronic lung infection acquired auxotrophy during the course of the disease.

Late *S. aureus* Clinical Isolates From Chronically Infected Patients Acquired Antibiotic Resistance

Genome analysis evidenced mutations in *thyA*, *gyrB*, and *rpsJ* genes, which are likely to explain SXT, fluoroquinolones, and cyclines resistance, respectively (Table 1). However, in the case of point mutations, a causal relation with phenotypic changes has to be experimentally verified. Thus, isolates from 3 of 4 patients with *S. aureus* chronic lung infection acquired antibiotic resistance consistent with administration of the corresponding drugs during the course of the disease.

Genomic, Proteomic, and Metabolomic Modifications Associated With *S. aureus* Adaptation During Chronic Lung Infection

In order to investigate the underlying genomic, proteomic, and metabolic modifications associated with the observed

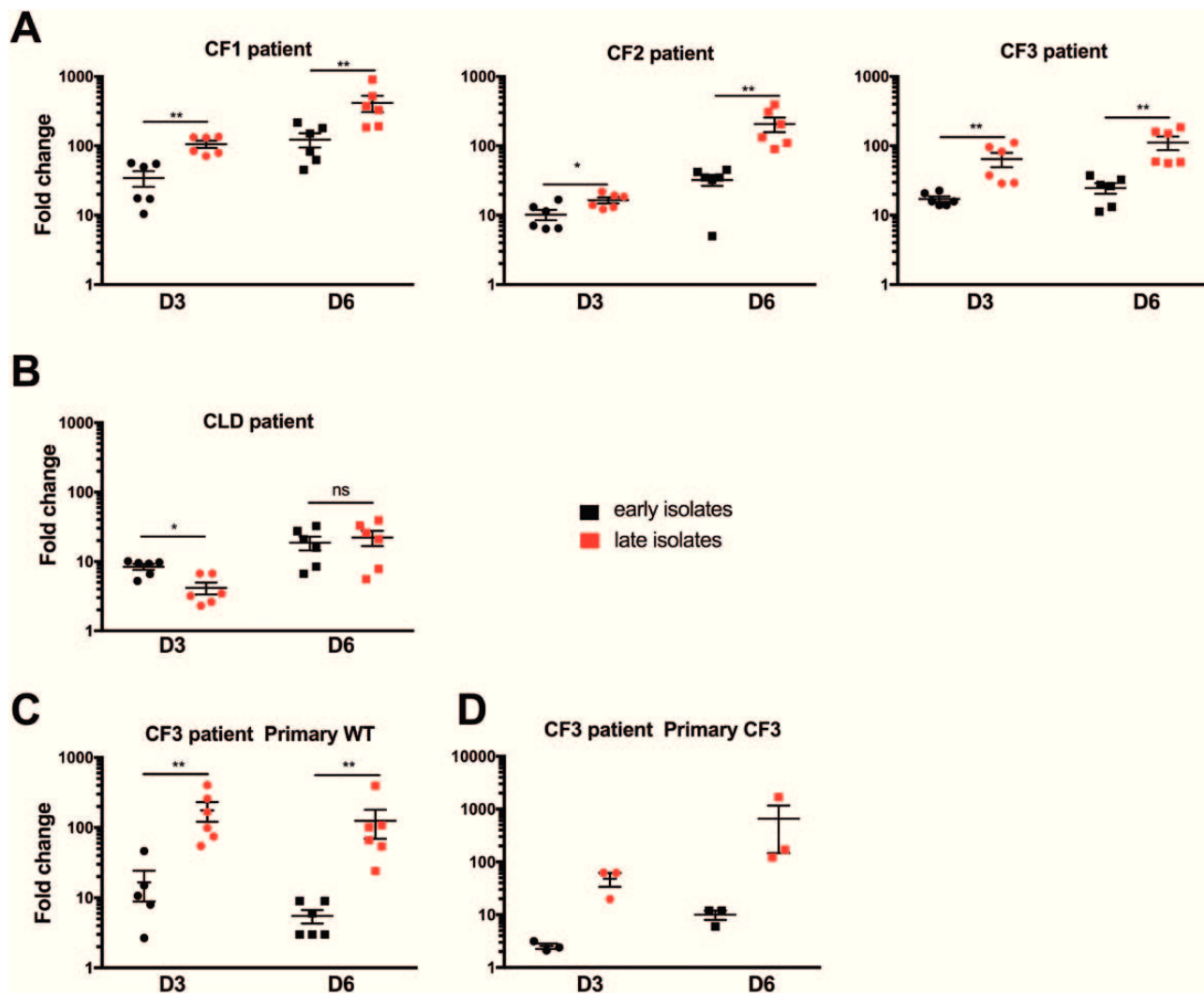


Figure 2. Intracellular persistence of *Staphylococcus aureus* clinical isolates in cystic fibrosis bronchial epithelial (CFBE)-F508del epithelial cell line and within primary patient cells. (A and B) Bronchial CFBE epithelial cell line (CFBE-F508del homozygous for the F508del-cystic fibrosis transmembrane conductance regulator [CFTR] mutation) was infected with the control strain USA300-LAC and clinical isolates from cystic fibrosis (CF) patients (A) or the chronic lung disease (CLD) patient (B). (C and D) Primary nasal epithelial cells retrieved from a healthy donor (“Primary WT”) (C) and from the CF3 patient (“Primary CF3” with F508del +/- CFTR mutation) (D) were infected with the control strain USA300-LAC and CF3 isolates. For all experiments, gentamicin was present throughout the experiment to prevent extracellular bacterial growth and new infection. Bacterial loads inside cells were evaluated by colony-forming unit (CFU) enumeration at 3 and 6 days after infection. Results are normalized with USA300-LAC strain as a reference and expressed as a fold change of CFUs. Results have been obtained from 2 independent experiments performed in triplicate for A, B, and C and 1 experiment for D. Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease; ns, not significant; WT, wild type. Statistical analysis was performed by Wilcoxon rank sum test: * $P < .05$; ** $P < .01$; $P > .05$.

phenotypic changes, we compared genomes, proteomes, and metabolomes of late compared to early isolates. The differences in proteomic and metabolic profiles between early and late isolates of patients are highlighted by heat maps shown in [Supplementary Figure S5](#).

Genomes of all clinical isolates were de novo assembled, and coding DNA sequences (CDSs) were annotated. Most of the single-nucleotide polymorphisms were missense variants occurring in CDSs ([Table 2](#)). Nonsynonymous mutations acquired by late isolates were found mainly in genes involved in metabolic processes ([Figure 5A](#)) and, more specifically, in the following functional categories: “amino acid transport and metabolism”

and “carbohydrate transport and metabolism”. In addition, the largest category of proteins to be differentially expressed for all pairs also comprised proteins related to metabolism processes (and more specifically to the “amino acid transport and metabolism” category) ([Figure 5B](#)). Concordant with genomic and proteomic results, the category “amino acids” was the most altered metabolite category in the late isolate of all patients compared to their cognate early isolates ([Figure 5C](#)). Of note, our proteomics data also indicated that there is no category in which the overall direction of change is the same in all patients ([Figure 5B](#)).

Many regulatory proteins were differentially expressed. Indeed, proteins of the Agr, Rot, Sae, Sar, or Fur regulatory

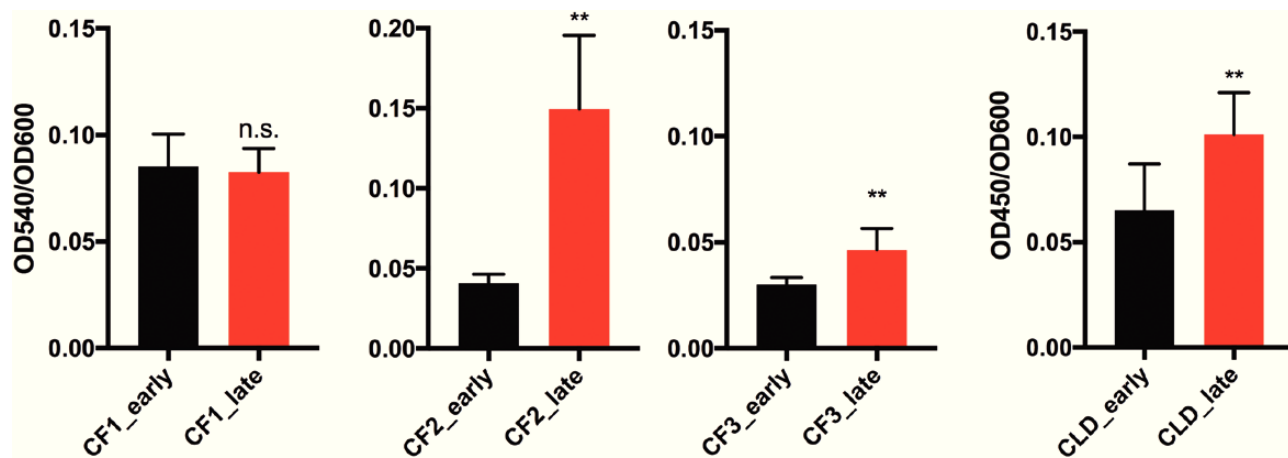


Figure 3. Quantification of biofilm formation of *Staphylococcus aureus* clinical isolates. Biofilm formation quantification was performed using the crystal violet microtiter assay in brain–heart infusion medium with 1% glucose. Results shown are the mean \pm standard deviation for 3 independent experiments performed in triplicate. Statistical significance was measured using a 2-tail Student *t* test when biofilm production of a late isolate was compared with biofilm production of a cognate early isolate from the same patient. Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease. ** indicates $P < .001$ and ns indicates $P > .05$.

networks were differently expressed in all late isolates. In CF1_late isolate, the premature stop codon identified in *agrC* (Table 1) is likely to profoundly impact the *agr* regulon and to explain a pleiotropic effect on the proteome (eg, downregulation of delta hemolysin and PSMB1 and upregulation of proteins encoded by *spa*, *sbi*, *fmbA*, *rot*, and *coa* genes). In addition, a missense

mutation in *saeR* regulatory gene (Table 1) could also affect *sae* regulon and modify the proteome. In CF2_late isolate, the *agr* regulon is also downregulated, suggesting an evolution toward low virulent and highly adhesive properties. The metabolite profiling of CF2_late isolate showed a decrease in adenosine diphosphate, which is well correlated with the

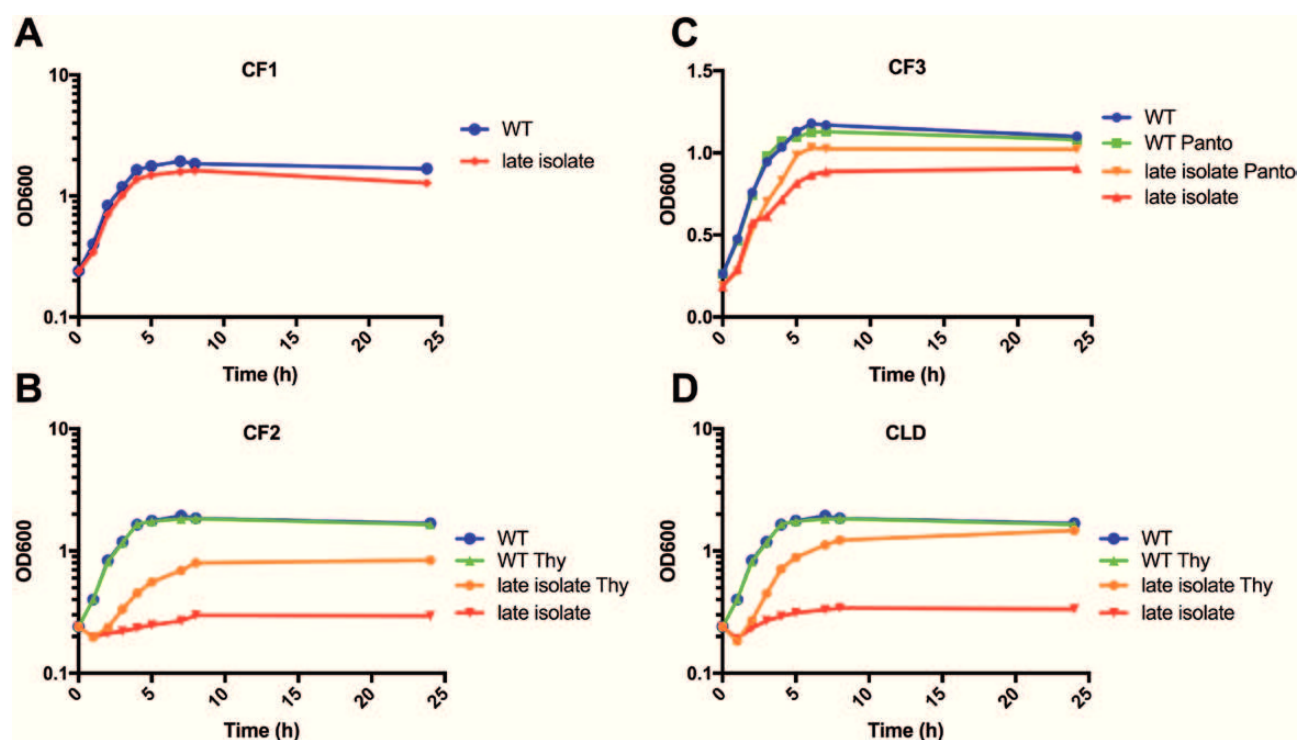


Figure 4. Growth of late *Staphylococcus aureus* clinical isolates in cystic fibrosis sputum medium. Growth curves were carried out in medium that mimics the respiratory fluid of cystic fibrosis patients, with or without the addition of thymidine or pantothenate. The results shown correspond to a representative experiment. The orange and green curves correspond to bacterial growth in media supplemented with either thymidine or pantothenate; the red and blue curves correspond to bacterial growth in medium without thymidine or pantothenate. WT, USA300-LAC. Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease; Panto, pantothenate; Thy, thymidine; WT, wild type.

Table 1. Mutations Likely Linked to Phenotypic Changes in Clinical Isolates

Isolate	Gene	Product	Mutation	Putative Associated Phenotype
CF1_late	<i>agrC</i>	Accessory gene regulator protein C	Premature stop codon (L193X)	Downregulation of toxins and upregulation of adhesins
CF1_late	<i>saeR</i>	<i>Staphylococcus</i> exoprotein expression protein R	Missense G179L	Upregulation of SdrD adhesin
CF2_late	<i>thyA</i>	Thymidylate synthase	Premature stop codon (W88X)	SXT resistance and thymidine auxotrophy
CF2_late	<i>rpsJ</i>	30S ribosomal protein S10	Missense K57M	Cyclines resistance
CF2_late	<i>thiM</i>	Hydroxyethylthiazole kinase	Frameshift	Decrease in adenosine diphosphate
CF2_late	<i>fakA</i>	Fatty acid kinase A	Missense G187D	Increased biofilm formation and intracellular persistency
CF3_late	<i>panB</i>	3-methyl-2-oxobutanoate hydroxymethyltransferase	Frameshift	Pantothenate auxotrophy
CF3_late	<i>fakA</i>	Fatty acid kinase A	Frameshift	Increased biofilm formation and intracellular persistency
CF3_late	<i>gyrB</i>	DNA topoisomerase subunit B	Missense F226S	Fluoroquinolones resistance
CF3_late	<i>saeR</i>	<i>Staphylococcus</i> exoprotein expression protein R	Missense A190T	Upregulation of SdrD adhesin
CLD_late	<i>thyA</i>	Thymidylate synthase	Missense P48R	SXT resistance and thymidine auxotrophy

Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease; SXT, sulfamethoxazole-trimethoprim.

lack of hydroxyethylthiazole kinase expression found in proteomic analysis due to a frameshift in *thiM* gene (Table 1). In CF3_late isolate, frameshifts in *fakA* and *panB* genes (Table 1) were associated with a lack of cognate protein expression in CF3_late isolate. In addition, adhesins encoded by *sdrD* and *sasF* were upregulated. Interestingly, an overproduction of penicillin-binding protein 2 encoded by *mecA* is correlated with the *saeR* mutation (Table 1) [33]. The metabolite profiling of CF3_late isolate revealed a drastic reduction in pantothenate, coenzyme A, and dephospho-coenzyme A, which is in line with the lack of expression of PanB and PanC proteins [34].

Of note, the non-CF control clone displays a different evolutionary trajectory. Indeed, *AgrA* and *AgrC* were upregulated in CLD_late isolate, suggesting that it has retained virulent properties.

SCVs have typically decreased ribosomal activity and DNA and amino acid synthesis. Indeed, we noticed that in CLD_late isolate, 2 ribosomal proteins as well as a ribosomal protein methyltransferase were less expressed compared to early isolate. In CF2_late, 1 ribosomal protein and a DNA topoisomerase were also less expressed compared to early isolate. Also, metabolic profiling suggests that all late isolates have evolved

a reduced citric acid cycle activity compared to cognate early isolates.

DISCUSSION

Here, we show that during chronic lung infection, *S. aureus* adapts through the acquisition of common adaptive traits including antibiotic resistances, auxotrophies, reduced citric acid cycle activity, increased biofilm, and intracellular persistence abilities that occurred irrespective of the clone type. Of particular interest, we report mutations in 2 master regulatory systems, *Agr* and *Sae*, likely to impact multiple protein expression and metabolite amounts.

The *agr*-defective mutants, such as CF1_late isolate, have been shown to arise during chronic infections and are better adapted to persistence within the infected host [27, 35, 36].

Genetic alterations that directly or indirectly target *SaeR* regulon were identified in the 3 CF patients. *SaeR* is directly involved in the regulation of more than 20 virulence factor genes [37] but is likely involved in the regulation of additional proteins by indirect mechanisms [38]. For instance, Cassat et al showed that 31 proteins, including *SdrD*, significantly increase

Table 2. General Features of Detected Mutations

Variable	CF1_late	CF2_late	CF3_late	CLD_late
Time since early isolate	2.8 years	6.7 years	9 years	4.4 years
Total polymorphisms	21	34	79	30
Single-nucleotide polymorphism	19	25	73	26
Insertion-deletion	2	9	6	4
Coding sequence	18	21	62	23
Nonsynonymous mutation (%)	14 (77.8)	16 (47.1)	51 (64.6)	17 (56.7)
Frameshift variant	2	4	4	2
Missense variant	10	11	47	14
Premature stop codon	1	1	0	1
Other	1	0	0	0
Synonymous mutation	4	5	11	6
Intergenic	3	13	17	7

Abbreviations: CF, cystic fibrosis; CLD, chronic lung disease.

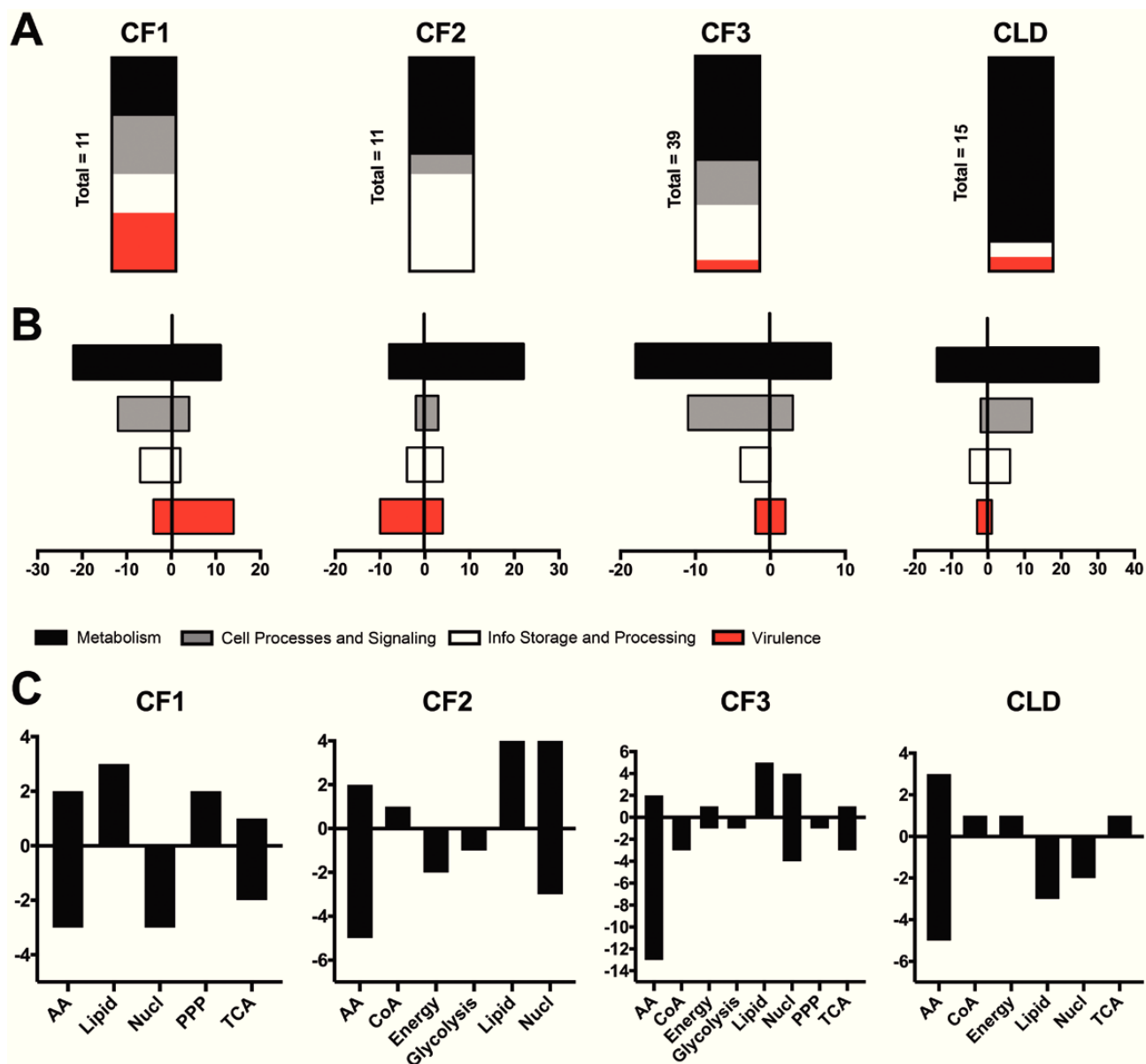


Figure 5. Proteogenomic and metabolomic analysis of the 4 pairs of *Staphylococcus aureus* isolates. (A) Vertical histograms show the functional classification of proteins encoded by genes with nonsynonymous mutations in the genomes of late isolates of *S. aureus* compared to early isolates. (B) Horizontal histograms show the functional classification of differentially expressed annotated proteins in late compared to early isolates of each patient. For each category, histograms represent the number of down- and upregulated proteins from proteomic analysis using the threshold of <2 and >2 , respectively. Only genes and proteins with functional annotation available are included. The “cellular processes and signaling” category encompasses regulatory proteins and proteins involved in cell wall and capsule synthesis. The “information storage and processing” category encompasses proteins involved in replication, translation, and repair processes. The “metabolism” category encompasses proteins involved in metabolism and transport. The “virulence” category encompasses exotoxins and proteins involved in adhesion, biofilm formation, and immunomodulation. (C) Categorization in 8 categories of an altered amount of metabolites in late compared to early isolates. Metabolites were detected by carrying out 2 independent experiments performed in triplicate. Abbreviations: AA, amino acids; CF, cystic fibrosis; CLD, chronic lung disease; CoA, coenzyme A; Nucl, nucleotides; PPP, pentose phosphate pathway; TCA, tricarboxylic acid cycle.

in abundance upon inactivation of *sae* [38]. Furthermore, SaeRS-deficient bacteria are less infective in animal models [39].

