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Influência do operão *nir* na adaptação de *Staphylococcus aureus* às condições do hospedeiro.

Influence of the *nir* operon in the adaptation of *Staphylococcus aureus* to the host environment

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Influência do operão *nir* na adaptação de *Staphylococcus aureus* às condições do hospedeiro.

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Lock up your libraries if you like; but there is no gate, no lock, no bolt that you can set upon the freedom of my mind.

Virginia Wolf

o júri

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abstract

Staphylococcus aureus is an opportunistic human pathogen, that commensally colonizes the human host and can cause a great number of severe diseases, that can be life-threatening. It is capable of infecting a wide range of tissues, from superficial skin to deeper tissues such as the gastrointestinal track, heart and bones. This variety of tissues show different oxygen concentrations, from highly oxygenated, like blood, to almost anaerobic environments, like the intestines. Little is known of the role played by genes involved in nitrite reduction (NirBD) and siroheme biosynthesis (sirABC) in the adaptation of *S. aureus* to less oxygenated environments and so it is important to study these genes and the response of this bacteria to the diverse environments found during the host infection.

In this work, through qRT-PCR, we observed that oxygen rich environments inhibited the expression of *nirR, nirB, sirA* and *sirC*. Hypoxia leads to the onset expression, culminating in higher levels of expression under anaerobic conditions. In anaerobic conditions, nitrates showed to decrease the expression of the *nir* operon genes and *sirC*, but their presence also presence improved growth.

The mutations Δ*nirB*, Δ*nirD* and Δ*nirR* weaken *S. aureus* growth, preventing the reduction of nitrites. The deletion of *sirC* also demonstrated effects, but since the process that is catabolized by this enzyme can occur naturally, but at a lower rate, the effects observed by the deletion of this gene resulted in slightly slower growth and consumption of nitrites, when compared to the wild-type.

In summary, *S. aureus* shows resilience, adapting and surviving, different oxygen environments. It was also showed that the presence of nitrates and oxygen are inhibitors of the expression of the *nir* and *sirC* operon genes, in which *sirC* has been shown to be an enzyme-encoding gene, that when deleted did not impair nitrite reduction by *S. aureus*.

.

Palavras-Chave   *Staphylococcus aureus*, condições fisiológicas, respiração anaeróbica, sirohemo,

Resumo   *Staphylococcus aureus* é patógeno oportunista do ser humano, que coloniza o seu hospedeiro comensalmente, tendo a capacidade de causar diversas doenças, que podem tornar-se mortais. É capaz de infetar uma grande panóplia de tecidos, desde altamente oxigenados, como o sangue, a quase anaeróbicos, como o intestino. Pouco se sabe relativamente ao papel de genes envolvidos na redução de nitritos (NirBD) e síntese de sirohemo (sirABC) na adaptação de *S. aureus* a ambientes menos oxigenados, sendo por isso importante estudar estes genes e a resposta da bactéria aos diversos ambientes encontrados aquando a infeção do hospedeiro.

Neste trabalho, observou-se através de qRT-PCR que ambientes ricos em oxigénio inibem a expressão de *nirR*, *nirB*, *sirA* e *sirC*. A hipóxia leva ao inicio de expressão destes genes, culminando em superiores níveis de expressão em condições de anaerobiose. Em condições de anaerobiose, nitratos inibem a transcrição de genes do operão *nir* e sirC, mas a sua presença levou a um maior crescimentos.

As mutações Δ*nirB*, Δ*nirD* e Δ*nirR* são debilitadores do crescimento de *S. aureus*, impedindo a redução de nitritos de uma forma completa. A deleção de *sirC*, o que resultou num crescimento e redução de nitritos semelhante ao do WT.

Em suma, *S.aureus,* demonstra uma incrível resiliência, adaptando-se e sobrevivendo a variadas concentrações de oxigênio. A presença de nitratos e oxigénio são inibidores da expressão dos genes do operão *nir* e *sirC*, no qual *sirC* demonstrou ser um gene que codifica uma enzima que quando deletado não impede a redução de nitritos por parte de *S. aureus*.

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List of Abbreviations and Acronyms

CA-MRSA – Community-Acquired Methicillin-Resistant *Staphylococcus aureus*

Coa - Coagulase

CT – Cycle Threshold

DNA - Deoxyribonucleic acid

EL - Early Log

EDTA - Ethylenediamine tetraacetic acid

FAD **-** flavin adenine dinucleotide

Fd-dependent - Ferredoxin-dependent

G/C – Guanine/Cytosine ratio

g/L – Gram per liter

H2O2 - Hydrogen peroxide

H_3PO_4 – Phosphoric acid

HA-MRSA – Hospital-Acquired Methicillin-Resistant *Staphylococcus aureus*

HCL – Hydrochloric acid

iNOS - inducible nitric oxide synthase

L - Liter

L-Arg - L-arginine

Late Log – LL

LB - Luria – Bertani

M – Molar

mg/L – Miligram per liter

$MgCl_2$ – Magnesium dichloride

ML - Middle Log

mM – Millimolar

MnSOD - Manganese-containing superoxide dismutase

MRSA – Methicillin-Resistant *Staphylococcus aureus*

NADH - Nicotinamide adenine dinucleotide

NADPH - Nicotinamide adenine dinucleotide phosphate

$NaNO_2$ – Sodium Nitrate

NaOH – Sodium Hydroxide

NEDD - Naphthylene diamine dihydrochloride

NH2OH, - Hydroxylamine

NiR - Nitrite reductase

NO˙ - Nitrogen oxide

NOX - Nicotinamide adenine dinucleotide phosphate

OD – Optical Density

PBP – Penicillin-binding proteins

PCR - Polymerase chain reaction

PN – Pattee and Neveln Medium

pO_2 – Oxygen pressure

PRRs - Pattern recognition receptors

qRT-PCR – Quantitative Real-Time PCR

RNA - Ribonucleic acid

RNS - Reactive nitrogen species

ROS - Reactive oxygen species

SCCmec – Staphylococcal cassette chromosome

SNM - Synthetic Nose Medium

$SO_3{}^2$ – Sulfite

SSTI – Soft Skin and Tissue Infection

TSB - Tryptic Soy Broth

Uro'gen III - Uroporphyrinogen III

UTI – Urinary tract infection

vWbp - von Willebrand factor–binding protein

μg – Microgram

μM - Micromolar

μm – Micrometer

Genus Staphylococcus

Initially, the genus *Staphylococcus*, was a member of the family *Micrococcaceae* and only later, through molecular and phylogenetic analysis, was perceptible that staphylococci were not genetically close to micrococci. Consequently, the genus *Staphylococcus* was separated from this family and inserted in a new one, to which was given the name *Staphylococcaceae* (1).

Since 1962, when only 3 species of *Staphylococcus* were described, extensive reviews to the taxonomy have been done. Nowadays, through molecular methods, there are about 45 species of *Staphylococcus* described, and 24 subspecies (2).

The identification of *Staphylococcus* species is a laborious process, that involves phenotypic methods that in many cases fail. For this reason, various methods of molecular biology were introduced in this process. These techniques are for example: sequencing specific genes, using hybridization probes and utilizing restriction enzymes, to name a few (3).

Staphylococci are Gram-positive bacteria that when analyzed through microscopy have a spherical structure, to which is given the name cocci. They possess the typical cellular wall of Gram-positive bacteria, composed by teichoic acid and peptidoglycan (4). They are a group of facultative anaerobic bacteria, with the exception of some anaerobic species, namely *S. saccharolyticus* e *S. aureus* subsp. *anaerobius*. Even though, in general, staphylococci are catalase-positive, some rare strains have been reported as catalase-negative. (5)

The percentage of G/C content in the chromosomal DNA in species of the genus *Staphylococcus* is between 30% and 40%. Most of the pathogenic species in this genus are called coagulase-positive (CoPS), as they possess a protein called coagulase, which is an enzyme capable of coagulating the plasm of rabbit, turning fibrinogen in fibrin. Members of this group are *S. delphini, S. intermedium* and *S. pseudintermedius*, which are species that have adapted to various hosts like mink, fox, pigeon and dog, but did not developed the ability to colonize or infect humans (6). In contrast, members of this genus that do not possess this gene, are classified as staphylococci coagulase-negative (CoNS), and are

classified as lesser pathogenic species, for example *S. epidermidis* (7), that usually causing opportunistic infection in immunocompromised hosts.

Staphylococcus aureus

1. General information

The genus as separated in two species: *S. aureus* and *S. albus*. This nomenclature was proposed by Rosenbach, he was the first to differentiate the two basing this on the pigmentation of the colonies (8). *S. albus* has since changed its nomenclature, being now called S. *epidermidis* (7).

Staphyloxanthin, a membrane-bound carotenoid produced by *S. aureus*, is responsible for the golden pigmentation of colonies. Additionally, the production of this pigment is also responsible for scavenging reactive oxygen species and protecting *S. aureus* from phagocytic killing (9).

It is, also, an organism that does not produce spores. When grown in various types of agar for 24 hours at 37ºC it organizes itself in individual and circular colonies, with about 2-3 millimeters of diameter and with a shiny and clean surface. The ability to clot human and animal blood or plasma is a hallmark of *S. aureus* and is mediated by two secreted products, coagulase (Coa) and von Willebrand factor–binding protein (vWbp), being one of the members of the *Staphylococcus* genus that is CoPS (7).

S. aureus strains possess two forms of coagulase. There is a bound coagulase that is connected to the cell wall and can convert fibrinogen into insoluble fibrin and cause the formation of the clump directly, and there is a free form coagulase that accomplishes the same result but by reacting with a globulin plasma factor, called coagulase-reacting factor, in order to generate staphylothrombin, and thrombin-like factors. This coagulase-reacting factor catalyzes the conversion of fibrinogen to insoluble fibrin (10).

It has been showed that Coa and vWbp play an essential role in *S. aureus* abscess formation and persistence in host tissues, by protecting the organisms from phagocytosis. The inhibition of these two key proteins prevents coagulase from binding to prothrombin

or fibrinogen and debilitates *S. aureus* ability of evading the immune system and compromises the ability of causing lethal sepsis (11).

Staphylococci produce a variety of other enzymes that hydrolyze host tissue components and aid in the spread of the bacteria. Hyaluronidase hydrolyzes hyaluronic acids, which is presented in the acellular matrix of connective tissue. Fibrinolysin, also named staphylokinase, has the ability to dissolve fibrin clots and help the spreading of bacteria. *S. aureus*, as many other strains, but all can produce several enzymes that can hydrolyze lipids, lipases, that play an important role in the survival of staphylococci in the sebaceous areas of the body. In order, to hydrolyze viscous DNA, *S. aureus* produces a thermostable nuclease (11).

S. aureus produces many toxins, including five cytolytic or membrane-damaging toxins (alpha, beta, delta, gamma, and Panton-Valentine [P-V] leukocidin), two exfoliative toxins (A and B), 18 enterotoxins (A to R), and toxic shock syndrome toxin-1 (TSST-1). (12).

2. Diseases

Even though colonization by *S. aureus* is typically not harmful to the host, since it is estimated that colonizes up to 50% of the healthy human adult population with 20% being persistently colonized (13), among the populations of patients that have an elevated risk of developing health care associated infections, with *S. aureus,* we have: very low birth-weight neonates, patients with indwelling catheters, endotracheal intubation, medical implantation of foreign bodies, trauma, surgical procedures, hemodialysis, peritoneal dialysis, diabetes and individuals who have undergone immuno-suppressive or cancer therapy.