In the 3 CF_{late} isolates, we observed an increase in the expression of the SdrD adhesin belonging to the SaeR regulon and involved in adhesion to human nasal epithelial cells and to

human keratinocytes [38, 40]. Our results suggest that SdrD is also important for long-term lung colonization.

In patients with chronic lung infections, SCV detection is most often the consequence of a long-term SXT treatment [41]. Mutations in the *thyA* gene, as found in CF2 and CLD_{late}

isolates, are known to lead to stable clinical SCVs that are no longer susceptible to SXT and are thymidine-auxotrophic (TA-SCV) [32, 41]. Since thymidine is assumed to be abundant during lung inflammation, TA-SCVs can still grow in the inflamed alveolar environment.

In CF3 late isolate, we observed a pantothenate auxotrophy, which has been previously associated with persistence in *Mycobacterium tuberculosis* [42]. The acquisition of pantothenate auxotrophy suggests that pantothenate could also be present in CF lungs. Thus, our data confirm that metabolic specialization is a common phenomenon among long-term colonizers [43].

Other striking traits of phenotypic convergent evolution of *S. aureus* identified in this work were the increased ability to form biofilm and to persist in the intracellular niche. For CF2 and CF3 patients, the increased biofilm ability of late isolates could be linked to a mutation in the *fakA* gene, encoding fatty acid kinase A (FakA). Indeed, several studies showed that FakA-null strains were proficient in biofilm formation [8] and deficient in the expression of virulence factors controlled by the SaeRS system [44]. Overexpression of adhesins detected in proteomic analysis could also ultimately lead to increase biofilm formation in clinical isolates.

Numerous studies have demonstrated the ability of *S. aureus* to persist within host cells [10–19]. Strikingly, for the 3 CF patients, the *S. aureus* late isolates showed a greater ability to persist within CFBE-F508del epithelial cells compared to the early ones at day 3 and day 6 post-infection. Of note, the late isolate of the CLD patient did not present an improved ability to persist intracellularly within CFBE-F508del epithelial cells, possibly due to the fact that it has adapted to a non-CF patient. However, it is not possible to draw conclusions on lung adaptation of *S. aureus* among patients with non-CF lung dysfunction based on a single patient.

In a previous study, Ziebandt and coworkers [45] reported that the protein patterns recorded within each pair of *S. aureus* isolates were very similar, whereas the pattern recorded between pairs of isolates belonging to different clonal groups were highly variable. Strikingly, in our study, marked differences in proteomic profiles between isolates belonging to the same clone were observed. These proteomic changes are likely the consequence of within-host adaptation. The fact that each patient's isolates exhibited specific proteomic changes could be correlated to host-specific factors or to clone-specific factors.

Our multi-omics approach allowed both confirmation of previously known mechanisms and identification of novel candidate genes and pathways involved in the persistence ability of clinical isolates. We now provide evidence that the *saeR/fakA* regulon and the pantothenate pathway could also be promising therapeutic targets to fight persistent *S. aureus* infections.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. X. T., M. C., X. N., A. C., and A. J. conceived and designed the study. X. T., E. R., M. D., D. E., F. T., J. M., and A. J. performed the experiments and analysis. I. N., C. C., I. C. G., A. F., and I. S. G. contributed with data and analysis. A. J., A. C., E. R., and X. T. wrote the manuscript, with contributions and comments from all authors.

Acknowledgments. The authors thank Aurélie Hatton, Charlotte Roy, and Zhicheng Zhou for their help with primary nasal epithelial cell cultures.

Disclaimer. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Financial support. This work was supported by a scholarship from the China Scholarship Council (CSC NO. 201508500097), provided to X. T.

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Wijers CD, Chmiel JE, Gaston BM. Bacterial infections in patients with primary ciliary dyskinesia: comparison with cystic fibrosis. *Chron Respir Dis* **2017**; 14:392–406.
2. Jesenak M, Banovcin P, Jesenakova B, Babusikova E. Pulmonary manifestations of primary immunodeficiency disorders in children. *Front Pediatr* **2014**; 2:77.
3. McAdam PR, Holmes A, Templeton KE, Fitzgerald JR. Adaptive evolution of *Staphylococcus aureus* during chronic endobronchial infection of a cystic fibrosis patient. *PLoS One* **2011**; 6:e24301.
4. López-Collazo E, Jurado T, de Dios Caballero J, et al. In vivo attenuation and genetic evolution of a ST247-SCCmecI MRSA clone after 13 years of pathogenic bronchopulmonary colonization in a patient with cystic fibrosis: implications of the innate immune response. *Mucosal Immunol* **2015**; 8:362–71.
5. Treffon J, Block D, Moche M, et al. Adaptation of *Staphylococcus aureus* to airway environments in patients with cystic fibrosis by upregulation of superoxide dismutase M and iron-scavenging proteins. *J Infect Dis* **2018**; 217:1453–61.
6. Langhanki L, Berger P, Treffon J, Catania F, Kahl BC, Mellmann A. In vivo competition and horizontal gene transfer among distinct *Staphylococcus aureus* lineages as major drivers for adaptational changes during long-term persistence in humans. *BMC Microbiol* **2018**; 18:152.
7. Zapotoczna M, O'Neill E, O'Gara JP. Untangling the diverse and redundant mechanisms of *Staphylococcus aureus* biofilm formation. *PLoS Pathog* **2016**; 12:e1005671.
8. Sabirova JS, Hernalsteens JP, De Backer S, et al. Fatty acid kinase A is an important determinant of biofilm formation in *Staphylococcus aureus* USA300. *BMC Genomics* **2015**; 16:861.
9. Schwartbeck B, Birtel J, Treffon J, et al. Dynamic in vivo mutations within the *ica* operon during persistence of *Staphylococcus aureus* in the airways of cystic fibrosis patients. *PLoS Pathog* **2016**; 12:e1006024.
10. Garzoni C, Francois P, Huyghe A, et al. A global view of *Staphylococcus aureus* whole genome expression upon internalization in human epithelial cells. *BMC Genomics* **2007**; 8:171.
11. Garzoni C, Kelley WL. *Staphylococcus aureus*: new evidence for intracellular persistence. *Trends Microbiol* **2009**; 17:59–65.
12. Kalinka J, Hachmeister M, Geraci J, et al. *Staphylococcus aureus* isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation. *Int J Med Microbiol* **2014**; 304:1038–49.
13. Mitchell G, Grondin G, Bilodeau G, Cantin AM, Malouin F. Infection of polarized airway epithelial cells by normal and small-colony variant strains of *Staphylococcus aureus* is increased in cells with abnormal cystic fibrosis transmembrane conductance regulator function and is influenced by NF- κ B. *Infect Immun* **2011**; 79:3541–51.

14. Proctor RA, von Eiff C, Kahl BC, et al. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat Rev Microbiol* **2006**; 4:295–305.
15. Rollin G, Tan X, Tros F, et al. Intracellular survival of *Staphylococcus aureus* in endothelial cells: a matter of growth or persistence. *Front Microbiol* **2017**; 8:1354.
16. Sendi P, Proctor RA. *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. *Trends Microbiol* **2009**; 17:54–8.
17. Tuchscher L, Heitmann V, Hussain M, et al. *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. *J Infect Dis* **2010**; 202:1031–40.
18. Tuchscher L, Medina E, Hussain M, et al. *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. *EMBO Mol Med* **2011**; 3:129–41.
19. von Eiff C, Becker K, Metze D, et al. Intracellular persistence of *Staphylococcus aureus* small-colony variants within keratinocytes: a cause for antibiotic treatment failure in a patient with Darier's disease. *Clin Infect Dis* **2001**; 32:1643–7.
20. Branger C, Gardye C, Lambert-Zechovsky N. Persistence of *Staphylococcus aureus* strains among cystic fibrosis patients over extended periods of time. *J Med Microbiol* **1996**; 45:294–301.
21. Kahl BC, Duebbers A, Lubritz G, et al. Population dynamics of persistent *Staphylococcus aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study. *J Clin Microbiol* **2003**; 41:4424–7.
22. Kriegeskorte A, Grubmüller S, Huber C, et al. *Staphylococcus aureus* small colony variants show common metabolic features in central metabolism irrespective of the underlying auxotrophism. *Front Cell Infect Microbiol* **2014**; 4:141.
23. Garcia-Betancur JC, Goni-Moreno A, Horger T, et al. Cell differentiation defines acute and chronic infection cell types in *Staphylococcus aureus*. *eLife* **2017**; 6:e28023.
24. Tuchscher L, Kreis CA, Hoerr V, et al. *Staphylococcus aureus* develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. *J Antimicrob Chemother* **2016**; 71:438–48.
25. Sadowska B, Bonar A, von Eiff C, et al. Characteristics of *Staphylococcus aureus* isolated from airways of cystic fibrosis patients, and their small colony variants. *FEMS Immunol Med Microbiol* **2002**; 32:191–7.
26. Das S, Lindemann C, Young BC, et al. Natural mutations in a *Staphylococcus aureus* virulence regulator attenuate cytotoxicity but permit bacteremia and abscess formation. *Proc Natl Acad Sci U S A* **2016**; 113:E3101–10.
27. Suligoy CM, Lattar SM, Noto Llana M, et al. Mutation of Agr is associated with the adaptation of *Staphylococcus aureus* to the host during chronic osteomyelitis. *Front Cell Infect Microbiol* **2018**; 8:18.
28. Tuchscher L, Löffler B. *Staphylococcus aureus* dynamically adapts global regulators and virulence factor expression in the course from acute to chronic infection. *Curr Genet* **2016**; 62:15–7.
29. Mäder U, Nicolas P, Depke M, et al. *Staphylococcus aureus* transcriptome architecture: from laboratory to infection-mimicking conditions. *PLoS Genet* **2016**; 12:e1005962.
30. Tuchscher L, Bischoff M, Lattar SM, et al. Sigma factor SigB is crucial to mediate *Staphylococcus aureus* adaptation during chronic infections. *PLoS Pathog* **2015**; 11:e1004870.
31. Deutsch EW, Csordas A, Sun Z, et al. The ProteomeXchange Consortium in 2017: supporting the cultural change in proteomics public data deposition. *Nucleic Acids Res* **2017**; 45:D1100–6.
32. Chatterjee I, Kriegeskorte A, Fischer A, et al. In vivo mutations of thymidylate synthase (encoded by thyA) are responsible for thymidine dependency in clinical small-colony variants of *Staphylococcus aureus*. *J Bacteriol* **2008**; 190:834–42.
33. Nygaard TK, Pallister KB, Ruzevich P, Griffith S, Vuong C, Voyich JM. SaeR binds a consensus sequence within virulence gene promoters to advance USA300 pathogenesis. *J Infect Dis* **2010**; 201:241–54.
34. Spry C, Kirk K, Saliba KJ. Coenzyme A biosynthesis: an antimicrobial drug target. *FEMS Microbiol Rev* **2008**; 32:56–106.
35. Painter KL, Krishna A, Wigneshwararaj S, Edwards AM. What role does the quorum-sensing accessory gene regulator system play during *Staphylococcus aureus* bacteremia? *Trends Microbiol* **2014**; 22:676–85.
36. Shopsin B, Eaton C, Wasserman GA, et al. Mutations in agr do not persist in natural populations of methicillin-resistant *Staphylococcus aureus*. *J Infect Dis* **2010**; 202:1593–9.
37. Liu Q, Yeo WS, Bae T. The SaeRS two-component system of *Staphylococcus aureus*. *Genes* **2016**; 7:81.
38. Cassat JE, Hammer ND, Campbell JP, et al. A secreted bacterial protease tailors the *Staphylococcus aureus* virulence repertoire to modulate bone remodeling during osteomyelitis. *Cell Host Microbe* **2013**; 13:759–72.
39. Montgomery CP, Boyle-Vavra S, Daum RS. Importance of the global regulators Agr and SaeRS in the pathogenesis of CA-MRSA USA300 infection. *PLoS One* **2010**; 5:e15177.
40. Askarian F, Ajayi C, Hanssen AM, et al. The interaction between *Staphylococcus aureus* SdrD and desmoglein 1 is important for adhesion to host cells. *Sci Rep* **2016**; 6:22134.
41. Kriegeskorte A, Lorè NI, Bragonzi A, et al. Thymidine-dependent *Staphylococcus aureus* small-colony variants are induced by trimethoprim-sulfamethoxazole (SXT) and have increased fitness during SXT challenge. *Antimicrob Agents Chemother* **2015**; 59:7265–72.
42. Sambandamurthy VK, Wang X, Chen B, et al. A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. *Nat Med* **2002**; 8:1171–4.
43. La Rosa R, Johansen HK, Molin S. Convergent metabolic specialization through distinct evolutionary paths in *Pseudomonas aeruginosa*. *mBio* **2018**; 9.
44. Ericson ME, Subramanian C, Frank MW, Rock CO. Role of fatty acid kinase in cellular lipid homeostasis and SaeRS-dependent virulence factor expression in *Staphylococcus aureus*. *mBio* **2017**; 8.
45. Ziebandt AK, Kusch H, Degner M, et al. Proteomics uncovers extreme heterogeneity in the *Staphylococcus aureus* exoproteome due to genomic plasticity and variant gene regulation. *Proteomics* **2010**; 10:1634–44.

1 **Supplementary Methods**

2 **Patients and bacterial isolates**

3 Serial isolates of *S. aureus* were obtained from airway secretions from four patients with
4 chronic lung infection at the Necker-Enfants Malades University Hospital, Paris, France.

5 The CF1 patient (F508del ++ CFTR mutation) (born in 2001) was treated repeatedly with
6 the association of rifampicine and fusidic acid for methicillin-resistant *S. aureus* (MRSA) and
7 ciprofloxacin, ceftazidime and meropenem together with inhaled tobramycin or colimycin
8 for *P. aeruginosa*. We selected an early isolate (CF1_early) from 2012 and a late isolate
9 (CF2_late) from 2015.

10 The CF2 patient (F508del/R347P CFTR mutation) (born in 1997) was treated with
11 association of amoxicillin-clavulanate and trimethoprim-sulfamethoxazole (SXT) and also
12 received occasionally minocycline and streptogramins during bronchial exacerbations. We
13 selected an early isolate (CF2_early) from 2009 and a late isolate (CF2_late) from 2015.

14 The CF3 patient (F508del ++ CFTR mutation) (born in 2002) displayed a coinfection with
15 *P. aeruginosa* requiring repeated intravenous antibiotics including ceftazidime, amikacin and
16 ciprofloxacin. We selected an early isolate (CF3_early) from 2008 and a late isolate
17 (CF3_late) from 2017.

18 The CLD patient (born in 1997) was not CFTR mutated but exhibited a primary humoral
19 immunodeficiency disorder with a mild IgG2 deficiency causing chronic bronchitis.
20 Bronchial exacerbations were treated with SXT association. We selected an early isolate
21 (CLD_early) from 2009 and a late isolate (CLD_late) from 2013.

22 Nasal epithelial cell (HNE cells) were sampled by nasal brushing of both nostrils after local
23 visualization of the nasal mucosa from one healthy volunteer and patient CF3.

24 The epidemic clone *S. aureus* USA300-LAC (designated WT) was provided by the
25 Biodefense and Emerging Infections Research Resources (BEI). A derivative of USA300-
26 LAC with a deletion in the haemin pathway (*ΔhemDBL* triple deletion mutant), involved in
27 the electron transport system, has been previously constructed to obtain a strain with a stable
28 SCV-phenotype [1].

29 *S. aureus* isolates were grown on brain heart infusion (BHI) broth and agar plates. Colony
30 morphotypes were assessed after 24h of growth on BHI or Mueller-Hinton agar plates.

31 When indicated, *S. aureus* isolates were cultured in medium mimicking the respiratory fluid
32 of cystic fibrosis patients (Cystic Fibrosis Sputum Medium or CFSM) [2] with or without the
33 addition of thymidine (100 µg/mL) or pantothenate (50 µg/mL).

34 **Whole-genome sequencing and analysis**

35 Genomic DNA was extracted using DNeasy Blood and Tissue Kit with 10 µg/mL lysostaphin
36 to facilitate cell lysis. Genomic libraries were prepared using a Nextera XT kit, multiplexed
37 and sequenced on an Illumina MiSeq instrument (2x150 paired-end sequencing).

38 The short reads were processed using the Nullarbor bioinformatic pipeline software v1.20
39 (Available from: <https://github.com/tseemann/nullarbor>). Briefly, raw reads were trimmed
40 using trimmomatic [3], de novo assembled using SPAdes 3.9.0 [4] and annotated using
41 Prokka v1.11 [5]. Single nucleotide polymorphisms (SNPs) and small indels were assessed
42 using Snippy v3.1 (<https://github.com/tseemann/snippy>). The Similar Genome Finder Service
43 of the PATRIC 3.5.2 on-line database was used to identify the most similar publically
44 available genome for each pair of clinical isolates
45 (<https://www.patricbrc.org/app/GenomeDistance>). Snippy was used both to map reads of late
46 isolates against the annotated assembly of the early isolate and to map reads of early and late
47 isolates against the publically available closest reference genome for the four pairs. Snippy

48 classifies variants by their predicted functional effect based on mapping to the annotated
49 reference genome in synonymous, nonsynonymous or truncating for protein-coding
50 sequences.

51 Assemblies generated by Nullarbor were uploaded in RAST server [6] and were also
52 annotated through the RAST annotation pipeline with ClassicRAST annotation scheme. The
53 ‘Sequence Based Comparison Tool’ of the RAST server, which is based on BLAST protein
54 similarity search, was used to identify large insertions and deletions of each late isolate
55 compared to early isolate as a reference.

56 **Quantification of biofilm formation**

57 To determine biofilm formation in static conditions, clinical isolates as well as reference
58 strains were cultured in Brain Heart Infusion (BHI) medium in the presence of 1% glucose.
59 The reference strains include USA300-LAC that is a weak biofilm producer and its *ΔagrC*
60 derivative that is a strong biofilm producer (obtained from the BEI Nebraska Transposon
61 Mutant Library). The over-night cultures were diluted to an OD_{600nm} of 0.05 in fresh BHI
62 medium supplemented with 1% glucose. A total of 200 μl of each diluted culture were
63 inoculated in two polystyrene 96-well plates in triplicate and incubated for 48 hours at 37 ° C
64 without shaking. After 24 h of growth, the medium was renewed. After 48 h, one of the 96-
65 well plates was used to assess bacterial growth by taking into account both bacteria in
66 suspension and adherent bacteria (resuspended mechanically) and measuring OD_{600nm}. The
67 other 96-well plate was used to quantify biofilm formation. The wells were washed three
68 times with distilled H₂O to remove unattached bacteria and the biofilm fixed at the bottom of
69 the wells was stained with 100 μl 2 % crystal violet solution for 15 min. After staining, wells
70 were washed three times with distilled H₂O. Crystal violet was solubilized using 150 μl of
71 acetic acid at 30 %. 100 μl of the solubilized dye was transferred to a new 96-well plate to

72 determine the absorbance at 540 nm.