S. aureus may be able to breach the innate immune system of the host and gain access to deeper tissues. Once there, it can be the cause of a vast number of invasive infections. Indeed, *S. aureus* is a leading cause of skin and soft tissue infections, which include superficial skin infections, such as impetigo and infected abrasions, as well, more complicated skin infections, such as, cellulitis, folliculitis, subcutaneous abscesses, and infected ulcers and wounds. (14, 15). It is also responsible for more rare but severe

infections in the community, such as, pyomyositis (13), necrotizing fasciitis (13, 16) and necrotizing pneumonia (17, 18). In nosocomial settings, *S. aureus* can initiate infections at surgical sites or from implanted medical devices including artificial heart valves, catheters, prosthetic joints and orthopedic implants (13, 19, 20). It can also infect blood, bacteremia, and once in the blood it can propagate to other vital organs (21), resulting in disseminated infections such as endocarditis, osteomyelitis and descending urinary tract infections (16, 22). The environment versatility of this pathogen results in a strong capability to infect a wide variety of host niches ranging from skin (23, 24) to abiotic devices (25) and deep-seated tissues this makes it difficult to eradicate which can result in recurrent infections
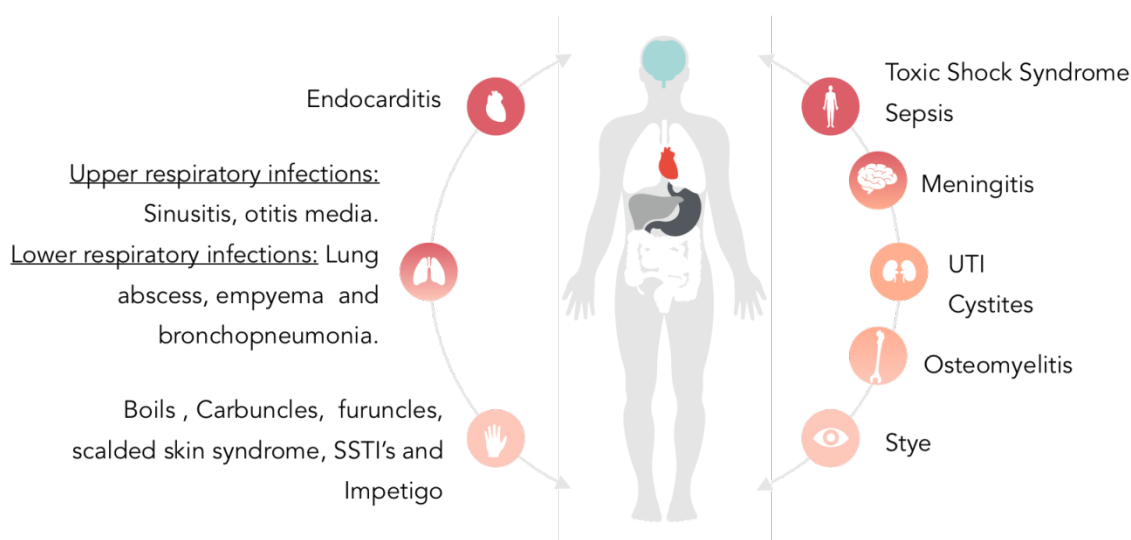
Figure 1: Representation of diseases inflicted by infections of *S. aureus*. (Balasubramanian, D. *et al* 2017; Park MK, *et al* 1992; Brezis M., *et al* 1995; Brooks M, *et al* 2004; Carreau A, *et al* 2011; Hatzenbuehler J, *et al* 2011; Spencer JA, *et al* 2014.)

(26).

3. Clinical Challenges

S. aureus pathogenicity is effectively enhanced by its antibiotic-resistant ability, and methicillin-resistant *S. aureus* (MRSA), is one of the most common types (27).

MRSA presents a great challenge given its high mortality and limited therapeutic treatment (28) . Recent estimates show that *S. aureus* MRSA causes approximately 95 000 invasive infections and 19 000 deaths per year (29).

Table 1: Mechanism of resistance by *S. aureus* to different therapeutically used antibiotics (Franklin D. Lowy *et al* 2017).

Antibiotic	Resistance genes	Gene products	Mechanism(s) of resistance	Location(s)
B-Lactams	1) *blaZ* 2) *mecA*	1) B-Lactamase 2) PBP2a	1) Enzymatic hydrolysis of B-lactam nucleus. 2) Reduced affinity for PBP	1) Pl: TN 2) C:SCC*mec*
Glycopeptides	1) Unknown (VISA) 2)	1) Altered peptidoglycan 2) D-Ala-D-Lac	1) Trapping of vancomycin in the cell wall 2) 2)Synthesis of dipeptide with reduced affinity for vancomycin	1) C 2) Pl: Tn
Quinolones	1) *parC* 2) *gyrA* or *gyrB*	1) ParC (or GrlA) component of topoisomerase IV 2) GyrA or GyrB components of gyrase	1, 2) Mutations in the QRDR region, reducing affinity of enzyme -DNA complex for quinolones	1) C 2) C
Aminoglycosides (e.g. gentamicin)	Aminoglycoside-modifying enzymes	Acetyltransferase, Phosphotransferase	Acetylating and/or phosphorylating enzymes modify aminoglycosides	Pl, Pl: Tn
Trimethoprim-sulfamethoxazole (TMP-SMZ)	1) Sulfonamide: *sulA* 2) TMP: *dfrB*	1) Dihydropteroate synthase 2) Dihydrofolate reductase(DHFR)	1) Overproduction of *p*-aminobenzoic acid by enzyme 2) Reduced affinity for DHFR	1) C 2) C
Oxazolidinones	*rrn*	23S rNA	Mutations in domain V of rRNA component of the 50S ribosome. Interferes with ribosomal binding	C
Quinupristin-d alfopristin	1) Q: *ermA, ermB, ermC* 2) D: *vat, vatB*	1) Ribosomal methylases 2) Acetyltransferases	1) Reduce binding to the 23S ribosomal subunit 2) Enzymatic modification of dalfopristin	1) Pl, C 2) C

MRSA is resistant to other β-lactam antibiotics, such as, oxacillin, nafcillin, dicloxacillin, and cefazolin (30). Usually, oxacillin and/or cefoxitin are used in susceptibility testing. For methicillin-susceptible *S. aureus*, ß-lactams are able to bind to the penicillin-binding proteins (PBP) which are essential for cell wall biosynthesis. Furthermore, the peptidoglycan crosslink formation is also inhibited. Therefore, this results in lysis of the bacterial cells. However, MRSA has a mobile genetic element called staphylococcal cassette chromosome (SCCmec). The SCCmec carries the *mecA* gene to encode altered PBP (PBP2a) so that the affinity to β-lactam antibiotics is decreased prominently. Consequently, the inhibition of cell wall biosynthesis fails, and MRSA strains are capable of surviving even in the presence of β-lactam antibiotics (31).

MRSA strains are the most common bacteria among those healthcare-associated patients, and they are named as HA-MRSA (32). Then, the first report about community-associated MRSA (CA-MRSA) infections was published at the beginning of the 20th century, the infections happened on healthy individuals (with no risk factors of HA-MRSA infections), injection drug users, incarcerated people, and athletes (33).

The USA300, was firstly isolated as a CA-MRSA, and has a close relationship with military personnel, prisoners, athletes, intravenous drug users, the homeless, urban populations and men who have sex with men, but soon it has expanded quickly to the general population (34).

MRSA isolates account for > 60% of clinical *S. aureus* infections in Japan, >50% in Italy and Portugal, and >30% in the United States (48). The use of vancomycin as the antibiotic of last-resort against MRSA has selected for the emergence of vancomycin-resistant *S. aureus* and is also responsible for the frequent occurrence of strains with intermediate resistance (35).

4. Host Conditions and *Staphylococcus aureus* responses

As a highly adaptive commensal organism, *S. aureus* possesses an array of genes that allow the bacterium to survive and grow in a wide variety of niches. This ability to survive was developed from the close association and evolution with humans and has

allowed *S. aureus* to become a leading cause of nosocomial and community-acquired infections (36).

4.1. Oxygen levels in host

Both in *in vitro* and in host tissues, molecular oxygen plays a critical role in *S. aureus* growth. In the host the concentration of oxygen tends to vary according to the tissue (37). Blood can be separated in two types that differ in the oxygen concentration, namely the arterial blood that has a 68-95 mmHg of oxygen, which is one of the most oxygenated tissue in the human body, while the second type is venous blood that has an oxygen content of 40 mmHg (38). Skin possesses a wide range of oxygen content that varies from 8-35 mmHg with the depth from the surface. The intestinal lumen is one peculiar place containing < 2 mmHg oxygen, making it almost anaerobic (39). Some critical organs show high levels of oxygen, such as kidneys (50-70 mmHg) and liver (30-40 mmHg)(40, 41).

During infection the recruitment of immune cells, like neutrophils, is a rapid process that increases the oxygen demand by more than 50-fold (42, 43) which can result in a state of hypoxia, a state of oxygen deficiency, in infection sites (39, 44). Besides neutrophils, macrophages resident in tissues, dendritic cells and T cells induce inflammation, altering vascular structures and leading to a restricted blood flow to tissues and consequently, reducing the oxygen concentrations of infections sites (43).

Table 2: Oxygen concentration in mmHg and percentage for the tissues: skin, blood, liver, kidney, bone marrow and bone; in the human body.

Tissue	pO_2 (mmHg)	pO_2 (%)
Skin	8 - 35	1.0 – 4.6
Arterial Blood	68 -95	8.0 – 12.0
Venous Blood	40	5.2
Liver and Kidney	30 - 40	3.9 – 5.2
Bone Marrow	Intravascular – 20.4 extravascular - 18	2.3 – 2.7
Bone	9.9 – 17.7	1.3 – 2.3

In particular, osteomyelitis is a low oxygen and biofilm-associated infection (45 – 47). In general, bone and bone marrow are considered to be hypoxic tissues caused by the low blood flow that reaches these tissues, since they are not well vascularized (48, 49). During infection with *S. aureus* the concentration of oxygen decreases even more (50).

In vitro studies have shown that hypoxic environments increase *S. aureus* cytotoxin production, which indicates that oxygen starvation may promote pathogenesis of *S. aureus* (50). It was also shown that this bacterium is capable of inducing hypoxia in tissues that have higher concentration level of oxygen, for example, in kidneys, (51) where micro-environments such as abscesses have low oxygen levels. From these abscesses *Staphylococcus aureus* can disseminate, infect blood, bacteremia, and reach other vital organs (52 - 55).

In summary, tissues contain a wide range of oxygen concentrations, from anaerobic environments, like the intestines, to comparatively oxygen replete sites, such as blood rich tissues, in which *S. aureus* is capable of striving. Also, *S. aureus* furthermore seems to be able to promote hypoxia in tissues, which plays a critical part in signaling for enhanced virulence.

4.2 Nutrients and metabolic signals

Carbohydrates (carbon sources) are critical for cellular growth and metabolism. Glycolysis, pentose phosphate pathway and citric acid (Krebs) cycle are some of the pathways in which carbohydrates play important roles as precursors and metabolic intermediates.