73 **Antimicrobial susceptibility**

74 Antimicrobial susceptibility of isolates was evaluated by disc diffusion method. Etests were
75 used to determine minimal inhibitory concentrations to gentamicin. Susceptibility tests were
76 carried out on BHI agar plates to allow the growth of auxotrophic isolates.

77 **Cell culture experiments**

78 Cystic Fibrosis Bronchial Epithelial cell line CFBE41o- that is an immortalized cell line
79 homozygous for the delF508 mutation was generously provided by Dieter Gruenert
80 (University of California, San Francisco) [7]. Cells were cultured in Minimum Essential
81 Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1%
82 penicillin/streptomycin and 1% amphotericin B.

83 Primary nasal epithelial cell (HNE) were obtained as described in [8] and cultured in
84 Dulbecco's Modified Eagle's medium mixed 1:1 with Ham's F-12 (DMEM:F12)
85 supplemented with 10% FBS, 1%v/v non-essential amino acids, 90 µg/10 µg/ml
86 Piperacillin/Tazobactam and 1% amphotericin B.

87 Prior infection assays, epithelial cells were seeded in uncoated wells for CFBE and wells
88 coated with collagen for HNE and all antibiotics were removed from culture medium.
89 Epithelial cells were infected with a multiplicity of infection (MOI) of 100 using an inoculum
90 taken from cultures of *S. aureus* grown in BHI until exponential growth phase. After 1h30,
91 each well was washed three times with 1 mL of phosphate buffer saline (PBS) containing 300
92 µg/mL gentamicin, to remove extracellular bacteria. Washing and addition of fresh medium
93 containing 50 µg/mL gentamycin were repeated at day 3. Gentamicin has a high bactericidal
94 effect on *S. aureus* USA300 (minimum inhibitory concentrations (MIC) = 2 µg.mL⁻¹) and a
95 very poor penetration inside eukaryotic cells. In addition, the measured MIC of gentamicin of

96 all clinical strains was below or equal to $2 \mu\text{g}\cdot\text{mL}^{-1}$. Infected cells were kept in a humidified
97 5% CO₂ atmosphere at 37°C for 6 days. To enumerate viable intracellular persisting bacteria,
98 the culture medium containing gentamicin was removed from infected cells and wells were
99 washed three times with antibiotic free PBS. Epithelial cells were lysed by scrapping with
100 1mL of distilled water during 15 minutes and serial dilutions of the cell lysates were plated on
101 BHI agar. Colony forming units were numerated after 48 h at 37°C. Results shown are
102 normalized with USA300-LAC strain as a reference and expressed as a fold change of CFUs.

103 **Proteomic analyses**

104 *S. aureus* isolates were grown in 50 ml of CFMSM with addition of thymidine (100
105 $\mu\text{g}/\text{mL}$) in technical triplicates and collected after 19h of growth at 37°C with agitation (150
106 RPM). These growth conditions allow the bacteria to reach post-exponential growth phase as
107 observed in the corresponding growth curves shown in Figure 4. Bacteria were lysed in PBS
108 containing CaCl₂ 1 mM, MgCl₂ 1 mM and 10 $\mu\text{g}/\text{mL}$ lysostaphin.

109 Proteins were digested and analyzed by liquid chromatography coupled with tandem mass
110 spectrometry (nanoLC-MS/MS). Filter Aided Sample Preparation (FASP) was used for
111 protein digestion [9, 10]. Proteins were then reduced in 100 mM DTT for 30 min at 60°C,
112 mixed with 8 M urea buffer, loaded on Microcon 30kDa centrifugal filters (Millipore) and
113 alkylated with 50 mM iodoacetamide. Filters were washed twice with 8 M urea and twice
114 with 50 mM ammonium bicarbonate. Following an overnight trypsin digestion at 37°C,
115 samples were vacuum dried, and kept at -20 °C until MS analysis.

116 MS analysis was performed as previously published [10]. Dried peptides were resuspended in
117 10% ACN, 0.1% TFA. For each run, 5 μl (corresponding approximately to 1 μg of peptides)
118 were injected in a nanoRSLC-Q Exactive PLUS MS (Dionex RSLC Ultimate 3000, Thermo
119 Scientific, Waltham, MA). Peptides were separated on a 50 cm reversed-phase liquid

120 chromatographic column (Pepmap C18, Dionex). Chromatography solvents were: (A) 0.1%
121 formic acid in water, and (B) 80% acetonitrile with 0.08% formic acid. Peptides were eluted
122 from the column with the following gradient: 5% to 40% of B for 120 min and then 40% to
123 80% of B in 1 min. At 125 min, the gradient was reversed to 5% to allow re-equilibration of
124 the column for 20 min before the next injection. One blank with two 30 minutes linear
125 gradients was run between triplicates to prevent sample carryover.

126 Peptides eluted from the column were analyzed by data dependent MS/MS, using the top-10
127 acquisition method. Briefly, the instrument settings were as follows: resolution was set to
128 70,000 for MS scans and 17,500 for the data dependent MS/MS scans in order to increase
129 speed. The MS AGC target was set to $3 \cdot 10^6$ counts, whereas the MS/MS AGC target was set
130 to $5 \cdot 10^4$. The MS scan range was from 400 to 2000 m/z. MS and MS/MS scans were recorded
131 in profile mode. Dynamic exclusion was set to 20 s duration. Each sample was analyzed in
132 three technical triplicates by nanoLC/MS/MS. Raw MS files were processed with the
133 MaxQuant software version 1.5.8.3 and searched with the Andromeda search engine against a
134 custom protein database containing 3 457 protein sequences. To build this database we pooled
135 protein sequences from both PROKKA and RAST annotations of all clinical isolates and the
136 MAGE annotation of USA300 (NC_007793.1) [11]. Redundancy of the sequence set was
137 reduced using CD-HIT v4.6 [12] with a 80% identity threshold. To search for parent mass and
138 fragment ions, we set the mass deviation at 4.5 ppm and 20 ppm, respectively. The minimum
139 peptide length was set to seven amino acids and strict specificity for trypsin cleavage was
140 required, allowing up to two missed cleavage sites. Match between runs was allowed.
141 Carbamidomethylation (Cys) was set as fixed modification, whereas oxidation (Met) and
142 protein N-terminal acetylation were set as variable modifications. The false discovery rates
143 (FDRs) at the protein and peptide level were set to 1%. Scores were calculated in MaxQuant
144 as described previously [13]. The reverse and common contaminants hits were removed from

145 MaxQuant output. Proteins were quantified according to the MaxQuant label-free algorithm
146 using normalized label free quantification (LFQ) intensities. Protein quantification was
147 obtained using at least two peptides per protein.

148 Statistical and bioinformatic analysis, including heatmaps and clustering, were performed
149 with Perseus software (version 1.6.0.7) freely available at www.perseus-framework.org [14].

150 EggNOG annotations of all proteins were retrieved from webserver (last accessed 2017-12-
151 15) [15]. Gene names were retrieved from AureoWiki [16] (last accessed 2017-12-15).

152 Extraction of annotations GO, Keywords, EggNOG and Kegg pathway were performed using
153 Perseus.

154 For accurate quantification, we analysed each isolate separately (3 samples from early and 3
155 samples from late collection). We filtered the data to keep only proteins with at least 3 valid
156 values in at least one group (late or early). Next, the data were imputed to fill missing data
157 points by creating a Gaussian distribution of random numbers with a standard deviation of
158 33% relative to the standard deviation of the measured values and a 2.5 standard deviation
159 downshift of the mean to simulate the distribution of low signal values. To discriminate
160 differential protein amongst early and late for each isolate we performed a t-test, $S_0=0.1$, and
161 retained only proteins with an $FDR<0.05$. Hierarchical clustering of significantly different
162 proteins was performed in Perseus on logarithmic LFQ intensities after z-score normalization
163 of the data, using Euclidean distances and default parameters. We also performed a T Test,
164 $S_0=0.1$, $FDR=0.001$ to discriminate differential proteins between late and early.

165 **Metabolomic analyses**

166 Metabolite profiling of *S. aureus* isolates was performed by liquid chromatography–mass
167 spectrometry (LC-MS) as described [17]. Two independent experiments with three biological
168 replicates were performed for each isolate. Briefly, *S. aureus* isolates were grown in CF5M

169 with addition of thymidine in the same conditions as for the proteomic analyses. Metabolic
170 activity was blocked by immersion in liquid nitrogen for 10 sec.

171 Metabolites were extracted using a solvent mixture of Methanol/ACN/H₂O (50:30:20) at
172 -20 °C. Samples were vortexed for 5 min at 4°C, and then centrifuged at 16,000 g for 15
173 minutes at 4°C. The supernatants were collected and analysed by LC-MS using SeQuant ZIC-
174 pHilic column (Millipore) for the liquid chromatography separation [17]. The aqueous
175 mobile-phase solvent was 20 mM ammonium carbonate plus 0.1% ammonium hydroxide
176 solution and the organic mobile phase was acetonitrile. The metabolites were separated over a
177 linear gradient from 80% organic to 80% aqueous phase for 15 min. The column temperature
178 was 48 °C and the flow rate was 200 µl/min. The metabolites were detected across a mass
179 range of 75–1,000 m/z using the Q-Exactive Plus mass spectrometer (Thermo) at a resolution
180 of 35,000 (at 200 m/z) with electrospray ionization and polarity switching mode. Lock masses
181 were used to insure mass accuracy below 5 ppm. The peak areas of different metabolites were
182 determined using TraceFinder software (Thermo) using the exact mass of the singly charged
183 ion and known retention time on the HPLC column. By this approach we were able to identify
184 and measure: Glucose, Glucose-6P (G6P), UDP-glucose (UDP-Glc), Sedoheptulose-7-P (SH-
185 7P), Ribose 5-phosphate, Glyceraldehyde 3-phosphate, Lactate, Pyruvate, Phospho-Enol-
186 Pyruvate (PEP), Citrate (Cit), cis-aconitate (cis-aco), Succinate (Suc), Fumarate (Fum),
187 Malate (Mal), alpha ketoglutarate (aKG), dihydroxyacetone phosphate (DHAP), N-
188 carbamoyl-L-aspartate, Nicotinamide adenine dinucleotide (NAD), NADH, Nicotinamide
189 adenine dinucleotide phosphate (NADP), NADPH, Adenine, Adenosine, Adenosine Mono, Di
190 and Tri-phosphate (AMP, ADP, ATP), Cytidine, Cytidine Mono, Di and Tri-phosphate
191 (CMP, CDP, CTP), Uridine Mono, Di and Tri-phosphate (UMP, UDP, UTP), Hypoxanthine,
192 Guanine, Guanosine, Guanidine Mono and Di-phosphate (GMP, GDP), Inosine
193 monophosphate (IMP), Pantothenate, Dephospho-Coenzyme A, Coenzyme A (CoA), Flavin

194 adenine dinucleotide (FAD), Niacin, Nicotinamide, 3-hydroxybutyrate, Urate, 5-oxo-L-
195 Proline, Acetyl-aspartate, Acetyl-glutamine, Alanine, Arginine, Argininosuccinate,
196 Asparagine, Aspartate, Betaine, Citrulline, Cystathionine, Cystine, Glutamine, Glutamate,
197 Glycine, Histidine, IsoLeucine, Leucine, Sarcosine, Lysine, Methionine, Ornithine,
198 Phenylalanine, Proline, Serine, Taurine, S-Adenosyl-L-methionine, Threonine, Tryptophan,
199 Tyrosine, Valine, Butyric acid, Carnitine, Decanoic acid, Lauric acid, Hexanoic acid, Linoleic
200 acid, Myristic acid, Octanoic acid, Oleic acid, Palmitic acid.

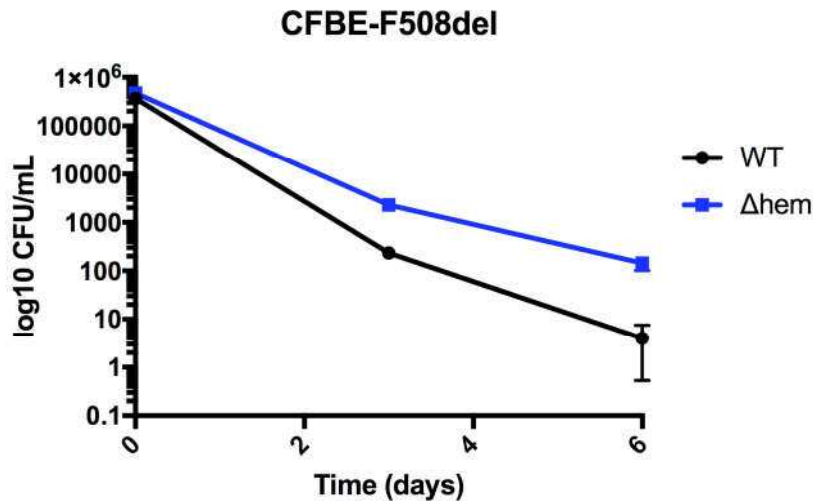
201 Statistical analyses were performed using MetaboAnalyst 4.0 software [18]. The algorithm for
202 heatmap clustering was based on the Pearson distance measure for similarity and the Ward
203 linkage method for biotype clustering. Metabolites with similar abundance patterns were
204 positioned closer together.

205

- 206 1. Rollin G, Tan X, Tros F, et al. Intracellular Survival of *Staphylococcus aureus* in
207 Endothelial Cells: A Matter of Growth or Persistence. *Frontiers in microbiology* **2017**;
208 8: 1354.
- 209 2. Palmer KL, Aye LM, Whiteley M. Nutritional cues control *Pseudomonas aeruginosa*
210 multicellular behavior in cystic fibrosis sputum. *Journal of bacteriology* **2007**;
211 189(22): 8079-87.
- 212 3. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina
213 sequence data. *Bioinformatics* **2014**; 30(15): 2114-20.
- 214 4. Bankevich A, Nurk S, Antipov D, et al. SPAdes: a new genome assembly algorithm
215 and its applications to single-cell sequencing. *Journal of computational biology : a*
216 *journal of computational molecular cell biology* **2012**; 19(5): 455-77.
- 217 5. Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* **2014**;
218 30(14): 2068-9.
- 219 6. Overbeek R, Olson R, Pusch GD, et al. The SEED and the Rapid Annotation of
220 microbial genomes using Subsystems Technology (RAST). *Nucleic acids research*
221 **2014**; 42(Database issue): D206-14.
- 222 7. Ehrhardt C, Collnot EM, Baldes C, et al. Towards an in vitro model of cystic fibrosis
223 small airway epithelium: characterisation of the human bronchial epithelial cell line
224 CFBE41o. *Cell and tissue research* **2006**; 323(3): 405-15.
- 225 8. Pranke IM, Hatton A, Simonin J, et al. Correction of CFTR function in nasal epithelial
226 cells from cystic fibrosis patients predicts improvement of respiratory function by
227 CFTR modulators. *Scientific reports* **2017**; 7(1): 7375.

- 228 9. Wisniewski JR, Zougman A, Mann M. Combination of FASP and StageTip-based
229 fractionation allows in-depth analysis of the hippocampal membrane proteome.
230 *Journal of proteome research* **2009**; 8(12): 5674-8.
- 231 10. Lipecka J, Chhuon C, Bourderioux M, et al. Sensitivity of mass spectrometry analysis
232 depends on the shape of the filtration unit used for filter aided sample preparation
233 (FASP). *Proteomics* **2016**; 16(13): 1852-7.
- 234 11. Vallenet D, Labarre L, Rouy Z, et al. MaGe: a microbial genome annotation system
235 supported by synteny results. *Nucleic acids research* **2006**; 34(1): 53-65.
- 236 12. Fu L, Niu B, Zhu Z, Wu S, Li W. CD-HIT: accelerated for clustering the next-
237 generation sequencing data. *Bioinformatics* **2012**; 28(23): 3150-2.
- 238 13. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized
239 p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature*
240 *biotechnology* **2008**; 26(12): 1367-72.
- 241 14. Tyanova S, Temu T, Sinitcyn P, et al. The Perseus computational platform for
242 comprehensive analysis of (prote)omics data. *Nature methods* **2016**; 13(9): 731-40.
- 243 15. Huerta-Cepas J, Forslund K, Coelho LP, et al. Fast Genome-Wide Functional
244 Annotation through Orthology Assignment by eggNOG-Mapper. *Molecular biology*
245 *and evolution* **2017**; 34(8): 2115-22.
- 246 16. Fuchs S, Mehlan H, Bernhardt J, et al. AureoWiki The repository of the
247 *Staphylococcus aureus* research and annotation community. *International journal of*
248 *medical microbiology : IJMM* **2017**.
- 249 17. Mackay GM, Zheng L, van den Broek NJ, Gottlieb E. Analysis of Cell Metabolism
250 Using LC-MS and Isotope Tracers. *Methods in enzymology* **2015**; 561: 171-96.
- 251 18. Xia J, Wishart DS. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data
252 Analysis. *Current protocols in bioinformatics* **2016**; 55: 14 0 1- 0 91.
- 253
254
255
256

1 Supplementary figures



2

3 **Figure S1. Intracellular growth curves of reference *S. aureus* isolates in CFBE**

4 **epithelial cell line.** Bronchial CFBE epithelial cell line (CFBE-F508del homozygous

5 for the F508del-CFTR mutation) was infected with the control strain USA300-LAC

6 (WT) and a stable SCV mutant altered in the haemin pathway (Δ hem). Gentamicin

7 was present throughout the experiment to prevent extracellular bacterial growth and

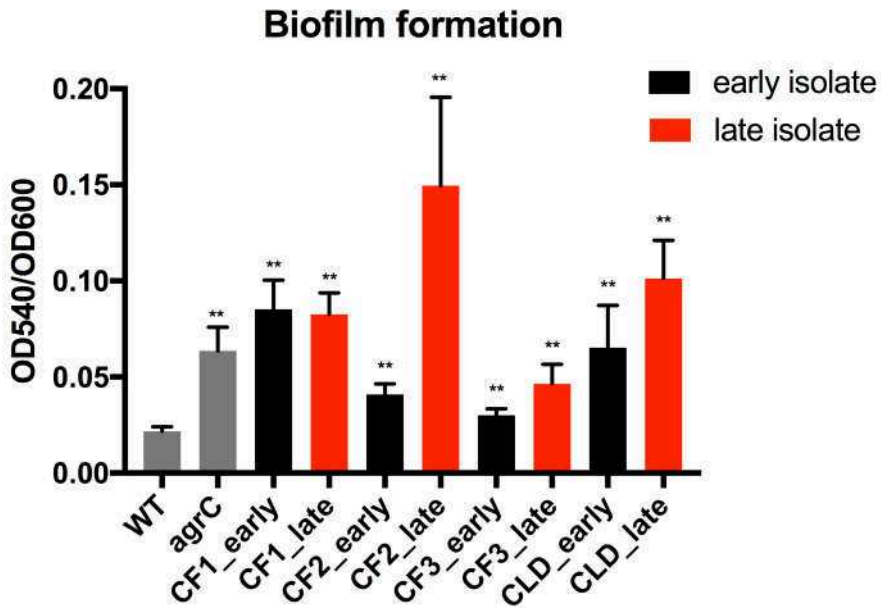
8 new infection. Bacterial load inside cells were evaluated by CFU enumeration at 3

9 and 6 days after infection. Results shown are the mean \pm SD for four experiments

10 performed in triplicate.

11

12



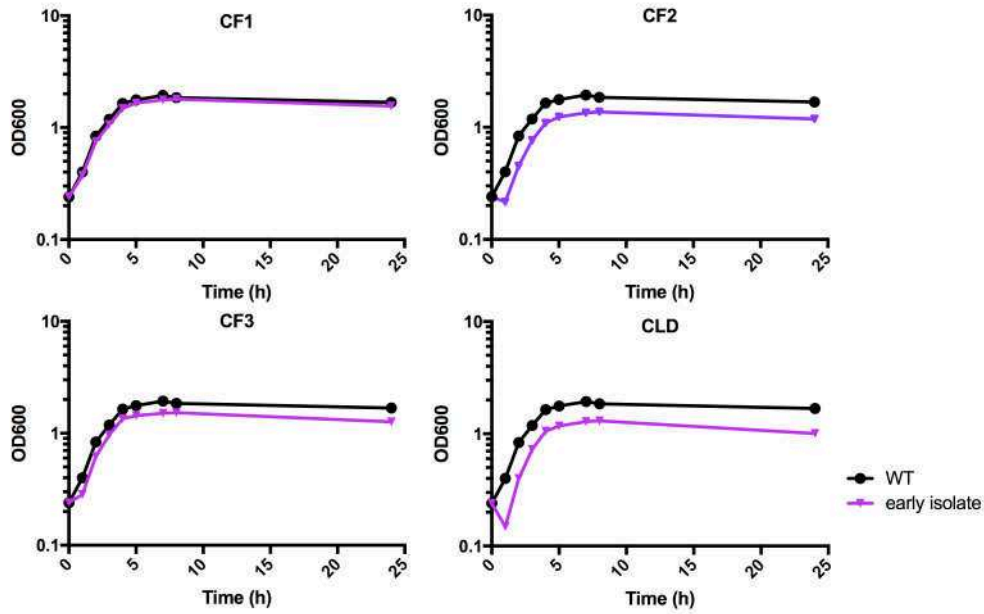
13

14 **Figure S2. Quantification of biofilm formation of *S. aureus* clinical isolates.**

15 Biofilm formation was monitored using the crystal violet microtiter assay in BHI
 16 medium with 1% glucose. USA300-LAC strain (WT) was used as a reference for
 17 weak biofilm production whereas its *agrC* derivative obtained from the Nebraska
 18 Transposon Mutant Library was used as a reference for strong biofilm production.
 19 Results shown are the mean \pm SD for three independent experiments performed in
 20 triplicate. Statistical significance was measured using one-way ANOVA with multiple
 21 comparisons (Dunnett's correction) performed on the dataset as a whole, with each
 22 value compared to the WT. ** indicates p-value of <0.001.