For most organisms, glucose is the preferred carbon source (56). Humans produce glucose and store it in the liver until it is transported to the bloodstream in order to be distributed throughout the body. It serves as a great energy source for cells of many types, and as a result its presence and homeostasis are carefully maintained in such a form that glucose is the most abundant free carbohydrate present in the human serum (57).

For humans, the blood glucose levels are in the range of 80-130 mg/dL range, with < 70 mg/dL being considered hypoglycemia and > 200 mg/dL as hyperglycemia (58). To survive and prosper in these diverse nutritional conditions, *S. aureus* regulates and modulates gene expression coordinated by particular environmental cues (59). One

example of this control and modulation are the hypoxic states, in which *S. aureus* increases its glycolytic flux to balance insufficient fermentation of carbohydrates and modulates the uptake of glucose, increasing it (60).

S. aureus pathogenesis appears to be linked to glucose availability *in vitro* and, in humans, this is supported by the fact that biofilm production is enhanced by addition of glucose to media (61). Also, *in vivo* studies have shown that diabetic mice are more susceptible to infection by *S. aureus* and are deficient in clearing *S. aureus* compared to their non-diabetic counter parts (62). Likewise, diabetic patients have been shown to be at a higher risk for *S. aureus* pneumonia (63) and also, more susceptible to *S. aureus* - mediated foot infections (64). Importantly it has been linked a higher risk of *S. aureus* infection to patients who shown to be hyperglycemic (65).

In conditions of low glucose content, *S. aureus* assumes a low-energy state, also known as starvation state (66). It was found that in this condition 99% of *S. aureus* cells lose viability, because of glucose depletion, during the first few days of culture. The bacterial population that survives has the capability of remaining viable for months. These cells, that are under a starvation state for a long period of time, are smaller and denser than cells grown in the presence of glucose. In the early stages of starvation, changes in RNA and protein synthesis profiles can also be observed (66). When supplemented with glucose after starvation, it can recover from that state, giving rise to an increase in RNA synthesis and protein production, that supports growth (66).

S. aureus adapts to nutritionally diverse environments by prioritizing the use of primary versus secondary carbon sources, and this process is known as catabolite repression (CCR) (67, 68).

In summary, *S. aureus* conforms and adapts to diverse host environments in which levels of carbon-based nutrients vary greatly. Either in the process of or as a result of changes of the metabolic state, *S. aureus* differentially regulates virulence factor expression, thereby modifying its pathogenesis.

4.3. Iron Availability

Iron is a vital nutrient across all domains of life. Although iron limitation inhibits cellular processes, iron abundance is toxic due to formation of reactive oxygen species (ROS). As a result, iron metabolism in mammalian cells and in bacteria is tightly regulated to maintain homeostasis. In vertebrates, iron exists in mainly three forms: the heme in hemoglobin; iron-sulfur clusters of several critical enzymes; and as storage molecules, like transferrin, and ferritin (69 – 71).

The most predominant form that retains 90% of the iron present in the host, is in heme. As a result, free extracellular iron in human tissues is estimated to be around 10^{-18}M (72), which is a concentration well below of the one required for microbial life. Additionally, infection-induced inflammation leads to the rapid decline of iron levels in blood serum (73, 74). Extracellular iron is often scavenged by host glycoproteins, which restricts even more the iron availability for microbes during infection (70). The process of depriving microbes of iron has been coined as 'nutritional immunity' (69, 70).

S. aureus most likely encounters a gradient of iron concentrations when it travels through different tissues. Under iron-starved conditions, *S. aureus* upregulates siderophore and heme acquisition pathways and represses virulence. When iron is abundant, this can be either due to the natural reservoir of iron in the tissue or caused by an efficient acquisition of iron, *S. aureus* switches to a more pathogenic behavior (70).

4.4. pH level and temperature

S. aureus can grow between pH 4.5 and 9.3. However, the growth is higher between pH 7 and 7.5 (75) but growth, at any pH, can be influenced by other environmental conditions. *In vitro*, for example, it depends also on the acid and base used to adjust the pH throughout the growth (76).

S. aureus grows also within temperature range from 6.7 to 45.6 °C, depending on other environmental conditions (77) with an optimum at 37°C. *S. aureus*, unlike its toxins, is not heat resistant. The optimum temperature for enterotoxin production is slightly higher than that for growth (78).
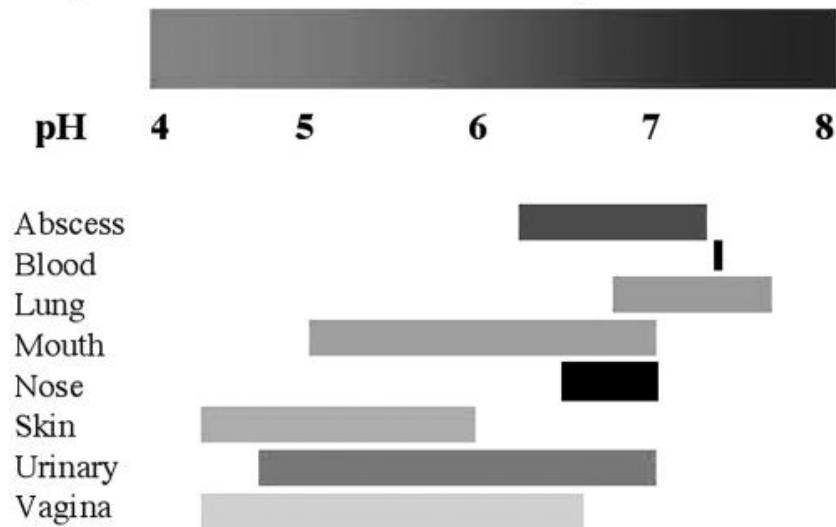
Figure 2: pH ranges of various niches *S. aureus* can colonize in the human host. Blood, pH 7.4 (Robinson 1975); vagina, 4.2 to 6.6 (Wagner and Ottesen, 1982); abscess, 6.2 to 7.3 (Bessman, Page *et al.* 1989); urinary tract (UT), 4.6 to 7 (McClatchey,1994); lung, 6.8 to 7.6 (Cheng, Rodriguez *et al.* 1998); mouth, 5 to 7 (Dong, Pearce *et al.* 1999); nose, 6.5 to 7 (England, Homer *et al.* 1999); skin, 4.2 to 5.9 (Ehlers, Ivens *et al.* 2001). (from Weinrick, Brian *et al.* 2005).

5. *Staphylococcus aureus* response to the immune system

For the host, resident phagocytes and epithelial cells in the skin or mucosal tissue respond to either bacterial products or tissue injury by activation of the immune system. *S. aureus* peptidoglycan and lipoprotein are sensed by host pattern recognition molecules (79, 80), hyaluronan breakdown products (79), and endogenous toll like receptor ligands (RNA, DNA, HMGB1) released by necrotic tissues (80, 81). During infection further augment pro-inflammatory signaling leading to local immune cell activation, and neutrophil and macrophage recruitment.

Macrophages are leukocytes involved in the first line of defense that have the role of primary professional scavenger cells. They can engulf microorganisms and other smaller cells using several mechanisms, such as a Fc receptor and complement-mediated phagocytosis, pinocytosis and endocytosis (82). *S. aureus* has the ability to survive inside and outside of the host cells. Once in the extracellular milieu, this bacterium expresses on its surface a clumping factor A, also named protein A, and other complement inhibitors in order to evade opsonization induced by the complement and antibodies. These inhibitors inactive or prevent the binding of opsonins to the target bacteria (83, 84). Also, to evade

death, *S. aureus* can find shelter intercellularly in cells like, epithelial cells, endothelial cells, and even macrophages (85).

Macrophages are known to produce various molecules such as nitric oxide (NO˙) and reactive oxygen species (ROS) during phagocytosis and ligands of pattern recognition receptors (PRRs) (86). The ROS and reactive nitrogen species (RNS) are produced by nicotinamide adenine dinucleotide phosphate oxidase (NOX) and inducible nitric oxide synthase (iNOS), respectively (87).

Activated macrophages produce NO˙ by inducible NO˙ synthase (iNOS), encoded by the NOS2 gene (88), by two successive monooxygenations of L-arginine (L-Arg) (89, 102). Hydrogen peroxide (H_2O_2) is one of the most active oxygen species, which is produced in the mitochondria by MnSOD (manganese-containing superoxide dismutase, SOD2) as an end product of plasma membrane associated-reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase during the respiratory burst in activated macrophage (90). NO˙ and H_2O_2 are two compounds that have an essential role as cell-signaling molecules for process like the microbicidal and cytotoxic response of macrophages (91). *S. aureus* cells can protect themselves against microbicidal agents generated by phagocytes by the expression of detoxifying enzymes (92).

The most abundant leukocyte population in the blood are the neutrophils, which are a crucial defense against *S. aureus* (93). So, in order to protect itself, *Staphylococcus aureus* will interfere with all the stages of neutrophil recruitment in infection and the neutrophil effector functions. *S. aureus* deploys a number of strategies to resist neutrophil killing. *S. aureus* can survive intracellularly within phagocytes including neutrophils (98) and macrophages (99). *S. aureus* ability to cleave the heavy chains of opsonic antibodies is an important mechanism to resist phagocytosis (100). This and other proteolytic enzymes may also be able to degrade the host antimicrobial peptide agents and tissue components (101).

Beyond provisions to escape from innate immunity to ensure staphylococcal survival in host tissues, *S. aureus* secretes toxins to disrupt epithelial or endothelial surfaces and to trigger lysis of immune cells.

A severe bacterial infection normally induces the host to mount an adaptive immune response within seven to ten days to limit the ongoing infection and prevent future reinfections. The mechanism underlying evasion of adaptive immune response is less understood (94, 95).
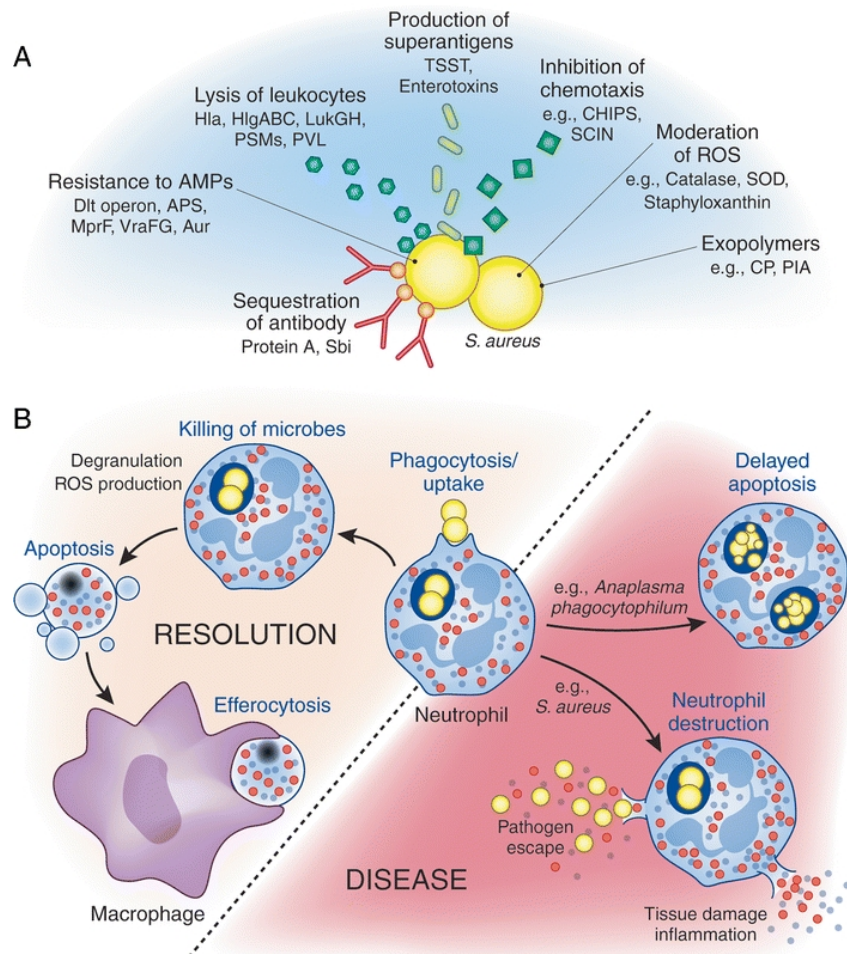
Figure 3: *S. aureus* **immune evasion mechanisms and possible outcomes of bacteria–neutrophil interaction.** Immune evasion by *S. aureus* includes strategies that serve to prevent recognition, inhibit chemotaxis, moderate ROS, protect against AMPs, and directly damage immune cells. B Phagocytic uptake of bacteria triggers production of ROS and degranulation, working collectively to kill ingested bacteria, after which neutrophils undergo apoptosis to be removed by macrophages and promote healthy resolution of infection. Alternatively, bacterial pathogens can alter normal neutrophil turnover, promoting either a delay in neutrophil apoptosis or an accelerated neutrophil lysis. Alteration of normal neutrophil turnover facilitates pathogen survival and promotion of disease. (Kevin M. Rigby et al. 2012)