23

24

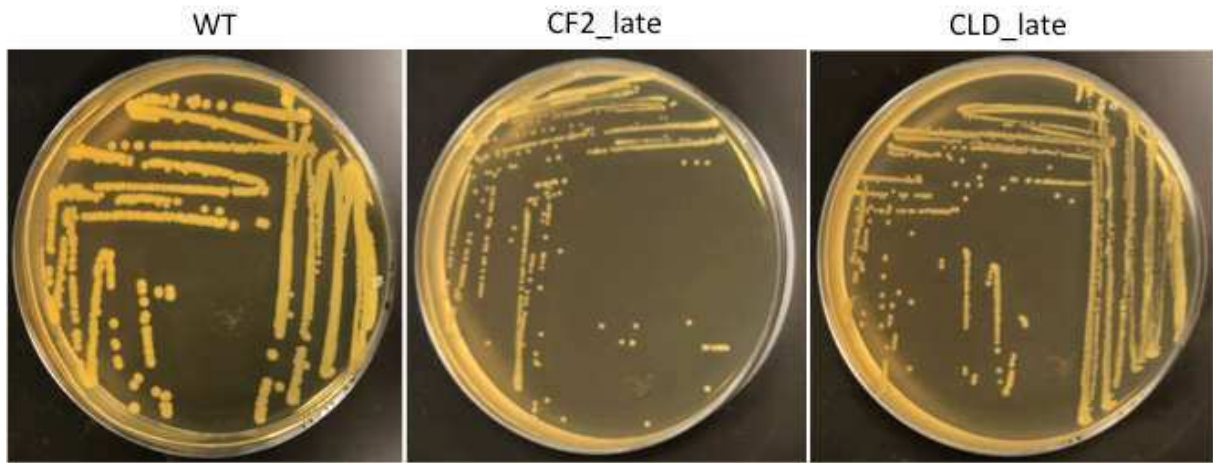


25

26 **Figure S3. Growth curves of early *S. aureus* clinical isolates in CF1, CF2, CF3, and CLD media.** Growth
 27 curves were carried out in CF1, CF2, CF3, and CLD media. The results shown correspond to a representative
 28 experiment. WT, USA300-LAC.

29

30



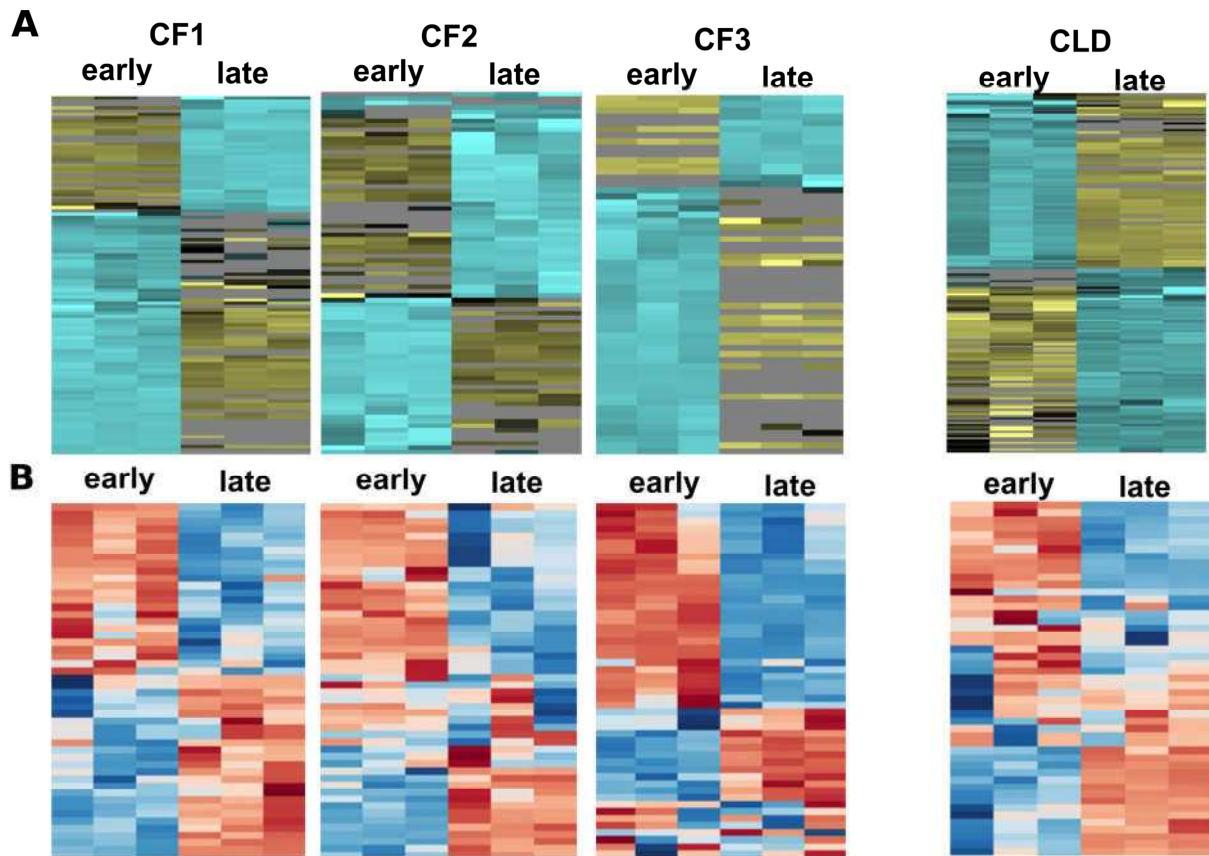
31

32 **Figure S4. Colony morphology of USA300-LAC (WT), CF2_late and CLD_late**

33 **isolates.** Bacteria were grown on BHI agar incubated 12h at 37°C.

34

35



36

37 **Figure S5. Heatmaps showing comparison of protein and metabolite profiles of**

38 **early/late *S. aureus* isolates.** The isolates were cultured to the stationary phase in

39 medium mimicking the respiratory fluid of cystic fibrosis patients (CFSM) with the

40 addition of thymidine. **A)** Heatmap visualization and hierarchical clustering analysis of

41 the proteomic profiling in the late isolate compared to the early isolate of each

42 patient. One experiment with three biological replicates performed for each isolate is

43 shown. Rows: proteins; columns: samples; color key indicates protein relative

44 concentration value (yellow: lowest; blue: highest). **B)** Heatmap visualization and

45 hierarchical clustering analysis of the metabolite profiling in the late isolate compared

46 to the early isolate of each patient. The top 50 most changing compounds of one

47 representative experiment with three biological replicates performed for each isolate

48 are presented. Rows: metabolites; columns: samples; color key indicates metabolite

49 relative concentration value (blue: lowest; red: highest).

Paper 3 Chronic *Staphylococcus aureus* lung infection correlates with proteogenomic and metabolic adaptations leading to an increased intracellular persistence

Contributions by Xin TAN

Method

-Biofilm assay

-*in vitro* infection assay with clinical isolates in CFTR cell lines

Results

Figure 2, Figure 3 and Figure S2

Discussion and perspectives

S. aureus is generally considered as an extracellular pathogen. However, more and more studies have demonstrated that *S. aureus* was able to invade a variety of non-professional phagocytic cells (NPPCs) such as endothelial and epithelial cells, osteoblasts, fibroblasts, keratinocytes ... (Strobel, Pfortner *et al.* 2016; Moldovan and Fraunholz 2019). The intracellular environment could protect staphylococci from host's immune system and antibiotics and, thus, constitute a reservoir likely to play an important role for bacterial persistence in the host and relapse of infection. During this thesis, we addressed different aspects of *S. aureus* persistence, using different set up and models but with a common idea: understand the importance of metabolic adaptations during chronic persistence.

In the first article, we confirmed the increased intracellular persistence of *S. aureus* USA300 in endothelial cells as compared to macrophages. Furthermore, using time-lapse microscopy, we could show that the initial persistence of wild-type *S. aureus* in endothelial cells corresponded to distinct simultaneous situations at the single cell level, notably including a prolonged bacterial persistence without apparent morphological cellular damage. Our results and those of others indicate that, within the first hours after internalization, bacteria tend to actively multiply inside human cells (and ultimately lyse them) whereas infected cells attempt to restrict their proliferation. This selective pressure on *S. aureus* may favor the emergence of transient persisters which are able to survive into host cells for several days, without multiplication, before re-emerging in the extracellular compartment where they can rapidly proliferate and infect neighboring cells.

Two main outcomes of host cell invasion by *S. aureus* are generally described: (i) rapid host cell lysis, triggered by the secretion of toxins and other pro-inflammatory factors, which induce strong inflammatory and cytotoxic effects; (ii) persistence within morphologically-intact host cells for extended periods of time, where secretion of virulence factors is either down-regulated or not expressed (Proctor, Kriegeskorte *et al.* 2014). Of note, these persisting bacteria generally correspond to so-called “small colony variants” (SCV). The association between *S. aureus* SCVs and persistent infection was first reported by Proctor and co-workers in 1995 (Proctor, van Langevelde *et al.* 1995) and several studies have shown that the impaired growth of *S. aureus* SCVs was due to decreased metabolic activity (Amato, Fazen *et al.* 2014). Since a number of other pathogenic bacteria have been shown to persist within their host as SCVs (Kahl,

Becker *et al.* 2016), it may be considered as a major survival strategy in chronic bacterial infections. However, summarizing the prolific literature addressing *S. aureus* intracellular persistence is quite complicated and may even be misleading. Indeed, the comparison of results obtained in one experimental setup with those obtained in another one is quite often impossible due to the different cell types and *S. aureus* strains which have been used. Our study reveals two major outcomes of intracellular *S. aureus* by Time-lapse microscopy. However, the molecular mechanism remains poorly understood. Next, we plan to investigate the transcriptional level of intracellular *S. aureus* on single cell level to better understand these distinct behaviors.

In the second article, we studied the role of the metabolic Pentose phosphate pathway (PPP) during the intracellular persistence of *S. aureus*, by focusing on transketolase (TKT), one key enzyme of this pathway. We showed that inactivation of the unique gene *tkt*, encoding transketolase in *S. aureus* USA300, conferred a slow-growth phenotype and was associated with important metabolomic alterations during planktonic growth. In infected cells, *tkt* inactivation also decreased bacterial intracellular persistence, most likely because of a drastic reduction of transcription of Sigma B- and RpiRc-encoding genes.

These data unraveled potential novel connections between the PPP and other regulatory circuits controlling chronic bacterial intracellular persistence. *S. aureus* SCVs have been particularly used as a model to study persistence (Proctor, von Eiff *et al.* 2006; Garzoni and Kelley 2009; Sendi and Proctor 2009; Mitchell, Gattuso *et al.* 2011; Tuchscher, Medina *et al.* 2011; Kalinka, Hachmeister *et al.* 2014; Strobel, Pfortner *et al.* 2016). As recalled earlier, the SCV phenotype is generally associated with the formation of tiny colonies on plates, increased biofilm capacity and antibiotic resistance. Furthermore, supernatants from SCVs cultures generally show reduced cytotoxicity on cell cultures. The Δtkt mutant of *S. aureus* USA300 shared the common properties of SCVs in planktonic conditions. The behavior of the Δtkt mutant of *S. aureus* USA300 was also similar to a slow-growing strain (here $\Delta hemDBL$) in time-lapse video microscopy, at the single cell level over a 24 hours-period. Somewhat unexpectedly, bacterial counts revealed that after seven days, the wild-type strain and the Δtkt mutant showed similar intracellular survival decrease, ultimately leading to their complete elimination whereas the $\Delta hemDBL$ strain was still clearly detected even after ten days inside cells. Confocal microscopy analyses did not reveal any phagosomal escape defect of the Δtkt mutant.

Hence, although non-proliferation is generally associated to prolonged persistence, the *Δtkk* mutant appeared to be a non-proliferative and non-persister strain. Among the most striking transcriptional changes recorded in the *Δtkk* mutant compared to WT *S. aureus* USA300, was an increase in RNAIII transcription at day 1 and day 3 in infected cells. Simultaneously, the *Δtkk* mutant showed a severe decreased transcription of *rpiRc* and *sigB* genes. In general, very low levels of RNAIII, either due to an inactivated *agrBDC*A operon or to an increased RNAIII degradation, constitute a hallmark of electron transport chain related SCVs (Felden, Vandenesch *et al.* 2011; Kriegeskorte, König *et al.* 2011; Romilly, Lays *et al.* 2014; Roux, Todd *et al.* 2014). The *agr* system constitutes one of the major and best-characterized players in *S. aureus* intracellular survival. Indeed, the up regulation of the *agr* locus, that triggers the expression of secreted virulence factors such as hemolysins and leukocidins, is unfavorable for intracellular persistence (Tuchscher, Heitmann *et al.* 2010). RpiRc, one of the three members of the transcriptional regulator RpiR family controlling enzymes involved in sugar catabolism in *S. aureus*, appears to function as a metabolite-responsive modulator of the *agr* system, that also negatively-controls RNAIII transcription (Balasubramanian, Ohneck *et al.* 2016). Of note, RpiRc has been shown to repress RNAIII (Zhu, Nandakumar *et al.* 2011), supporting the notion of a direct connection between the PPP and the regulation of *S. aureus* virulence. The expression of SigB has also been shown to play a crucial role during persistence possibly by down-regulating pro-inflammatory virulence factors and increasing the expression of factors promoting persistence (Tuchscher, Bischoff *et al.* 2015) and *ΔsigB* mutants are completely cleared by the host cells and fail to form SCVs (Tuchscher and Löffler 2016). TKT inactivation which triggers the up-regulation of RNAIII and down-regulation of *sigB* and *rpiRc* appears thus to confer a non-persister phenotype during chronic infection of host cells.

A number of “Omics” approaches have been carried out to explain why *S. aureus* SCVs have a reduced metabolic activity and how these metabolic changes affect persistence (Seggewiss, Becker *et al.* 2006; von Eiff, McNamara *et al.* 2006; Kriegeskorte, König *et al.* 2011). During its intracellular survival, a *Δtkk* mutant is likely to deal simultaneously with PPP blocking and altered levels of carbon-based nutrients. Our whole cell metabolomic analyses of planktonic-grown bacteria showed a huge increase in ribose-5-phosphate (R5P) intermediates in the *Δtkk* mutant compared to wild-type that could be explained by the impaired entry of R5P into glycolysis and a concomitant significant decrease in sedoheptulose-7-phosphate (SH7P) relative concentration. We also observed significant changes in the relative concentrations of TCA cycle intermediates and TCA-related amino acids compared to WT strain. For example,

we observed a decrease in citrate and cis-aconitase amounts, corresponding to the very first TCA intermediates, in *Atkt* mutant compared to wild-type, which could account for a limited fueling of the TCA cycle at this stage. In contrast, we noted a significant increase in the relative concentrations of following TCA cycle intermediates such as α -ketoglutarate, succinate, fumarate and malate in *Atkt* mutant that could be due to an increased import of proline and glutamine. The TCA cycle plays a central role in maintaining the bacterial metabolic status and has been repeatedly implicated in the regulation of staphylococcal virulence (Somerville, Chaussee *et al.* 2002; Somerville, Cockayne *et al.* 2003; Ding, Liu *et al.* 2014). Activity of TCA cycle enzymes, the amounts of biosynthetic intermediates and generated ATP can be affected by a number of conditions, including: i) nutrients availability, ii) a stressful environment (e.g., an intracellular environment), or iii) stress-inducing stimuli that alter carbon flux or here the PPP. These changes induce alterations of the overall metabolome of the bacterium that, in turn, may modulate the activity of metabolite-responsive global regulators such as SigB or RpiR.

We also found that bacteria grown in CDM supplemented with ribose or fructose had reduced *sigB* expression during stationary phase. We thus hypothesized that the modification of carbon source availability inside infected cells might be a suitable strategy to act on *sigB* expression and therefore impair *S. aureus* intracellular persistence. We were not able to modify sufficiently the carbon source availability in the cytoplasm of infected endothelial cell, using DMEM supplementation with either ribose or fructose, to abrogate *S. aureus* USA300 intracellular persistence. A modest reduction of *S. aureus* intracellular persistence was however recorded with both sugars. At any rate, impaired bacterial intracellular survival might also be due to a global modification of the endothelial cell physiology. For instance, fructose uptake by cells may be linked to oxidative stress, inflammation and autophagy (Zhang, Jiao *et al.* 2017). Other carbon sources, non-toxic to the cell and with better efficacy on *S. aureus* intracellular survival control, could be the focus of future research. Ribose is present in the 100 μ M range in human blood (Gross and Zollner 1991) and R5P concentration inside human lymphoblast is 70 μ M (Pilz, Willis *et al.* 1984). It is thus possible that the availability of ribose in the tissues may directly affects *S. aureus* pathogenesis by influencing bacterial growth. Our work highlighted an unprecedentedly reported role of transketolase in *S. aureus* intracellular persistence. Modulation of the pentose phosphate pathway activity may represent an interesting mean to impair *S. aureus* survival during chronic infections.

In the third article, we showed that during chronic infection of patients with the chronic lung disease (CLD) including cystic fibrosis (CF), *S. aureus* adapts through the acquisition of common adaptive traits including antibiotic resistances, auxotrophies, reduced citric acid cycle activity, increased biofilm, and intracellular persistence abilities that occurred irrespective of the clone type. Of particular interest, we report mutations in two master regulatory systems, Agr and Sae, likely to impact multiple protein expression and metabolite amounts.

The *agr*-defective mutants, such as CF1_late isolate, have been shown to arise during chronic infections and are better adapted to persistence within the infected host (Shopsin, Drlica-Wagner *et al.* 2008; Painter, Krishna *et al.* 2014; Suligoy, Lattar *et al.* 2018). Genetic alterations, that directly or indirectly target SaeR regulon, were identified in the 3 CF patients. SaeR is directly involved in the regulation of more than 20 virulence factor genes (Liu, Yeo *et al.* 2016) but is likely involved in the regulation of additional proteins by indirect mechanisms (Cassat, Hammer *et al.* 2013). For instance, Cassat *et al.* showed that 31 proteins, including SdrD, significantly increase in abundance upon inactivation of *sae* (Cassat, Hammer *et al.* 2013). Furthermore, SaeRS-deficient bacteria are less infective in animal models (Montgomery, Boyle-Vavra *et al.* 2010). In the 3 CF_late isolates, we observed an increased expression of the SdrD adhesin belonging to the SaeR regulon (Cassat, Hammer *et al.* 2013; Askarian, Ajayi *et al.* 2016), suggesting that SdrD could also be important for long-term lung colonization.

In patients with chronic lung infections, SCV detection is most often the consequence of a long-term SXT treatment (Kriegeskorte, Lore *et al.* 2015). Mutations in the *thyA* gene, as found in CF2 and CLD_late isolates, are known to lead to stable clinical SCVs that are no longer susceptible to SXT and are thymidine-auxotrophic (TA-SCV) (Chatterjee, Kriegeskorte *et al.* 2008; Kriegeskorte, Lore *et al.* 2015). Since thymidine is assumed to be abundant during lung inflammation, TA-SCVs can still grow in the inflamed alveolar environment.

Altogether, our data confirm that metabolic specialization is a common phenomenon among long-term colonizers (La Rosa, Johansen *et al.* 2018). Other striking traits of phenotypic convergent evolution of *S. aureus* identified in this work were the increased ability of “adapted” isolates: i) to form biofilm and ii) to persist in the intracellular niche. Our multi-omics approach allowed both confirmation of previously known mechanisms and identification of novel candidate genes and pathways involved in the persistence ability of clinical isolates. We now provide evidence that the *saeR/fakA* regulon and the pantothenate pathway could also be promising therapeutic targets to fight persistent *S. aureus* infections.

The study of *S. aureus* adaptation in the CF model allowed us to better understand the importance of *S. aureus* adaptation during a chronic infection. However, our first study of *S. aureus* adaptation in CF patients was limited to samples collected from three patients. So, we plan to increase the number of patients in our cohort study and include bacterial isolates collected from patients since their very early period of life (at-birth) to follow the evolution of *S. aureus* from their primo-implantation in the lungs of CF patients.

The characteristic gel-like property of the mucus of CF patients is believed to be mainly due to the presence of high molecular weight, polydisperse glycoproteins, or mucins. Besides, the thick mucus is also an ideal breeding ground for bacteria (Paranjape and Mogayzel 2014). How *S. aureus* interacts with the mucus components in CF patients is poorly understood.