6. *Staphylococcus aureus* response to nitrogen species

Nitric Oxide Synthase (iNOS) enzymes are ubiquitous to all domains of life and are critical for a great number of biological processes (103). Mammalian NOS proteins contain both oxygenase and reductase domains that catalyze the two-step oxidation of L-arginine NO$^{\cdot}$ and L-citrulline (104).

Alternatively, most bacterial NOS enzymes only contain an oxygenase domain and require a separate cellular reductase partner (105). Bacterial iNOS proteins are primarily found in Gram-positive like *Bacillus* and *Staphylococcus* (106).

NO$^{\cdot}$ is a highly reactive free radical gas (107), which at low concentrations can act as a signaling molecule (108), and can also promote nitrosative stress when NO$^{\cdot}$ levels are high (109).

RNS are generated by oxidation of the compound NO$^{\cdot}$ and typically, target heme, iron-sulfur clusters, redox-active thiols, lipids, and DNA (110-114). When exposed to NO$^{\cdot}$, bacteria will suffer a variety of biochemical and metabolic stress, for example, DNA damage, altered metal homeostasis, and disrupted metabolic enzymes, including components of the respiratory chain.

At high NO$^{\cdot}$ concentrations, the reversible binding of NO$^{\cdot}$ to cytochrome heme iron results in respiration inhibition (114). NO$^{\cdot}$ and its derivatives can also cause DNA damage, lipid peroxidation, and nitration of tyrosine residues (115).

A major factor contributing to *S. aureus* persistence within mammalian hosts is resistance to the antimicrobial radical NO$^{\cdot}$ (116). Production of NO$^{\cdot}$ is important for limiting bacterial proliferation in multiple infection models, so the ability to continue growth in the presence of this broad-spectrum antimicrobial confers *S. aureus* with a major pathogenic advantage. *S. aureus* NO$^{\cdot}$ resistance is a highly unique trait, as even closely related Staphylococci (117, 118). Additionally, NO$^{\cdot}$ resistance is important for *S. aureus* persistence.

Several components of the known *S. aureus* NO$^{\cdot}$ response are controlled by as-yet-unidentified regulatory mechanisms, suggesting that additional NO$^{\cdot}$-responsive regulators exist beyond those currently characterized (119).

7. *Staphylococcus aureus* response to anaerobic environment

S. aureus is versatile in its responses to many types of environments, but is a less versatile facultative anaerobic bacteria, when compared, for example, with *Escherichia coli*, not only because it possesses a less complex fermentative pathway, but also, because it lacks the enzyme cytochrome oxidase. (121)

However, it has a great array of sensors that allow it to recognize promptly hypoxia and anaerobiosis environments. Under anaerobic conditions, *S. aureus* upregulates certain pathways like glycolysis, anaerobic respiration and fermentation; and downregulate genes involved in the Krebs cycle, which is a major pathway for nicotinamide adenine dinucleotide (NADH) regeneration (121).

The inability to replenish the NADH/NAD+ pools and inefficient ATP synthesis are the two major challenges that *S. aureus* faces under hypoxic or anaerobic conditions (129).

During anaerobic respiration, *S. aureus* uses nitrates and nitrites as electron acceptors (120-124). In this pathway, nitrate is reduced to nitrite by nitrate reductase (NarGHI) this process leads to the accumulation of nitrite that is consequently reduced, by nitrite reductase (NirBD), to ammonia (126 -128). When there is an absence of oxygen as electron acceptor, the bacteria switches to a fermentative metabolism (120, 125).

Bacterial nitrite reductases, also known as NiRs, are cytoplasmic enzymes that catalyze the six-electron reduction involved in the process of turning NO_2 to NH^{4+}. There are two types of NiRs, they can be differentiated by the electron donor they have affinity with. Ferredoxin (Fd)-dependent NiRs are one of the two types of NiR and are characteristic of both eukaryotic and prokaryotic photosynthetic organisms. The second one is NAD(P)H-dependent NiRs which are present in most heterotrophic bacteria (129). Both types of NiRs contain a siroheme cofactor that transfers six electrons to nitrite, which is then reduced to ammonia.