Our laboratory has very recently initiated a collaboration with the team of C. Robbe (Institut Pasteur, Lille) who is expert on mucins. Preliminary observations of her group indicate an increased sialylation of mucins in CF airways both *in vivo* and *ex vivo*. Intriguingly, *S. aureus* USA300 strain appears to strongly adhere on mucins from CFTR^{-/-} piglets airways while binding was lost after chemical desialylation of mucins. At this stage, one may hypothesize that increased sialylation function could be a convergent adaption for *S. aureus* in CF patient. Further studies are underway to precise the role of sialylations on *S. aureus* persistence in the context of the CF disease.

Reference

- Abdelnour, A., S. Arvidson, T. Bremell, C. Ryden and A. Tarkowski (1993). "The accessory gene regulator (*agr*) controls *Staphylococcus aureus* virulence in a murine arthritis model." *Infect Immun* **61**(9): 3879-3885.
- Amato, S. M., C. H. Fazen, T. C. Henry, W. W. Mok, M. A. Orman, E. L. Sandvik, K. G. Volzing and M. P. Brynildsen (2014). "The role of metabolism in bacterial persistence." *Front Microbiol* **5**: 70.
- Andersen, D. H. (1938). "Cystic fibrosis of the pancreas and its relation to celiac disease: a clinical and pathologic study." *Am J Dis Child* **56**(2): 344-399.
- Anderson, M., P. J. Sansonetti and B. S. Marteyn (2016). "Shigella Diversity and Changing Landscape: Insights for the Twenty-First Century." *Front Cell Infect Microbiol* **6**: 45.
- Andersson, J. O. and S. G. Andersson (1999). "Insights into the evolutionary process of genome degradation." *Curr Opin Genet Dev* **9**(6): 664-671.
- Armstrong, D. S., K. Grimwood, J. B. Carlin, R. Carzino, J. P. Gutierrez, J. Hull, A. Olinsky, E. M. Phelan, C. F. Robertson and P. D. Phelan (1997). "Lower airway inflammation in infants and young children with cystic fibrosis." *Am J Respir Crit Care Med* **156**(4 Pt 1): 1197-1204.
- Askarian, F., C. Ajayi, A. M. Hanssen, N. M. van Sorge, I. Pettersen, D. B. Diep, J. U. Sollid and M. Johannessen (2016). "The interaction between *Staphylococcus aureus* SdrD and desmoglein 1 is important for adhesion to host cells." *Sci Rep* **6**: 22134.
- Assari, T. (2006). "Chronic Granulomatous Disease; fundamental stages in our understanding of CGD." *Med Immunol* **5**: 4.
- Atalla, H., B. Wilkie, C. Gyles, K. Leslie, L. Mutharia and B. Mallard (2010). "Antibody and cell-mediated immune responses to *Staphylococcus aureus* small colony variants and their parental strains associated with bovine mastitis." *Dev Comp Immunol* **34**(12): 1283-1290.
- Bakare, N., V. Rickerts, J. Bargon and G. Just-Nubling (2003). "Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis." *Mycoses* **46**(1-2): 19-23.
- Balasubramanian, D., E. A. Ohneck, J. Chapman, A. Weiss, M. K. Kim, T. Reyes-Robles, J. Zhong, L. N. Shaw, D. S. Lun, B. Ueberheide, B. Shopsin and V. J. Torres (2016). "Staphylococcus aureus Coordinates Leukocidin Expression and Pathogenesis by Sensing Metabolic Fluxes via RpiRc." *MBio* **7**(3).
- Bargon, J., N. Dauletbaev, B. Kohler, M. Wolf, H. G. Posselt and T. O. Wagner (1999). "Prophylactic antibiotic therapy is associated with an increased prevalence of *Aspergillus* colonization in adult cystic fibrosis patients." *Respir Med* **93**(11): 835-838.
- Bauckman, K. A., N. Owusu-Boaitey and I. U. Mysorekar (2015). "Selective autophagy: xenophagy." *Methods* **75**: 120-127.
- Baur, S., M. Rautenberg, M. Faulstich, T. Grau, Y. Severin, C. Unger, W. H. Hoffmann, T. Rudel, I. B. Autenrieth and C. Weidenmaier (2014). "A nasal epithelial receptor for *Staphylococcus aureus* WTA governs adhesion to epithelial cells and modulates nasal colonization." *PLoS Pathog* **10**(5): e1004089.
- Bayer, A. S., P. McNamara, M. R. Yeaman, N. Lucindo, T. Jones, A. L. Cheung, H. G. Sahl and R. A. Proctor (2006). "Transposon disruption of the complex I NADH oxidoreductase gene (*snoD*) in *Staphylococcus aureus* is associated with reduced susceptibility to the microbicidal activity of thrombin-induced platelet microbicidal protein 1." *J Bacteriol* **188**(1): 211-222.
- Becker, D., M. Selbach, C. Rollenhagen, M. Ballmaier, T. F. Meyer, M. Mann and D. Bumann (2006). "Robust *Salmonella* metabolism limits possibilities for new antimicrobials." *Nature* **440**(7082): 303-307.

Bera, A., S. Herbert, A. Jakob, W. Vollmer and F. Gotz (2005). "Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*." *Mol Microbiol* **55**(3): 778-787.

Berglund, C. and B. Soderquist (2008). "The origin of a methicillin-resistant *Staphylococcus aureus* isolate at a neonatal ward in Sweden-possible horizontal transfer of a staphylococcal cassette chromosome mec between methicillin-resistant *Staphylococcus haemolyticus* and *Staphylococcus aureus*." *Clin Microbiol Infect* **14**(11): 1048-1056.

Bergsbaken, T., S. L. Fink and B. T. Cookson (2009). "Pyroptosis: host cell death and inflammation." *Nat Rev Microbiol* **7**(2): 99-109.

Berube, B. J. and J. Bubeck Wardenburg (2013). "Staphylococcus aureus alpha-toxin: nearly a century of intrigue." *Toxins (Basel)* **5**(6): 1140-1166.

Besier, S., J. Zander, B. C. Kahl, P. Kraiczky, V. Brade and T. A. Wichelhaus (2008). "The thymidine-dependent small-colony-variant phenotype is associated with hypermutability and antibiotic resistance in clinical *Staphylococcus aureus* isolates." *Antimicrob Agents Chemother* **52**(6): 2183-2189.

Bigger, J. (1944). "TREATMENT OF STAPHYLOCOCCAL INFECTIONS WITH PENICILLIN BY INTERMITTENT STERILISATION." *The Lancet* **244**(6320): 497-500.

Bischoff, M., J. M. Entenza and P. Giachino (2001). "Influence of a functional sigB operon on the global regulators sar and agr in *Staphylococcus aureus*." *J Bacteriol* **183**(17): 5171-5179.

Blattner, S., S. Das, K. Paprotka, U. Eilers, M. Krischke, D. Kretschmer, C. W. Remmele, M. Dittrich, T. Muller, C. Schuelein-Voelk, T. Hertlein, M. J. Mueller, B. Huettel, R. Reinhardt, K. Ohlsen, T. Rudel and M. J. Fraunholz (2016). "Staphylococcus aureus Exploits a Non-ribosomal Cyclic Dipeptide to Modulate Survival within Epithelial Cells and Phagocytes." *PLoS Pathog* **12**(9): e1005857.

Bloemendaal, A. L., E. C. Brouwer and A. C. Fluit (2010). "Methicillin resistance transfer from *Staphylococcus epidermidis* to methicillin-susceptible *Staphylococcus aureus* in a patient during antibiotic therapy." *PLoS One* **5**(7): e11841.

Boles, B. R. and A. R. Horswill (2008). "Agr-mediated dispersal of *Staphylococcus aureus* biofilms." *PLoS Pathog* **4**(4): e1000052.

Boucher, H. W. and G. R. Corey (2008). "Epidemiology of methicillin-resistant *Staphylococcus aureus*." *Clin Infect Dis* **46 Suppl 5**: S344-349.

Branger, C., C. Gardye and N. Lambert-Zechovsky (1996). "Persistence of *Staphylococcus aureus* strains among cystic fibrosis patients over extended periods of time." *J Med Microbiol* **45**(4): 294-301.

Broms, J. E., A. Sjostedt and M. Lavander (2010). "The Role of the Francisella Tularensis Pathogenicity Island in Type VI Secretion, Intracellular Survival, and Modulation of Host Cell Signaling." *Front Microbiol* **1**: 136.

Bronner, S., H. Monteil and G. Prevost (2004). "Regulation of virulence determinants in *Staphylococcus aureus*: complexity and applications." *FEMS Microbiol Rev* **28**(2): 183-200.

Bubeck Wardenburg, J., R. J. Patel and O. Schneewind (2007). "Surface proteins and exotoxins are required for the pathogenesis of *Staphylococcus aureus* pneumonia." *Infect Immun* **75**(2): 1040-1044.

Burns, J. L., J. Emerson, J. R. Stapp, D. L. Yim, J. Krzewinski, L. Louden, B. W. Ramsey and C. R. Clausen (1998). "Microbiology of sputum from patients at cystic fibrosis centers in the United States." *Clin Infect Dis* **27**(1): 158-163.

Canton, J., R. Khezri, M. Glogauer and S. Grinstein (2014). "Contrasting phagosome pH regulation and maturation in human M1 and M2 macrophages." *Mol Biol Cell* **25**(21): 3330-3341.

Casadevall, A. (2008). "Evolution of intracellular pathogens." *Annu Rev Microbiol* **62**: 19-33.

Cassat, J. E., N. D. Hammer, J. P. Campbell, M. A. Benson, D. S. Perrien, L. N. Mrak, M. S. Smeltzer, V. J. Torres and E. P. Skaar (2013). "A secreted bacterial protease tailors the *Staphylococcus aureus* virulence repertoire to modulate bone remodeling during osteomyelitis." Cell Host Microbe **13**(6): 759-772.

Cassat, J. E. and E. P. Skaar (2013). "Iron in infection and immunity." Cell Host Microbe **13**(5): 509-519.

Cazalet, C., C. Rusniok, H. Bruggemann, N. Zidane, A. Magnier, L. Ma, M. Tichit, S. Jarraud, C. Bouchier, F. Vandenesch, F. Kunst, J. Etienne, P. Glaser and C. Buchrieser (2004). "Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity." Nat Genet **36**(11): 1165-1173.

Celli, J. (2015). "The changing nature of the *Brucella*-containing vacuole." Cell Microbiol **17**(7): 951-958.

Celli, J. and T. C. Zahrt (2013). "Mechanisms of *Francisella tularensis* intracellular pathogenesis." Cold Spring Harb Perspect Med **3**(4): a010314.

Chang, S., D. M. Sievert, J. C. Hageman, M. L. Boulton, F. C. Tenover, F. P. Downes, S. Shah, J. T. Rudrik, G. R. Pupp, W. J. Brown, D. Cardo, S. K. Fridkin and T. Vancomycin-Resistant *Staphylococcus aureus* Investigative (2003). "Infection with vancomycin-resistant *Staphylococcus aureus* containing the *vanA* resistance gene." N Engl J Med **348**(14): 1342-1347.

Chatterjee, I., M. Herrmann, R. A. Proctor, G. Peters and B. C. Kahl (2007). "Enhanced post-stationary-phase survival of a clinical thymidine-dependent small-colony variant of *Staphylococcus aureus* results from lack of a functional tricarboxylic acid cycle." J Bacteriol **189**(7): 2936-2940.

Chatterjee, I., A. Kriegeskorte, A. Fischer, S. Deiwick, N. Theimann, R. A. Proctor, G. Peters, M. Herrmann and B. C. Kahl (2008). "In vivo mutations of thymidylate synthase (encoded by *thyA*) are responsible for thymidine dependency in clinical small-colony variants of *Staphylococcus aureus*." J Bacteriol **190**(3): 834-842.

Chatterjee, S. S., H. Hossain, S. Otten, C. Kuenne, K. Kuchmina, S. Machata, E. Domann, T. Chakraborty and T. Hain (2006). "Intracellular gene expression profile of *Listeria monocytogenes*." Infect Immun **74**(2): 1323-1338.

Chatterjee, S. S., H. S. Joo, A. C. Duong, T. D. Dieringer, V. Y. Tan, Y. Song, E. R. Fischer, G. Y. Cheung, M. Li and M. Otto (2013). "Essential *Staphylococcus aureus* toxin export system." Nat Med **19**(3): 364-367.

Chen, X. and F. Alonzo, 3rd (2019). "Bacterial lipolysis of immune-activating ligands promotes evasion of innate defenses." Proc Natl Acad Sci U S A **116**(9): 3764-3773.

Cheung, A. L., K. J. Eberhardt, E. Chung, M. R. Yeaman, P. M. Sullam, M. Ramos and A. S. Bayer (1994). "Diminished virulence of a *sar*-/*agr*- mutant of *Staphylococcus aureus* in the rabbit model of endocarditis." J Clin Invest **94**(5): 1815-1822.

Cheung, G. Y., H. S. Joo, S. S. Chatterjee and M. Otto (2014). "Phenol-soluble modulins--critical determinants of staphylococcal virulence." FEMS Microbiol Rev **38**(4): 698-719.

Cheung, G. Y., R. Wang, B. A. Khan, D. E. Sturdevant and M. Otto (2011). "Role of the accessory gene regulator *agr* in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis." Infect Immun **79**(5): 1927-1935.

Cheung, J. C. and C. M. Deber (2008). "Misfolding of the cystic fibrosis transmembrane conductance regulator and disease." Biochemistry **47**(6): 1465-1473.

Chi, C. Y., C. C. Lin, I. C. Liao, Y. C. Yao, F. C. Shen, C. C. Liu and C. F. Lin (2014). "Panton-Valentine leukocidin facilitates the escape of *Staphylococcus aureus* from human keratinocyte endosomes and induces apoptosis." J Infect Dis **209**(2): 224-235.

Clauditz, A., A. Resch, K. P. Wieland, A. Peschel and F. Gotz (2006). "Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress." Infect Immun **74**(8): 4950-4953.

Clemens, D. L., B. Y. Lee and M. A. Horwitz (2005). "Francisella tularensis enters macrophages via a novel process involving pseudopod loops." Infect Immun **73**(9): 5892-5902.

Clemens, D. L., B. Y. Lee and M. A. Horwitz (2018). "The Francisella Type VI Secretion System." Front Cell Infect Microbiol **8**: 121.

Cohen, T. S. and A. Prince (2012). "Cystic fibrosis: a mucosal immunodeficiency syndrome." Nat Med **18**(4): 509-519.

Cooper, V. S. and R. E. Lenski (2000). "The population genetics of ecological specialization in evolving *Escherichia coli* populations." Nature **407**(6805): 736-739.

Cosgrove, K., G. Coutts, I. M. Jonsson, A. Tarkowski, J. F. Kokai-Kun, J. J. Mond and S. J. Foster (2007). "Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*." J Bacteriol **189**(3): 1025-1035.

Cossart, P. and C. R. Roy (2010). "Manipulation of host membrane machinery by bacterial pathogens." Curr Opin Cell Biol **22**(4): 547-554.

Craven, R. R., X. Gao, I. C. Allen, D. Gris, J. Bubeck Wardenburg, E. McElvania-Tekippe, J. P. Ting and J. A. Duncan (2009). "Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells." PLoS One **4**(10): e7446.

Cui, L., H. M. Neoh, A. Iwamoto and K. Hiramatsu (2012). "Coordinated phenotype switching with large-scale chromosome flip-flop inversion observed in bacteria." Proc Natl Acad Sci U S A **109**(25): E1647-1656.

Daly, S. M., B. O. Elmore, J. S. Kavanaugh, K. D. Triplett, M. Figueroa, H. A. Raja, T. El-Elimat, H. A. Crosby, J. K. Femling, N. B. Cech, A. R. Horswill, N. H. Oberlies and P. R. Hall (2015). "omega-Hydroxyemodin limits staphylococcus aureus quorum sensing-mediated pathogenesis and inflammation." Antimicrob Agents Chemother **59**(4): 2223-2235.

Dandekar, T., A. Fieselmann, E. Fischer, J. Popp, M. Hensel and J. Noster (2014). "Salmonella-how a metabolic generalist adopts an intracellular lifestyle during infection." Front Cell Infect Microbiol **4**: 191.

DeLeo, F. R. and H. F. Chambers (2009). "Reemergence of antibiotic-resistant *Staphylococcus aureus* in the genomics era." J Clin Invest **119**(9): 2464-2474.

DeLeo, F. R., M. Otto, B. N. Kreiswirth and H. F. Chambers (2010). "Community-associated methicillin-resistant *Staphylococcus aureus*." Lancet **375**(9725): 1557-1568.

Denamur, E. and I. Matic (2006). "Evolution of mutation rates in bacteria." Mol Microbiol **60**(4): 820-827.

Derichs, N. (2013). "Targeting a genetic defect: cystic fibrosis transmembrane conductance regulator modulators in cystic fibrosis." Eur Respir Rev **22**(127): 58-65.

Desjardins, M., L. A. Huber, R. G. Parton and G. Griffiths (1994). "Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus." J Cell Biol **124**(5): 677-688.

Deutscher, J., C. Francke and P. W. Postma (2006). "How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria." Microbiol Mol Biol Rev **70**(4): 939-1031.

Deutscher, J., E. Kuster, U. Bergstedt, V. Charrier and W. Hillen (1995). "Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in gram-positive bacteria." Mol Microbiol **15**(6): 1049-1053.

Diep, B. A., S. R. Gill, R. F. Chang, T. H. Phan, J. H. Chen, M. G. Davidson, F. Lin, J. Lin, H. A. Carleton, E. F. Mongodin, G. F. Sensabaugh and F. Perdreau-Remington (2006). "Complete

genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*." Lancet **367**(9512): 731-739.

Ding, Y., X. Liu, F. Chen, H. Di, B. Xu, L. Zhou, X. Deng, M. Wu, C. G. Yang and L. Lan (2014). "Metabolic sensor governing bacterial virulence in *Staphylococcus aureus*." Proc Natl Acad Sci U S A **111**(46): E4981-4990.

Dinges, M. M., P. M. Orwin and P. M. Schlievert (2000). "Exotoxins of *Staphylococcus aureus*." Clin Microbiol Rev **13**(1): 16-34, table of contents.

Dziewanowska, K., J. M. Patti, C. F. Deobald, K. W. Bayles, W. R. Trumble and G. A. Bohach (1999). "Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells." Infect Immun **67**(9): 4673-4678.

Edwards, A. M. (2012). "Phenotype switching is a natural consequence of *Staphylococcus aureus* replication." J Bacteriol **194**(19): 5404-5412.

Edwards, A. M., U. Potter, N. A. Meenan, J. R. Potts and R. C. Massey (2011). "*Staphylococcus aureus* keratinocyte invasion is dependent upon multiple high-affinity fibronectin-binding repeats within FnBPA." PLoS One **6**(4): e18899.

Eisenreich, W., T. Dandekar, J. Heesemann and W. Goebel (2010). "Carbon metabolism of intracellular bacterial pathogens and possible links to virulence." Nat Rev Microbiol **8**(6): 401-412.

Eisenreich, W., J. Heesemann, T. Rudel and W. Goebel (2015). "Metabolic Adaptations of Intracellular Bacterial Pathogens and their Mammalian Host Cells during Infection ("Pathometabolism")." Microbiol Spectr **3**(3).

Eriksson, S., S. Lucchini, A. Thompson, M. Rhen and J. C. Hinton (2003). "Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*." Mol Microbiol **47**(1): 103-118.

Essmann, F., H. Bantel, G. Totzke, I. H. Engels, B. Sinha, K. Schulze-Osthoff and R. U. Janicke (2003). "*Staphylococcus aureus* alpha-toxin-induced cell death: predominant necrosis despite apoptotic caspase activation." Cell Death Differ **10**(11): 1260-1272.

Fabrega, A. and J. Vila (2013). "*Salmonella enterica* serovar Typhimurium skills to succeed in the host: virulence and regulation." Clin Microbiol Rev **26**(2): 308-341.

Fang, F. C., S. J. Libby, M. E. Castor and A. M. Fung (2005). "Isocitrate lyase (AceA) is required for *Salmonella* persistence but not for acute lethal infection in mice." Infect Immun **73**(4): 2547-2549.

Farrell, P. M. (2008). "The prevalence of cystic fibrosis in the European Union." J Cyst Fibros **7**(5): 450-453.

Felden, B., F. Vandenesch, P. Bouloc and P. Romby (2011). "The *Staphylococcus aureus* RNome and its commitment to virulence." PLoS Pathog **7**(3): e1002006.

Fernandez-Prada, C. M., D. L. Hoover, B. D. Tall, A. B. Hartman, J. Kopelowitz and M. M. Venkatesan (2000). "*Shigella flexneri* IpaH(7.8) facilitates escape of virulent bacteria from the endocytic vacuoles of mouse and human macrophages." Infect Immun **68**(6): 3608-3619.

Finlay, B. B. and S. Falkow (1988). "Comparison of the invasion strategies used by *Salmonella cholerae-suis*, *Shigella flexneri* and *Yersinia enterocolitica* to enter cultured animal cells: endosome acidification is not required for bacterial invasion or intracellular replication." Biochimie **70**(8): 1089-1099.

Flanagan, R. S., B. Heit and D. E. Heinrichs (2015). "Antimicrobial Mechanisms of Macrophages and the Immune Evasion Strategies of *Staphylococcus aureus*." Pathogens **4**(4): 826-868.

Flanagan, R. S., B. Heit and D. E. Heinrichs (2016). "Intracellular replication of *Staphylococcus aureus* in mature phagolysosomes in macrophages precedes host cell death, and bacterial escape and dissemination." Cell Microbiol **18**(4): 514-535.

Flannagan, R. S., R. C. Kuiack, M. J. McGavin and D. E. Heinrichs (2018). "Staphylococcus aureus Uses the GraXRS Regulatory System To Sense and Adapt to the Acidified Phagolysosome in Macrophages." *MBio* **9**(4).

Folkesson, A., L. Jelsbak, L. Yang, H. K. Johansen, O. Ciofu, N. Hoiby and S. Molin (2012). "Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective." *Nat Rev Microbiol* **10**(12): 841-851.

Foster, T. J., J. A. Geoghegan, V. K. Ganesh and M. Hook (2014). "Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*." *Nat Rev Microbiol* **12**(1): 49-62.

Foster, T. J. and M. Hook (1998). "Surface protein adhesins of *Staphylococcus aureus*." *Trends Microbiol* **6**(12): 484-488.

Foundation, C. F. (2017). "2017 Patient Registry Annual Data Report." from <https://www.cff.org/Research/Researcher-Resources/Patient-Registry/2017-Patient-Registry-Annual-Data-Report.pdf>.

Fowler, V. G., Jr., G. Sakoulas, L. M. McIntyre, V. G. Meka, R. D. Arbeit, C. H. Cabell, M. E. Stryjewski, G. M. Eliopoulos, L. B. Reller, G. R. Corey, T. Jones, N. Lucindo, M. R. Yeaman and A. S. Bayer (2004). "Persistent bacteremia due to methicillin-resistant *Staphylococcus aureus* infection is associated with agr dysfunction and low-level in vitro resistance to thrombin-induced platelet microbicidal protein." *J Infect Dis* **190**(6): 1140-1149.

Franchi, L., A. Amer, M. Body-Malapel, T. D. Kanneganti, N. Ozoren, R. Jagirdar, N. Inohara, P. Vandenabeele, J. Bertin, A. Coyle, E. P. Grant and G. Nunez (2006). "Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages." *Nat Immunol* **7**(6): 576-582.

Friedman, D. B., D. L. Stauff, G. Pishchany, C. W. Whitwell, V. J. Torres and E. P. Skaar (2006). "*Staphylococcus aureus* redirects central metabolism to increase iron availability." *PLoS Pathog* **2**(8): e87.

Fuchs, T. M., W. Eisenreich, J. Heesemann and W. Goebel (2012). "Metabolic adaptation of human pathogenic and related nonpathogenic bacteria to extra- and intracellular habitats." *FEMS Microbiol Rev* **36**(2): 435-462.

Fuchs, T. M., W. Eisenreich, T. Kern and T. Dandekar (2012). "Toward a Systemic Understanding of *Listeria monocytogenes* Metabolism during Infection." *Front Microbiol* **3**: 23.

Ganesh, V. K., E. M. Barbu, C. C. Deivanayagam, B. Le, A. S. Anderson, Y. V. Matsuka, S. L. Lin, T. J. Foster, S. V. Narayana and M. Hook (2011). "Structural and biochemical characterization of *Staphylococcus aureus* clumping factor B/ligand interactions." *J Biol Chem* **286**(29): 25963-25972.

Ganz, T. (2003). "Defensins: antimicrobial peptides of innate immunity." *Nat Rev Immunol* **3**(9): 710-720.

Garzoni, C., P. Francois, A. Huyghe, S. Couzinet, C. Tapparel, Y. Charbonnier, A. Renzoni, S. Lucchini, D. P. Lew, P. Vaudaux, W. L. Kelley and J. Schrenzel (2007). "A global view of *Staphylococcus aureus* whole genome expression upon internalization in human epithelial cells." *BMC Genomics* **8**: 171.

Garzoni, C. and W. L. Kelley (2009). "*Staphylococcus aureus*: new evidence for intracellular persistence." *Trends Microbiol* **17**(2): 59-65.

Gaupp, R., J. Wirf, B. Wonnemberg, T. Biegel, J. Eisenbeis, J. Graham, M. Herrmann, C. Y. Lee, C. Beisswenger, C. Wolz, T. Tschernig, M. Bischoff and G. A. Somerville (2016). "RpiRc Is a Pleiotropic Effector of Virulence Determinant Synthesis and Attenuates Pathogenicity in *Staphylococcus aureus*." *Infect Immun* **84**(7): 2031-2041.

Gautam, V., L. Singhal and P. Ray (2011). "Burkholderia cepacia complex: beyond pseudomonas and acinetobacter." *Indian J Med Microbiol* **29**(1): 4-12.

Genestier, A. L., M. C. Michallet, G. Prevost, G. Bellot, L. Chalabreysse, S. Peyrol, F. Thivolet, J. Etienne, G. Lina, F. M. Vallette, F. Vandenesch and L. Genestier (2005). "Staphylococcus aureus Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils." J Clin Invest **115**(11): 3117-3127.

Gertz, S., S. Engelmann, R. Schmid, A. K. Ziebandt, K. Tischer, C. Scharf, J. Hacker and M. Hecker (2000). "Characterization of the sigma(B) regulon in Staphylococcus aureus." J Bacteriol **182**(24): 6983-6991.

Gibson, R. L., J. L. Burns and B. W. Ramsey (2003). "Pathophysiology and management of pulmonary infections in cystic fibrosis." Am J Respir Crit Care Med **168**(8): 918-951.

Giese, B., F. Glowinski, K. Paprotka, S. Dittmann, T. Steiner, B. Sinha and M. J. Fraunholz (2011). "Expression of delta-toxin by Staphylococcus aureus mediates escape from phagosomes of human epithelial and endothelial cells in the presence of beta-toxin." Cell Microbiol **13**(2): 316-329.

Goerke, C., U. Fluckiger, A. Steinhuber, V. Bisanzio, M. Ulrich, M. Bischoff, J. M. Patti and C. Wolz (2005). "Role of Staphylococcus aureus global regulators sae and sigmaB in virulence gene expression during device-related infection." Infect Immun **73**(6): 3415-3421.

Goerke, C., S. Matias y Papenberg, S. Dasbach, K. Dietz, R. Ziebach, B. C. Kahl and C. Wolz (2004). "Increased frequency of genomic alterations in Staphylococcus aureus during chronic infection is in part due to phage mobilization." J Infect Dis **189**(4): 724-734.

Goetz, M., A. Bubert, G. Wang, I. Chico-Calero, J. A. Vazquez-Boland, M. Beck, J. Slaghuis, A. A. Szalay and W. Goebel (2001). "Microinjection and growth of bacteria in the cytosol of mammalian host cells." Proc Natl Acad Sci U S A **98**(21): 12221-12226.

Goss, C. H., K. Otto, M. L. Aitken and G. D. Rubenfeld (2002). "Detecting Stenotrophomonas maltophilia does not reduce survival of patients with cystic fibrosis." Am J Respir Crit Care Med **166**(3): 356-361.

Gotz, A., E. Eylert, W. Eisenreich and W. Goebel (2010). "Carbon metabolism of enterobacterial human pathogens growing in epithelial colorectal adenocarcinoma (Caco-2) cells." PLoS One **5**(5): e10586.

Gould, I. M., M. Z. David, S. Esposito, J. Garau, G. Lina, T. Mazzei and G. Peters (2012). "New insights into meticillin-resistant Staphylococcus aureus (MRSA) pathogenesis, treatment and resistance." Int J Antimicrob Agents **39**(2): 96-104.

Graczyk, J. P., C. J. Harvey, I. Laczovich and F. Alonzo, 3rd (2017). "A Lipoylated Metabolic Protein Released by Staphylococcus aureus Suppresses Macrophage Activation." Cell Host Microbe **22**(5): 678-687 e679.

Gross, M. and N. Zollner (1991). "Serum levels of glucose, insulin, and C-peptide during long-term D-ribose administration in man." Klin Wochenschr **69**(1): 31-36.

Grosz, M., J. Kolter, K. Paprotka, A. C. Winkler, D. Schafer, S. S. Chatterjee, T. Geiger, C. Wolz, K. Ohlsen, M. Otto, T. Rudel, B. Sinha and M. Fraunholz (2014). "Cytoplasmic replication of Staphylococcus aureus upon phagosomal escape triggered by phenol-soluble modulins alpha." Cell Microbiol **16**(4): 451-465.

Grubmüller, S., K. Schauer, W. Goebel, T. M. Fuchs and W. Eisenreich (2014). "Analysis of carbon substrates used by Listeria monocytogenes during growth in J774A.1 macrophages suggests a bipartite intracellular metabolism." Front Cell Infect Microbiol **4**: 156.

Grundmeier, M., L. Tuchscher, M. Bruck, D. Viemann, J. Roth, E. Willscher, K. Becker, G. Peters and B. Löffler (2010). "Staphylococcal strains vary greatly in their ability to induce an inflammatory response in endothelial cells." J Infect Dis **201**(6): 871-880.

Guani-Guerra, E., T. Santos-Mendoza, S. O. Lugo-Reyes and L. M. Teran (2010). "Antimicrobial peptides: general overview and clinical implications in human health and disease." Clin Immunol **135**(1): 1-11.

Hamosh, A., S. C. FitzSimmons, M. Macek, Jr., M. R. Knowles, B. J. Rosenstein and G. R. Cutting (1998). "Comparison of the clinical manifestations of cystic fibrosis in black and white patients." *J Pediatr* **132**(2): 255-259.

Hanssen, A. M., B. Kindlund, N. C. Stenklev, A. S. Furberg, S. Fismen, R. S. Olsen, M. Johannessen and J. U. Sollid (2017). "Localization of Staphylococcus aureus in tissue from the nasal vestibule in healthy carriers." *BMC Microbiol* **17**(1): 89.

Harraghy, N., M. Hussain, A. Hagggar, T. Chavakis, B. Sinha, M. Herrmann and J. I. Flock (2003). "The adhesive and immunomodulating properties of the multifunctional Staphylococcus aureus protein Eap." *Microbiology* **149**(Pt 10): 2701-2707.

Haslinger-Loffler, B., B. C. Kahl, M. Grundmeier, K. Strangfeld, B. Wagner, U. Fischer, A. L. Cheung, G. Peters, K. Schulze-Osthoff and B. Sinha (2005). "Multiple virulence factors are required for Staphylococcus aureus-induced apoptosis in endothelial cells." *Cell Microbiol* **7**(8): 1087-1097.

Haslinger, B., K. Strangfeld, G. Peters, K. Schulze-Osthoff and B. Sinha (2003). "Staphylococcus aureus alpha-toxin induces apoptosis in peripheral blood mononuclear cells: role of endogenous tumour necrosis factor-alpha and the mitochondrial death pathway." *Cell Microbiol* **5**(10): 729-741.

Haupt, K., M. Reuter, J. van den Elsen, J. Burman, S. Halbich, J. Richter, C. Skerka and P. F. Zipfel (2008). "The Staphylococcus aureus protein Sbi acts as a complement inhibitor and forms a tripartite complex with host complement Factor H and C3b." *PLoS Pathog* **4**(12): e1000250.

Hautefort, I., A. Thompson, S. Eriksson-Ygberg, M. L. Parker, S. Lucchini, V. Danino, R. J. Bongaerts, N. Ahmad, M. Rhen and J. C. Hinton (2008). "During infection of epithelial cells Salmonella enterica serovar Typhimurium undergoes a time-dependent transcriptional adaptation that results in simultaneous expression of three type 3 secretion systems." *Cell Microbiol* **10**(4): 958-984.

Hayes, S. M., R. Howlin, D. A. Johnston, J. S. Webb, S. C. Clarke, P. Stoodley, P. G. Harries, S. J. Wilson, S. L. Pender, S. N. Faust, L. Hall-Stoodley and R. J. Salib (2015). "Intracellular residency of Staphylococcus aureus within mast cells in nasal polyps: A novel observation." *J Allergy Clin Immunol* **135**(6): 1648-1651.

He, C. and D. J. Klionsky (2009). "Regulation mechanisms and signaling pathways of autophagy." *Annu Rev Genet* **43**: 67-93.

Helaine, S. and E. Kugelberg (2014). "Bacterial persisters: formation, eradication, and experimental systems." *Trends Microbiol* **22**(7): 417-424.

Herold, B. C., L. C. Immergluck, M. C. Maranan, D. S. Lauderdale, R. E. Gaskin, S. Boyle-Vavra, C. D. Leitch and R. S. Daum (1998). "Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk." *JAMA* **279**(8): 593-598.

Hirschhausen, N., T. Schlesier, M. A. Schmidt, F. Gotz, G. Peters and C. Heilmann (2010). "A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor." *Cell Microbiol* **12**(12): 1746-1764.

Hood, M. I. and E. P. Skaar (2012). "Nutritional immunity: transition metals at the pathogen-host interface." *Nat Rev Microbiol* **10**(8): 525-537.

Hybiske, K. and R. S. Stephens (2008). "Exit strategies of intracellular pathogens." *Nat Rev Microbiol* **6**(2): 99-110.

Jabado, N., A. Jankowski, S. Dougaparsad, V. Picard, S. Grinstein and P. Gros (2000). "Natural resistance to intracellular infections: natural resistance-associated macrophage protein 1 (Nramp1) functions as a pH-dependent manganese transporter at the phagosomal membrane." *J Exp Med* **192**(9): 1237-1248.

Jarry, T. M. and A. L. Cheung (2006). "Staphylococcus aureus escapes more efficiently from the phagosome of a cystic fibrosis bronchial epithelial cell line than from its normal counterpart." *Infect Immun* **74**(5): 2568-2577.

Jarry, T. M., G. Memmi and A. L. Cheung (2008). "The expression of alpha-haemolysin is required for Staphylococcus aureus phagosomal escape after internalization in CFT-1 cells." *Cell Microbiol* **10**(9): 1801-1814.

Jevon, M., C. Guo, B. Ma, N. Mordan, S. P. Nair, M. Harris, B. Henderson, G. Bentley and S. Meghji (1999). "Mechanisms of internalization of Staphylococcus aureus by cultured human osteoblasts." *Infect Immun* **67**(5): 2677-2681.

Joo, H. S. and M. Otto (2015). "Mechanisms of resistance to antimicrobial peptides in staphylococci." *Biochim Biophys Acta* **1848**(11 Pt B): 3055-3061.

Joseph, B., S. Mertins, R. Stoll, J. Schar, K. R. Umeha, Q. Luo, S. Muller-Altrock and W. Goebel (2008). "Glycerol metabolism and PrfA activity in Listeria monocytogenes." *J Bacteriol* **190**(15): 5412-5430.

Joseph, B., K. Przybilla, C. Stuhler, K. Schauer, J. Slaghuis, T. M. Fuchs and W. Goebel (2006). "Identification of Listeria monocytogenes genes contributing to intracellular replication by expression profiling and mutant screening." *J Bacteriol* **188**(2): 556-568.

Jubrail, J., P. Morris, M. A. Bewley, S. Stoneham, S. A. Johnston, S. J. Foster, A. A. Peden, R. C. Read, H. M. Marriott and D. H. Dockrell (2016). "Inability to sustain intraphagosomal killing of Staphylococcus aureus predisposes to bacterial persistence in macrophages." *Cell Microbiol* **18**(1): 80-96.

Kahl, B., M. Herrmann, A. S. Everding, H. G. Koch, K. Becker, E. Harms, R. A. Proctor and G. Peters (1998). "Persistent infection with small colony variant strains of Staphylococcus aureus in patients with cystic fibrosis." *J Infect Dis* **177**(4): 1023-1029.

Kahl, B. C. (2010). "Impact of Staphylococcus aureus on the pathogenesis of chronic cystic fibrosis lung disease." *Int J Med Microbiol* **300**(8): 514-519.

Kahl, B. C., K. Becker and B. Löffler (2016). "Clinical Significance and Pathogenesis of Staphylococcal Small Colony Variants in Persistent Infections." *Clin Microbiol Rev* **29**(2): 401-427.

Kahl, B. C., A. Duebbers, G. Lubritz, J. Haeberle, H. G. Koch, B. Ritzerfeld, M. Reilly, E. Harms, R. A. Proctor, M. Herrmann and G. Peters (2003). "Population dynamics of persistent Staphylococcus aureus isolated from the airways of cystic fibrosis patients during a 6-year prospective study." *J Clin Microbiol* **41**(9): 4424-4427.

Kahl, B. C., M. Goulian, W. van Wamel, M. Herrmann, S. M. Simon, G. Kaplan, G. Peters and A. L. Cheung (2000). "Staphylococcus aureus RN6390 replicates and induces apoptosis in a pulmonary epithelial cell line." *Infect Immun* **68**(9): 5385-5392.

Kahl, B. C., A. Mellmann, S. Deiwick, G. Peters and D. Harmsen (2005). "Variation of the polymorphic region X of the protein A gene during persistent airway infection of cystic fibrosis patients reflects two independent mechanisms of genetic change in Staphylococcus aureus." *J Clin Microbiol* **43**(1): 502-505.

Kalinka, J., M. Hachmeister, J. Geraci, D. Sordelli, U. Hansen, S. Niemann, S. Oetermann, G. Peters, B. Löffler and L. Tuchscher (2014). "Staphylococcus aureus isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation." *Int J Med Microbiol* **304**(8): 1038-1049.

Kane, T. L., K. E. Carothers and S. W. Lee (2018). "Virulence Factor Targeting of the Bacterial Pathogen Staphylococcus aureus for Vaccine and Therapeutics." *Curr Drug Targets* **19**(2): 111-127.

Kang, C. K., J. E. Cho, Y. J. Choi, Y. Jung, N. H. Kim, C. J. Kim, T. S. Kim, K. H. Song, P. G. Choe, W. B. Park, J. H. Bang, E. S. Kim, K. U. Park, S. W. Park, N. J. Kim, M. D. Oh and H. B. Kim (2015). "agr dysfunction affects staphylococcal cassette chromosome mec type-

dependent clinical outcomes in methicillin-resistant *Staphylococcus aureus* bacteremia." Antimicrob Agents Chemother **59**(6): 3125-3132.

Karavolos, M. H., M. J. Horsburgh, E. Ingham and S. J. Foster (2003). "Role and regulation of the superoxide dismutases of *Staphylococcus aureus*." Microbiology **149**(Pt 10): 2749-2758.

Katayama, Y., T. Baba, M. Sekine, M. Fukuda and K. Hiramatsu (2013). "Beta-hemolysin promotes skin colonization by *Staphylococcus aureus*." J Bacteriol **195**(6): 1194-1203.

Keeney, K., L. Colosi, W. Weber and M. O'Riordan (2009). "Generation of branched-chain fatty acids through lipoate-dependent metabolism facilitates intracellular growth of *Listeria monocytogenes*." J Bacteriol **191**(7): 2187-2196.

Kentner, D., G. Martano, M. Callon, P. Chiquet, M. Brodmann, O. Burton, A. Wahlander, P. Nanni, N. Delmotte, J. Grossmann, J. Limenitakis, R. Schlapbach, P. Kiefer, J. A. Vorholt, S. Hiller and D. Bumann (2014). "Shigella reroutes host cell central metabolism to obtain high-flux nutrient supply for vigorous intracellular growth." Proc Natl Acad Sci U S A **111**(27): 9929-9934.

Killackey, S. A., M. T. Sorbara and S. E. Girardin (2016). "Cellular Aspects of *Shigella* Pathogenesis: Focus on the Manipulation of Host Cell Processes." Front Cell Infect Microbiol **6**: 38.

Kirby, W. M. (1944). "Extraction of a Highly Potent Penicillin Inactivator from Penicillin Resistant *Staphylococci*." Science **99**(2579): 452-453.

Kohler, C., C. von Eiff, G. Peters, R. A. Proctor, M. Hecker and S. Engelmann (2003). "Physiological characterization of a heme-deficient mutant of *Staphylococcus aureus* by a proteomic approach." J Bacteriol **185**(23): 6928-6937.

Kriegeskorte, A., S. Konig, G. Sander, A. Pirkl, E. Mahabir, R. A. Proctor, C. von Eiff, G. Peters and K. Becker (2011). "Small colony variants of *Staphylococcus aureus* reveal distinct protein profiles." Proteomics **11**(12): 2476-2490.

Kriegeskorte, A., N. I. Lore, A. Bragonzi, C. Riva, M. Kelkenberg, K. Becker, R. A. Proctor, G. Peters and B. C. Kahl (2015). "Thymidine-Dependent *Staphylococcus aureus* Small-Colony Variants Are Induced by Trimethoprim-Sulfamethoxazole (SXT) and Have Increased Fitness during SXT Challenge." Antimicrob Agents Chemother **59**(12): 7265-7272.

Kubica, M., K. Guzik, J. Koziel, M. Zarebski, W. Richter, B. Gajkowska, A. Golda, A. Maciag-Gudowska, K. Brix, L. Shaw, T. Foster and J. Potempa (2008). "A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages." PLoS One **3**(1): e1409.

Kumar, Y. and R. H. Valdivia (2009). "Leading a sheltered life: intracellular pathogens and maintenance of vacuolar compartments." Cell Host Microbe **5**(6): 593-601.

La Rosa, R., H. K. Johansen and S. Molin (2018). "Convergent Metabolic Specialization through Distinct Evolutionary Paths in *Pseudomonas aeruginosa*." MBio **9**(2).

Lacoma, A., V. Cano, D. Moranta, V. Regueiro, D. Dominguez-Villanueva, M. Laabei, M. Gonzalez-Nicolau, V. Ausina, C. Prat and J. A. Bengoechea (2017). "Investigating intracellular persistence of *Staphylococcus aureus* within a murine alveolar macrophage cell line." Virulence **8**(8): 1761-1775.

Lai, X. H., I. Golovliov and A. Sjostedt (2001). "Francisella tularensis induces cytopathogenicity and apoptosis in murine macrophages via a mechanism that requires intracellular bacterial multiplication." Infect Immun **69**(7): 4691-4694.

Lai, X. H. and A. Sjostedt (2003). "Delineation of the molecular mechanisms of Francisella tularensis-induced apoptosis in murine macrophages." Infect Immun **71**(8): 4642-4646.

Lam, G. Y., J. Huang and J. H. Brumell (2010). "The many roles of NOX2 NADPH oxidase-derived ROS in immunity." Semin Immunopathol **32**(4): 415-430.

Lannergard, J., C. von Eiff, G. Sander, T. Cordes, J. Seggewiss, G. Peters, R. A. Proctor, K. Becker and D. Hughes (2008). "Identification of the genetic basis for clinical menadione-

auxotrophic small-colony variant isolates of *Staphylococcus aureus*." Antimicrob Agents Chemother **52**(11): 4017-4022.

Ledvina, H. E., K. A. Kelly, A. Eshraghi, R. L. Plemel, S. B. Peterson, B. Lee, S. Steele, M. Adler, T. H. Kawula, A. J. Merz, S. J. Skerrett, J. Celli and J. D. Mougous (2018). "A Phosphatidylinositol 3-Kinase Effector Alters Phagosomal Maturation to Promote Intracellular Growth of *Francisella*." Cell Host Microbe **24**(2): 285-295 e288.

Lewis, K. (2010). "Persister cells." Annu Rev Microbiol **64**: 357-372.

Lieberman, T. D., J. B. Michel, M. Aingaran, G. Potter-Bynoe, D. Roux, M. R. Davis, Jr., D. Skurnik, N. Leiby, J. J. LiPuma, J. B. Goldberg, A. J. McAdam, G. P. Priebe and R. Kishony (2011). "Parallel bacterial evolution within multiple patients identifies candidate pathogenicity genes." Nat Genet **43**(12): 1275-1280.

Liu, Q., W. S. Yeo and T. Bae (2016). "The SaeRS Two-Component System of *Staphylococcus aureus*." Genes (Basel) **7**(10).

Lowy, F. D. (1998). "Staphylococcus aureus infections." N Engl J Med **339**(8): 520-532.

Lucchini, S., H. Liu, Q. Jin, J. C. Hinton and J. Yu (2005). "Transcriptional adaptation of *Shigella flexneri* during infection of macrophages and epithelial cells: insights into the strategies of a cytosolic bacterial pathogen." Infect Immun **73**(1): 88-102.

Lugrin, J. and F. Martinon (2018). "The AIM2 inflammasome: Sensor of pathogens and cellular perturbations." Immunol Rev **281**(1): 99-114.

Mahenthiralingam, E., A. Baldwin and C. G. Dowson (2008). "*Burkholderia cepacia* complex bacteria: opportunistic pathogens with important natural biology." J Appl Microbiol **104**(6): 1539-1551.

Majerczyk, C. D., M. R. Sadykov, T. T. Luong, C. Lee, G. A. Somerville and A. L. Sonenshein (2008). "*Staphylococcus aureus* CodY negatively regulates virulence gene expression." J Bacteriol **190**(7): 2257-2265.

Man, S. M., R. Karki and T. D. Kanneganti (2017). "Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases." Immunol Rev **277**(1): 61-75.

Mao, X. J., Y. X. Huo, M. Buck, A. Kolb and Y. P. Wang (2007). "Interplay between CRP-cAMP and PII-Ntr systems forms novel regulatory network between carbon metabolism and nitrogen assimilation in *Escherichia coli*." Nucleic Acids Res **35**(5): 1432-1440.

Mariathasan, S. and D. M. Monack (2007). "Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation." Nat Rev Immunol **7**(1): 31-40.

Martinez-Cano, D. J., M. Reyes-Prieto, E. Martinez-Romero, L. P. Partida-Martinez, A. Latorre, A. Moya and L. Delaye (2014). "Evolution of small prokaryotic genomes." Front Microbiol **5**: 742.

Mathur, A., J. A. Hayward and S. M. Man (2018). "Molecular mechanisms of inflammasome signaling." J Leukoc Biol **103**(2): 233-257.

Maurelli, A. T., R. E. Fernandez, C. A. Bloch, C. K. Rode and A. Fasano (1998). "'Black holes' and bacterial pathogenicity: a large genomic deletion that enhances the virulence of *Shigella* spp. and enteroinvasive *Escherichia coli*." Proc Natl Acad Sci U S A **95**(7): 3943-3948.

Maurer, K., T. Reyes-Robles, F. Alonzo, 3rd, J. Durbin, V. J. Torres and K. Cadwell (2015). "Autophagy mediates tolerance to *Staphylococcus aureus* alpha-toxin." Cell Host Microbe **17**(4): 429-440.

McAdam, P. R., A. Holmes, K. E. Templeton and J. R. Fitzgerald (2011). "Adaptive evolution of *Staphylococcus aureus* during chronic endobronchial infection of a cystic fibrosis patient." PLoS One **6**(9): e24301.

Mellouk, N. and J. Enninga (2016). "Cytosolic Access of Intracellular Bacterial Pathogens: The *Shigella* Paradigm." Front Cell Infect Microbiol **6**: 35.

Mengaud, J., H. Ohayon, P. Gounon, R. M. Mege and P. Cossart (1996). "E-cadherin is the receptor for internalin, a surface protein required for entry of *L. monocytogenes* into epithelial cells." Cell **84**(6): 923-932.

Mercado-Lubo, R., M. P. Leatham, T. Conway and P. S. Cohen (2009). "Salmonella enterica serovar Typhimurium mutants unable to convert malate to pyruvate and oxaloacetate are avirulent and immunogenic in BALB/c mice." Infect Immun **77**(4): 1397-1405.

Merhej, V., E. Angelakis, C. Socolovschi and D. Raoult (2014). "Genotyping, evolution and epidemiological findings of *Rickettsia* species." Infect Genet Evol **25**: 122-137.

Mestre, M. B., C. M. Fader, C. Sola and M. I. Colombo (2010). "Alpha-hemolysin is required for the activation of the autophagic pathway in *Staphylococcus aureus*-infected cells." Autophagy **6**(1): 110-125.

Mitchell, G., E. Brouillette, D. L. Seguin, A. E. Asselin, C. L. Jacob and F. Malouin (2010). "A role for sigma factor B in the emergence of *Staphylococcus aureus* small-colony variants and elevated biofilm production resulting from an exposure to aminoglycosides." Microb Pathog **48**(1): 18-27.

Mitchell, G., M. Gattuso, G. Grondin, E. Marsault, K. Bouarab and F. Malouin (2011). "Tomatidine inhibits replication of *Staphylococcus aureus* small-colony variants in cystic fibrosis airway epithelial cells." Antimicrob Agents Chemother **55**(5): 1937-1945.

Moldovan, A. and M. J. Fraunholz (2019). "In or out: Phagosomal escape of *Staphylococcus aureus*." Cell Microbiol **21**(3): e12997.

Molofsky, A. B., B. G. Byrne, N. N. Whitfield, C. A. Madigan, E. T. Fuse, K. Tateda and M. S. Swanson (2006). "Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection." J Exp Med **203**(4): 1093-1104.

Montgomery, C. P., S. Boyle-Vavra and R. S. Daum (2010). "Importance of the global regulators Agr and SaeRS in the pathogenesis of CA-MRSA USA300 infection." PLoS One **5**(12): e15177.

Montgomery, C. P., S. Boyle-Vavra, A. Roux, K. Ebine, A. L. Sonenshein and R. S. Daum (2012). "CodY deletion enhances in vivo virulence of community-associated methicillin-resistant *Staphylococcus aureus* clone USA300." Infect Immun **80**(7): 2382-2389.

Munoz-Elias, E. J. and J. D. McKinney (2006). "Carbon metabolism of intracellular bacteria." Cell Microbiol **8**(1): 10-22.

Munoz-Planillo, R., L. Franchi, L. S. Miller and G. Nunez (2009). "A critical role for hemolysins and bacterial lipoproteins in *Staphylococcus aureus*-induced activation of the Nlrp3 inflammasome." J Immunol **183**(6): 3942-3948.

Mwangi, M. M., S. W. Wu, Y. Zhou, K. Sieradzki, H. de Lencastre, P. Richardson, D. Bruce, E. Rubin, E. Myers, E. D. Siggia and A. Tomasz (2007). "Tracking the in vivo evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing." Proc Natl Acad Sci U S A **104**(22): 9451-9456.

Nagaev, I., J. Bjorkman, D. I. Andersson and D. Hughes (2001). "Biological cost and compensatory evolution in fusidic acid-resistant *Staphylococcus aureus*." Mol Microbiol **40**(2): 433-439.

Naimi, T. S., K. H. LeDell, K. Como-Sabetti, S. M. Borchardt, D. J. Boxrud, J. Etienne, S. K. Johnson, F. Vandenesch, S. Fridkin, C. O'Boyle, R. N. Danila and R. Lynfield (2003). "Comparison of community- and health care-associated methicillin-resistant *Staphylococcus aureus* infection." JAMA **290**(22): 2976-2984.

Neumann, Y., S. A. Bruns, M. Rohde, T. K. Prajsnar, S. J. Foster and I. Schmitz (2016). "Intracellular *Staphylococcus aureus* eludes selective autophagy by activating a host cell kinase." Autophagy **12**(11): 2069-2084.

Ni Eidhin, D., S. Perkins, P. Francois, P. Vaudaux, M. Hook and T. J. Foster (1998). "Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*." Mol Microbiol **30**(2): 245-257.

Nielsen, A., M. Mansson, M. S. Bojer, L. Gram, T. O. Larsen, R. P. Novick, D. Frees, H. Frokiaer and H. Ingmer (2014). "Solonamide B inhibits quorum sensing and reduces *Staphylococcus aureus* mediated killing of human neutrophils." PLoS One **9**(1): e84992.

Nielsen, K. M., T. Bohn and J. P. Townsend (2014). "Detecting rare gene transfer events in bacterial populations." Front Microbiol **4**: 415.

Nimmo, G. R. (2012). "USA300 abroad: global spread of a virulent strain of community-associated methicillin-resistant *Staphylococcus aureus*." Clin Microbiol Infect **18**(8): 725-734.

Novick, R. P. and E. Geisinger (2008). "Quorum sensing in staphylococci." Annu Rev Genet **42**: 541-564.

Osterlund, A., R. Popa, T. Nikkila, A. Scheynius and L. Engstrand (1997). "Intracellular reservoir of *Streptococcus pyogenes* in vivo: a possible explanation for recurrent pharyngotonsillitis." Laryngoscope **107**(5): 640-647.

Paetzold, S., S. Lourido, B. Raupach and A. Zychlinsky (2007). "*Shigella flexneri* phagosomal escape is independent of invasion." Infect Immun **75**(10): 4826-4830.

Painter, K. L., A. Krishna, S. Wigneshweraraj and A. M. Edwards (2014). "What role does the quorum-sensing accessory gene regulator system play during *Staphylococcus aureus* bacteremia?" Trends Microbiol **22**(12): 676-685.

Pallen, M. J. and B. W. Wren (2007). "Bacterial pathogenomics." Nature **449**(7164): 835-842.

Palma, M. and A. L. Cheung (2001). "sigma(B) activity in *Staphylococcus aureus* is controlled by RsbU and an additional factor(s) during bacterial growth." Infect Immun **69**(12): 7858-7865.

Pandey, D. P. and K. Gerdes (2005). "Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes." Nucleic Acids Res **33**(3): 966-976.

Pane-Farre, J., B. Jonas, S. W. Hardwick, K. Gronau, R. J. Lewis, M. Hecker and S. Engelmann (2009). "Role of RsbU in controlling SigB activity in *Staphylococcus aureus* following alkaline stress." J Bacteriol **191**(8): 2561-2573.

Paranjape, S. M. and P. J. Mogayzel, Jr. (2014). "Cystic fibrosis." Pediatr Rev **35**(5): 194-205.

Park, J. Y., J. W. Kim, B. Y. Moon, J. Lee, Y. J. Fortin, F. W. Austin, S. J. Yang and K. S. Seo (2015). "Characterization of a novel two-component regulatory system, HptRS, the regulator for the hexose phosphate transport system in *Staphylococcus aureus*." Infect Immun **83**(4): 1620-1628.

Paulander, W., A. Nissen Varming, K. T. Baek, J. Haaber, D. Frees and H. Ingmer (2013). "Antibiotic-mediated selection of quorum-sensing-negative *Staphylococcus aureus*." MBio **3**(6): e00459-00412.

Peschel, A. (2002). "How do bacteria resist human antimicrobial peptides?" Trends Microbiol **10**(4): 179-186.

Peschel, A. and M. Otto (2013). "Phenol-soluble modulins and staphylococcal infection." Nat Rev Microbiol **11**(10): 667-673.

Pieper, R., C. R. Fisher, M. J. Suh, S. T. Huang, P. Parmar and S. M. Payne (2013). "Analysis of the proteome of intracellular *Shigella flexneri* reveals pathways important for intracellular growth." Infect Immun **81**(12): 4635-4648.

Pilz, R. B., R. C. Willis and G. R. Boss (1984). "The influence of ribose 5-phosphate availability on purine synthesis of cultured human lymphoblasts and mitogen-stimulated lymphocytes." J Biol Chem **259**(5): 2927-2935.

Pizarro-Cerda, J., A. Charbit, J. Enninga, F. Lafont and P. Cossart (2016). "Manipulation of host membranes by the bacterial pathogens *Listeria*, *Francisella*, *Shigella* and *Yersinia*." Semin Cell Dev Biol **60**: 155-167.

Pohl, K., P. Francois, L. Stenz, F. Schlink, T. Geiger, S. Herbert, C. Goerke, J. Schrenzel and C. Wolz (2009). "CodY in *Staphylococcus aureus*: a regulatory link between metabolism and virulence gene expression." *J Bacteriol* **191**(9): 2953-2963.

Pollitt, E. J. G., P. T. Szkuta, N. Burns and S. J. Foster (2018). "Staphylococcus aureus infection dynamics." *PLoS Pathog* **14**(6): e1007112.

Poteryaev, D., S. Datta, K. Ackema, M. Zerial and A. Spang (2010). "Identification of the switch in early-to-late endosome transition." *Cell* **141**(3): 497-508.

Powers, M. E., H. K. Kim, Y. Wang and J. Bubeck Wardenburg (2012). "ADAM10 mediates vascular injury induced by *Staphylococcus aureus* alpha-hemolysin." *J Infect Dis* **206**(3): 352-356.

Proctor, R. A., A. Kriegeskorte, B. C. Kahl, K. Becker, B. Löffler and G. Peters (2014). "Staphylococcus aureus Small Colony Variants (SCVs): a road map for the metabolic pathways involved in persistent infections." *Front Cell Infect Microbiol* **4**: 99.

Proctor, R. A., P. van Langevelde, M. Kristjansson, J. N. Maslow and R. D. Arbeit (1995). "Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*." *Clin Infect Dis* **20**(1): 95-102.

Proctor, R. A., C. von Eiff, B. C. Kahl, K. Becker, P. McNamara, M. Herrmann and G. Peters (2006). "Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections." *Nat Rev Microbiol* **4**(4): 295-305.

Queck, S. Y., M. Jameson-Lee, A. E. Villaruz, T. H. Bach, B. A. Khan, D. E. Sturdevant, S. M. Ricklefs, M. Li and M. Otto (2008). "RNAlII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*." *Mol Cell* **32**(1): 150-158.

Radoshevich, L. and P. Cossart (2018). "*Listeria monocytogenes*: towards a complete picture of its physiology and pathogenesis." *Nat Rev Microbiol* **16**(1): 32-46.

Randow, F. and C. Munz (2012). "Autophagy in the regulation of pathogen replication and adaptive immunity." *Trends Immunol* **33**(10): 475-487.

Ravanan, P., I. F. Srikumar and P. Talwar (2017). "Autophagy: The spotlight for cellular stress responses." *Life Sci* **188**: 53-67.

Ray, K., B. Marteyn, P. J. Sansonetti and C. M. Tang (2009). "Life on the inside: the intracellular lifestyle of cytosolic bacteria." *Nat Rev Microbiol* **7**(5): 333-340.

Recsei, P., B. Kreiswirth, M. O'Reilly, P. Schlievert, A. Gruss and R. P. Novick (1986). "Regulation of exoprotein gene expression in *Staphylococcus aureus* by agar." *Mol Gen Genet* **202**(1): 58-61.

Ren, C. L., W. J. Morgan, M. W. Konstan, M. S. Schechter, J. S. Wagener, K. A. Fisher, W. E. Regelman, Investigators and F. Coordinators of the Epidemiologic Study of Cystic (2007). "Presence of methicillin resistant *Staphylococcus aureus* in respiratory cultures from cystic fibrosis patients is associated with lower lung function." *Pediatr Pulmonol* **42**(6): 513-518.

Renesto, P., H. Ogata, S. Audic, J. M. Claverie and D. Raoult (2005). "Some lessons from *Rickettsia* genomics." *FEMS Microbiol Rev* **29**(1): 99-117.

Ribet, D. and P. Cossart (2015). "How bacterial pathogens colonize their hosts and invade deeper tissues." *Microbes Infect* **17**(3): 173-183.

Richardson, A. R., S. J. Libby and F. C. Fang (2008). "A nitric oxide-inducible lactate dehydrogenase enables *Staphylococcus aureus* to resist innate immunity." *Science* **319**(5870): 1672-1676.

Rink, J., E. Ghigo, Y. Kalaidzidis and M. Zerial (2005). "Rab conversion as a mechanism of progression from early to late endosomes." *Cell* **122**(5): 735-749.

Riordan, J. R., J. M. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J. L. Chou and et al. (1989). "Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA." *Science* **245**(4922): 1066-1073.

Roetzer, A., G. Haller, J. Beyerly, C. B. Geier, H. M. Wolf, C. S. Gruener, N. Model and M. M. Eibl (2016). "Genotypic and phenotypic analysis of clinical isolates of *Staphylococcus aureus* revealed production patterns and hemolytic potentials unlinked to gene profiles and source." BMC Microbiol **16**: 13.

Rohlf, E. M., Z. Zhou, R. A. Heim, N. Nagan, L. S. Rosenblum, K. Flynn, T. Scholl, V. R. Akmaev, D. A. Sirko-Osadsa, B. A. Allitto and E. A. Sugarman (2011). "Cystic fibrosis carrier testing in an ethnically diverse US population." Clin Chem **57**(6): 841-848.

Rolain, J. M., S. M. Diene, M. Kempf, G. Gimenez, C. Robert and D. Raoult (2013). "Real-time sequencing to decipher the molecular mechanism of resistance of a clinical pan-drug-resistant *Acinetobacter baumannii* isolate from Marseille, France." Antimicrob Agents Chemother **57**(1): 592-596.

Romilly, C., C. Lays, A. Tomasini, I. Caldelari, Y. Benito, P. Hammann, T. Geissmann, S. Boisset, P. Romby and F. Vandenesch (2014). "A non-coding RNA promotes bacterial persistence and decreases virulence by regulating a regulator in *Staphylococcus aureus*." PLoS Pathog **10**(3): e1003979.

Rooijackers, S. H., M. Ruyken, A. Roos, M. R. Daha, J. S. Presanis, R. B. Sim, W. J. van Wamel, K. P. van Kessel and J. A. van Strijp (2005). "Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases." Nat Immunol **6**(9): 920-927.

Roux, A., D. A. Todd, J. V. Velazquez, N. B. Cech and A. L. Sonenshein (2014). "CodY-mediated regulation of the *Staphylococcus aureus* Agr system integrates nutritional and population density signals." J Bacteriol **196**(6): 1184-1196.

Rubinsztein, D. C., P. Codogno and B. Levine (2012). "Autophagy modulation as a potential therapeutic target for diverse diseases." Nat Rev Drug Discov **11**(9): 709-730.

Runyen-Janecky, L. J. and S. M. Payne (2002). "Identification of chromosomal *Shigella flexneri* genes induced by the eukaryotic intracellular environment." Infect Immun **70**(8): 4379-4388.

Saitoh, T. and S. Akira (2010). "Regulation of innate immune responses by autophagy-related proteins." J Cell Biol **189**(6): 925-935.

Santic, M., R. Asare, I. Skrobonja, S. Jones and Y. Abu Kwaik (2008). "Acquisition of the vacuolar ATPase proton pump and phagosome acidification are essential for escape of *Francisella tularensis* into the macrophage cytosol." Infect Immun **76**(6): 2671-2677.

Santos, J. C. and J. Enninga (2016). "At the crossroads: communication of bacteria-containing vacuoles with host organelles." Cell Microbiol **18**(3): 330-339.

Schauer, K., J. Stolz, S. Scherer and T. M. Fuchs (2009). "Both thiamine uptake and biosynthesis of thiamine precursors are required for intracellular replication of *Listeria monocytogenes*." J Bacteriol **191**(7): 2218-2227.

Schlumberger, M. C. and W. D. Hardt (2006). "Salmonella type III secretion effectors: pulling the host cell's strings." Curr Opin Microbiol **9**(1): 46-54.

Schnaith, A., H. Kashkar, S. A. Leggio, K. Addicks, M. Kronke and O. Krut (2007). "*Staphylococcus aureus* subvert autophagy for induction of caspase-independent host cell death." J Biol Chem **282**(4): 2695-2706.

Schroder, A., R. Kland, A. Peschel, C. von Eiff and M. Aepfelbacher (2006). "Live cell imaging of phagosome maturation in *Staphylococcus aureus* infected human endothelial cells: small colony variants are able to survive in lysosomes." Med Microbiol Immunol **195**(4): 185-194.

Seggewiss, J., K. Becker, O. Kotte, M. Eisenacher, M. R. Yazdi, A. Fischer, P. McNamara, N. Al Laham, R. Proctor, G. Peters, M. Heinemann and C. von Eiff (2006). "Reporter metabolite analysis of transcriptional profiles of a *Staphylococcus aureus* strain with normal phenotype and its isogenic hemB mutant displaying the small-colony-variant phenotype." J Bacteriol **188**(22): 7765-7777.

Sendi, P. and R. A. Proctor (2009). "Staphylococcus aureus as an intracellular pathogen: the role of small colony variants." *Trends Microbiol* **17**(2): 54-58.

Senn, M. M., P. Giachino, D. Homerova, A. Steinhuber, J. Strassner, J. Kormanec, U. Fluckiger, B. Berger-Bachi and M. Bischoff (2005). "Molecular analysis and organization of the sigmaB operon in Staphylococcus aureus." *J Bacteriol* **187**(23): 8006-8019.

Shen, Y., M. Naujokas, M. Park and K. Ireton (2000). "InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase." *Cell* **103**(3): 501-510.

Shenoy, A. T., T. Brissac, R. P. Gilley, N. Kumar, Y. Wang, N. Gonzalez-Juarbe, W. S. Hinkle, S. C. Daugherty, A. C. Shetty, S. Ott, L. J. Tallon, J. Deshane, H. Tettelin and C. J. Orihuela (2017). "Streptococcus pneumoniae in the heart subvert the host response through biofilm-mediated resident macrophage killing." *PLoS Pathog* **13**(8): e1006582.

Shopsin, B., A. Drlica-Wagner, B. Mathema, R. P. Adhikari, B. N. Kreiswirth and R. P. Novick (2008). "Prevalence of agr dysfunction among colonizing Staphylococcus aureus strains." *J Infect Dis* **198**(8): 1171-1174.

Shopsin, B., C. Eaton, G. A. Wasserman, B. Mathema, R. P. Adhikari, S. Agolory, D. R. Altman, R. S. Holzman, B. N. Kreiswirth and R. P. Novick (2010). "Mutations in agr do not persist in natural populations of methicillin-resistant Staphylococcus aureus." *J Infect Dis* **202**(10): 1593-1599.

Sinha, B., P. Francois, Y. A. Que, M. Hussain, C. Heilmann, P. Moreillon, D. Lew, K. H. Krause, G. Peters and M. Herrmann (2000). "Heterologously expressed Staphylococcus aureus fibronectin-binding proteins are sufficient for invasion of host cells." *Infect Immun* **68**(12): 6871-6878.

Sivaraman, K., N. Venkataraman and A. M. Cole (2009). "Staphylococcus aureus nasal carriage and its contributing factors." *Future Microbiol* **4**(8): 999-1008.

Sokurenko, E. V., D. L. Hasty and D. E. Dykhuizen (1999). "Pathoadaptive mutations: gene loss and variation in bacterial pathogens." *Trends Microbiol* **7**(5): 191-195.

Somerville, G. A., M. S. Chaussee, C. I. Morgan, J. R. Fitzgerald, D. W. Dorward, L. J. Reitzer and J. M. Musser (2002). "Staphylococcus aureus aconitase inactivation unexpectedly inhibits post-exponential-phase growth and enhances stationary-phase survival." *Infect Immun* **70**(11): 6373-6382.

Somerville, G. A., A. Cockayne, M. Durr, A. Peschel, M. Otto and J. M. Musser (2003). "Synthesis and deformylation of Staphylococcus aureus delta-toxin are linked to tricarboxylic acid cycle activity." *J Bacteriol* **185**(22): 6686-6694.

Somerville, G. A. and R. A. Proctor (2009). "At the crossroads of bacterial metabolism and virulence factor synthesis in Staphylococci." *Microbiol Mol Biol Rev* **73**(2): 233-248.

Somerville, G. A., B. Said-Salim, J. M. Wickman, S. J. Raffel, B. N. Kreiswirth and J. M. Musser (2003). "Correlation of acetate catabolism and growth yield in Staphylococcus aureus: implications for host-pathogen interactions." *Infect Immun* **71**(8): 4724-4732.

Sonenshein, A. L. (2007). "Control of key metabolic intersections in Bacillus subtilis." *Nat Rev Microbiol* **5**(12): 917-927.

Soong, G., J. Chun, D. Parker and A. Prince (2012). "Staphylococcus aureus activation of caspase 1/calpain signaling mediates invasion through human keratinocytes." *J Infect Dis* **205**(10): 1571-1579.

Stapels, D. A., K. X. Ramyar, M. Bischoff, M. von Kockritz-Blickwede, F. J. Milder, M. Ruyken, J. Eisenbeis, W. J. McWhorter, M. Herrmann, K. P. van Kessel, B. V. Geisbrecht and S. H. Rooijackers (2014). "Staphylococcus aureus secretes a unique class of neutrophil serine protease inhibitors." *Proc Natl Acad Sci U S A* **111**(36): 13187-13192.

Steeb, B., B. Claudi, N. A. Burton, P. Tienz, A. Schmidt, H. Farhan, A. Maze and D. Bumann (2013). "Parallel exploitation of diverse host nutrients enhances Salmonella virulence." *PLoS Pathog* **9**(4): e1003301.

- Stenmark, H. (2009). "Rab GTPases as coordinators of vesicle traffic." Nat Rev Mol Cell Biol **10**(8): 513-525.
- Stoll, R. and W. Goebel (2010). "The major PEP-phosphotransferase systems (PTSs) for glucose, mannose and cellobiose of *Listeria monocytogenes*, and their significance for extra- and intracellular growth." Microbiology **156**(Pt 4): 1069-1083.
- Strobel, M., H. Pfortner, L. Tuchscher, U. Volker, F. Schmidt, N. Kramko, H. J. Schnittler, M. J. Fraunholz, B. Löffler, G. Peters and S. Niemann (2016). "Post-invasion events after infection with *Staphylococcus aureus* are strongly dependent on both the host cell type and the infecting *S. aureus* strain." Clin Microbiol Infect **22**(9): 799-809.
- Stryjewski, M. E. and G. R. Corey (2014). "Methicillin-resistant *Staphylococcus aureus*: an evolving pathogen." Clin Infect Dis **58 Suppl 1**: S10-19.
- Sturgill-Koszycki, S., P. H. Schlesinger, P. Chakraborty, P. L. Haddix, H. L. Collins, A. K. Fok, R. D. Allen, S. L. Gluck, J. Heuser and D. G. Russell (1994). "Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase." Science **263**(5147): 678-681.
- Suligoy, C. M., S. M. Lattar, M. Noto Llana, C. D. Gonzalez, L. P. Alvarez, D. A. Robinson, M. I. Gomez, F. R. Buzzola and D. O. Sordelli (2018). "Mutation of Agr Is Associated with the Adaptation of *Staphylococcus aureus* to the Host during Chronic Osteomyelitis." Front Cell Infect Microbiol **8**: 18.
- Sully, E. K., N. Malachowa, B. O. Elmore, S. M. Alexander, J. K. Femling, B. M. Gray, F. R. DeLeo, M. Otto, A. L. Cheung, B. S. Edwards, L. A. Sklar, A. R. Horswill, P. R. Hall and H. D. Gresham (2014). "Selective chemical inhibition of agr quorum sensing in *Staphylococcus aureus* promotes host defense with minimal impact on resistance." PLoS Pathog **10**(6): e1004174.
- Sun, Y. and M. X. O'Riordan (2010). "Branched-chain fatty acids promote *Listeria monocytogenes* intracellular infection and virulence." Infect Immun **78**(11): 4667-4673.
- Surewaard, B. G., J. F. Deniset, F. J. Zemp, M. Amrein, M. Otto, J. Conly, A. Omri, R. M. Yates and P. Kubes (2016). "Identification and treatment of the *Staphylococcus aureus* reservoir in vivo." J Exp Med **213**(7): 1141-1151.
- Tan, K., S. P. Conway, K. G. Brownlee, C. Etherington and D. G. Peckham (2002). "Alcaligenes infection in cystic fibrosis." Pediatr Pulmonol **34**(2): 101-104.
- Tattoli, I., M. T. Sorbara, C. Yang, S. A. Tooze, D. J. Philpott and S. E. Girardin (2013). "*Listeria* phospholipases subvert host autophagic defenses by stalling pre-autophagosomal structures." EMBO J **32**(23): 3066-3078.
- Taylor, R. C., S. P. Cullen and S. J. Martin (2008). "Apoptosis: controlled demolition at the cellular level." Nat Rev Mol Cell Biol **9**(3): 231-241.
- Tchawa Yimga, M., M. P. Leatham, J. H. Allen, D. C. Laux, T. Conway and P. S. Cohen (2006). "Role of gluconeogenesis and the tricarboxylic acid cycle in the virulence of *Salmonella enterica* serovar Typhimurium in BALB/c mice." Infect Immun **74**(2): 1130-1140.
- Tesse, R., F. Cardinale, T. Santostasi, A. Polizzi, A. Manca, L. Mappa, G. Iacoviello, F. De Robertis, V. P. Logrillo and L. Armenio (2008). "Association of beta-defensin-1 gene polymorphisms with *Pseudomonas aeruginosa* airway colonization in cystic fibrosis." Genes Immun **9**(1): 57-60.
- Thammavongsa, V., D. M. Missiakas and O. Schneewind (2013). "*Staphylococcus aureus* degrades neutrophil extracellular traps to promote immune cell death." Science **342**(6160): 863-866.
- Thwaites, G. E. and V. Gant (2011). "Are bloodstream leukocytes Trojan Horses for the metastasis of *Staphylococcus aureus*?" Nat Rev Microbiol **9**(3): 215-222.
- Traber, K. E., E. Lee, S. Benson, R. Corrigan, M. Cantera, B. Shopsin and R. P. Novick (2008). "agr function in clinical *Staphylococcus aureus* isolates." Microbiology **154**(Pt 8): 2265-2274.

Tranchemontagne, Z. R., R. B. Camire, V. J. O'Donnell, J. Baugh and K. M. Burkholder (2016). "Staphylococcus aureus Strain USA300 Perturbs Acquisition of Lysosomal Enzymes and Requires Phagosomal Acidification for Survival inside Macrophages." *Infect Immun* **84**(1): 241-253.

Tristan, A., M. Bes, H. Meugnier, G. Lina, B. Bozdogan, P. Courvalin, M. E. Reverdy, M. C. Enright, F. Vandenesch and J. Etienne (2007). "Global distribution of Panton-Valentine leukocidin--positive methicillin-resistant Staphylococcus aureus, 2006." *Emerg Infect Dis* **13**(4): 594-600.

Tuchscher, L., M. Bischoff, S. M. Lattar, M. Noto Llana, H. Pfortner, S. Niemann, J. Geraci, H. Van de Vyver, M. J. Fraunholz, A. L. Cheung, M. Herrmann, U. Volker, D. O. Sordelli, G. Peters and B. Loffler (2015). "Sigma Factor SigB Is Crucial to Mediate Staphylococcus aureus Adaptation during Chronic Infections." *PLoS Pathog* **11**(4): e1004870.

Tuchscher, L., V. Heitmann, M. Hussain, D. Viemann, J. Roth, C. von Eiff, G. Peters, K. Becker and B. Loffler (2010). "Staphylococcus aureus small-colony variants are adapted phenotypes for intracellular persistence." *J Infect Dis* **202**(7): 1031-1040.

Tuchscher, L. and B. Loffler (2016). "Staphylococcus aureus dynamically adapts global regulators and virulence factor expression in the course from acute to chronic infection." *Curr Genet* **62**(1): 15-17.

Tuchscher, L., E. Medina, M. Hussain, W. Volker, V. Heitmann, S. Niemann, D. Holzinger, J. Roth, R. A. Proctor, K. Becker, G. Peters and B. Loffler (2011). "Staphylococcus aureus phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection." *EMBO Mol Med* **3**(3): 129-141.

van Belkum, A., D. C. Melles, J. Nouwen, W. B. van Leeuwen, W. van Wamel, M. C. Vos, H. F. Wertheim and H. A. Verbrugh (2009). "Co-evolutionary aspects of human colonisation and infection by Staphylococcus aureus." *Infect Genet Evol* **9**(1): 32-47.

Vandenesch, F., T. Naimi, M. C. Enright, G. Lina, G. R. Nimmo, H. Heffernan, N. Liassine, M. Bes, T. Greenland, M. E. Reverdy and J. Etienne (2003). "Community-acquired methicillin-resistant Staphylococcus aureus carrying Panton-Valentine leukocidin genes: worldwide emergence." *Emerg Infect Dis* **9**(8): 978-984.

von Eiff, C., C. Heilmann, R. A. Proctor, C. Woltz, G. Peters and F. Gotz (1997). "A site-directed Staphylococcus aureus hemB mutant is a small-colony variant which persists intracellularly." *J Bacteriol* **179**(15): 4706-4712.

von Eiff, C., P. McNamara, K. Becker, D. Bates, X. H. Lei, M. Ziman, B. R. Bochner, G. Peters and R. A. Proctor (2006). "Phenotype microarray profiling of Staphylococcus aureus menD and hemB mutants with the small-colony-variant phenotype." *J Bacteriol* **188**(2): 687-693.

Waldron, D. E. and J. A. Lindsay (2006). "Saul: a novel lineage-specific type I restriction-modification system that blocks horizontal gene transfer into Staphylococcus aureus and between S. aureus isolates of different lineages." *J Bacteriol* **188**(15): 5578-5585.

Waligora, E. A., C. R. Fisher, N. J. Hanovice, A. Rodou, E. E. Wyckoff and S. M. Payne (2014). "Role of intracellular carbon metabolism pathways in Shigella flexneri virulence." *Infect Immun* **82**(7): 2746-2755.

Wang, B., A. Zhao, R. P. Novick and T. W. Muir (2014). "Activation and inhibition of the receptor histidine kinase AgrC occurs through opposite helical transduction motions." *Mol Cell* **53**(6): 929-940.

Wang, L., C. Quan, W. Xiong, X. Qu, S. Fan and W. Hu (2014). "New insight into transmembrane topology of Staphylococcus aureus histidine kinase AgrC." *Biochim Biophys Acta* **1838**(3): 988-993.

Wang, R., K. R. Braughton, D. Kretschmer, T. H. Bach, S. Y. Queck, M. Li, A. D. Kennedy, D. W. Dorward, S. J. Klebanoff, A. Peschel, F. R. DeLeo and M. Otto (2007). "Identification

of novel cytolytic peptides as key virulence determinants for community-associated MRSA." *Nat Med* **13**(12): 1510-1514.

Weddle, E. and H. Agaisse (2018). "Principles of intracellular bacterial pathogen spread from cell to cell." *PLoS Pathog* **14**(12): e1007380.

Weidenmaier, C., J. F. Kokai-Kun, S. A. Kristian, T. Chanturiya, H. Kalbacher, M. Gross, G. Nicholson, B. Neumeister, J. J. Mond and A. Peschel (2004). "Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections." *Nat Med* **10**(3): 243-245.

Weigel, L. M., D. B. Clewell, S. R. Gill, N. C. Clark, L. K. McDougal, S. E. Flannagan, J. F. Kolonay, J. Shetty, G. E. Killgore and F. C. Tenover (2003). "Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*." *Science* **302**(5650): 1569-1571.

Weinrick, B., P. M. Dunman, F. McAleese, E. Murphy, S. J. Projan, Y. Fang and R. P. Novick (2004). "Effect of mild acid on gene expression in *Staphylococcus aureus*." *J Bacteriol* **186**(24): 8407-8423.

Weiss, G. and U. E. Schaible (2015). "Macrophage defense mechanisms against intracellular bacteria." *Immunol Rev* **264**(1): 182-203.

Wernegreen, J. J. (2015). "Endosymbiont evolution: predictions from theory and surprises from genomes." *Ann N Y Acad Sci* **1360**: 16-35.

Wertheim, H. F., D. C. Melles, M. C. Vos, W. van Leeuwen, A. van Belkum, H. A. Verbrugh and J. L. Nouwen (2005). "The role of nasal carriage in *Staphylococcus aureus* infections." *Lancet Infect Dis* **5**(12): 751-762.

Witte, W., C. Cuny, I. Klare, U. Nubel, B. Strommenger and G. Werner (2008). "Emergence and spread of antibiotic-resistant Gram-positive bacterial pathogens." *Int J Med Microbiol* **298**(5-6): 365-377.

Worlitzsch, D., R. Tarran, M. Ulrich, U. Schwab, A. Cekici, K. C. Meyer, P. Birrer, G. Bellon, J. Berger, T. Weiss, K. Botzenhart, J. R. Yankaskas, S. Randell, R. C. Boucher and G. Doring (2002). "Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients." *J Clin Invest* **109**(3): 317-325.

Wu, S., H. de Lencastre and A. Tomasz (1996). "Sigma-B, a putative operon encoding alternate sigma factor of *Staphylococcus aureus* RNA polymerase: molecular cloning and DNA sequencing." *J Bacteriol* **178**(20): 6036-6042.

Xu, S. X. and J. K. McCormick (2012). "Staphylococcal superantigens in colonization and disease." *Front Cell Infect Microbiol* **2**: 52.

Yamamoto, H., M. Serizawa, J. Thompson and J. Sekiguchi (2001). "Regulation of the *glv* operon in *Bacillus subtilis*: YfiA (GlvR) is a positive regulator of the operon that is repressed through CcpA and cre." *J Bacteriol* **183**(17): 5110-5121.

Yang, L., L. Jelsbak, R. L. Marvig, S. Damkiaer, C. T. Workman, M. H. Rau, S. K. Hansen, A. Folkesson, H. K. Johansen, O. Ciofu, N. Hoiby, M. O. Sommer and S. Molin (2011). "Evolutionary dynamics of bacteria in a human host environment." *Proc Natl Acad Sci U S A* **108**(18): 7481-7486.

Yoong, P. and V. J. Torres (2013). "The effects of *Staphylococcus aureus* leukotoxins on the host: cell lysis and beyond." *Curr Opin Microbiol* **16**(1): 63-69.

Yoshikawa, Y., M. Ogawa, T. Hain, M. Yoshida, M. Fukumatsu, M. Kim, H. Mimuro, I. Nakagawa, T. Yanagawa, T. Ishii, A. Kakizuka, E. Sztul, T. Chakraborty and C. Sasakawa (2009). "*Listeria monocytogenes* ActA-mediated escape from autophagic recognition." *Nat Cell Biol* **11**(10): 1233-1240.

Zapotoczna, M., Z. Jevnikar, H. Miajlovic, J. Kos and T. J. Foster (2013). "Iron-regulated surface determinant B (IsdB) promotes *Staphylococcus aureus* adherence to and internalization by non-phagocytic human cells." *Cell Microbiol* **15**(6): 1026-1041.

Zautner, A. E., M. Krause, G. Stropahl, S. Holtfreter, H. Frickmann, C. Maletzki, B. Kreikemeyer, H. W. Pau and A. Podbielski (2010). "Intracellular persisting *Staphylococcus aureus* is the major pathogen in recurrent tonsillitis." PLoS One **5**(3): e9452.

Zhang, D. M., R. Q. Jiao and L. D. Kong (2017). "High Dietary Fructose: Direct or Indirect Dangerous Factors Disturbing Tissue and Organ Functions." Nutrients **9**(4).

Zhu, Y., R. Nandakumar, M. R. Sadykov, N. Madayiputhiya, T. T. Luong, R. Gaupp, C. Y. Lee and G. A. Somerville (2011). "RpiR homologues may link *Staphylococcus aureus* RNAIII synthesis and pentose phosphate pathway regulation." J Bacteriol **193**(22): 6187-6196.

Zielenski, J. (2000). "Genotype and phenotype in cystic fibrosis." Respiration **67**(2): 117-133.

Zimmermann, M. and M. A. Fischbach (2010). "A family of pyrazinone natural products from a conserved nonribosomal peptide synthetase in *Staphylococcus aureus*." Chem Biol **17**(9): 925-930.

Zong, Y., Y. Xu, X. Liang, D. R. Keene, A. Hook, S. Gurusiddappa, M. Hook and S. V. Narayana (2005). "A 'Collagen Hug' model for *Staphylococcus aureus* CNA binding to collagen." EMBO J **24**(24): 4224-4236.