Fd-NiRs, are monomers that contain as prosthetic groups, namely, a [4Fe4S] cluster and siroheme. The physiological electron donor is reduced FAD or Flavodoxin (Fld). These NiRs have been shown to have biochemical properties similar to those present in plants and algae (130).

As described for Fd-NiRs, NO and NH_2OH, are two intermediate, that can be formed in the catalytic cycle (130).

$$Fd\ \{[2Fe - 2S]\} \rightarrow \{[4Fe - 4S \rightarrow siroheme]\} \rightarrow NO_2^-$$
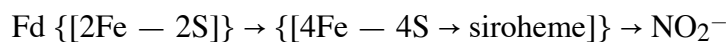
Most information about NADH-NiRs of heterotrophic bacteria, such as *Klebsiella oxytoca* and *Bacillus subtilis*, has been deduced by DNA sequence analysis or biochemical studies. The bacterial NADH-NiRs also share a high C-terminal sequence similarity with sulfite reductases (131).

The NADH-NiRs contain a noncovalently bound FAD, a [4Fe4S] cluster, and siroheme as prosthetic groups. The FAD- and NADH-binding sites are located at the N-terminal extension that is not present in the Fd-dependent NiR (132).

$$NADH \rightarrow \{FAD \rightarrow [2Fe - 2S] \rightarrow [4Fe - 4S] \rightarrow siroheme\} \rightarrow NO_2^-$$
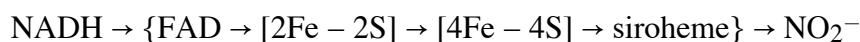
Siroheme

Sirohemes are Fe complexes of sirohydrochlorin, a class of hydroporphyrins with eight carboxylic acid side chains (133).
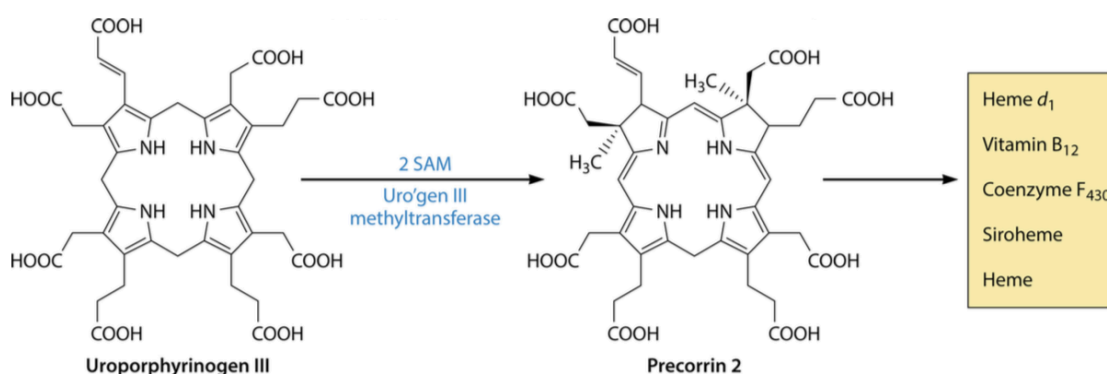
Figure 4: Transformation of uroporphyrinogen III (Uro'gen III) into precorrin-2, a key precursor in the biogenesis of heme d_1, vitamin B_{12}, coenzyme F_{430}, siroheme, and heme. Precorrin-2 is synthesized from uroporphyrinogen III by the action of the enzyme uroporphyrinogen III methyltransferase, which adds two *S*-adenosylmethionine-derived methyl groups to C-2 and C-7 of the macrocycle (Harry A. Dailey *et al* 2017).

In higher plants tetrapyrrole synthesis occurs in plastids (134, 135). The process initiates by the reduction of the glutamyl moiety of glutamyl-tRNA to glutamate-1-semialdehyde. Intermolecular transamination of glutamate semialdehyde generates 5-aminolevulinic acid (ALA). ALA is then transformed into uroporphyrinogen III (uro'gen III) by three enzymes: ALA dehydratase, porphobilinogen deaminase and uroporphyrinogen III synthase (136-138).
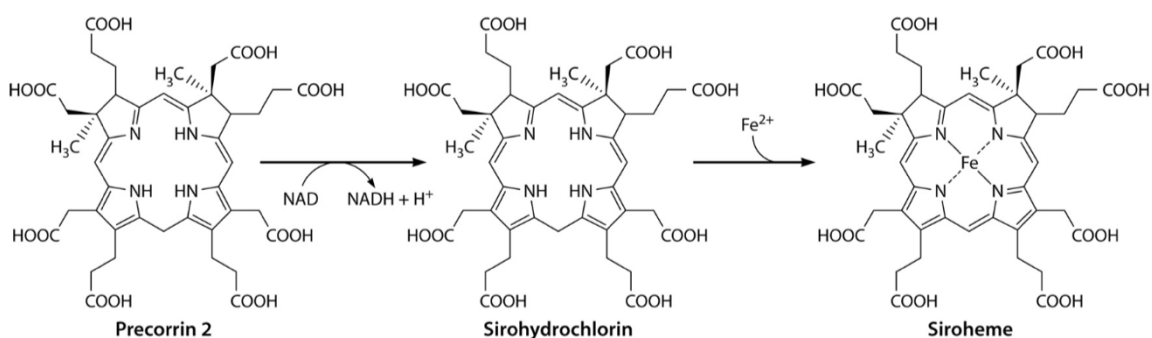
Figure 5: Transformation of precorrin 2 intosiroheme. Precorrin-2 is converted into siroheme via sirohydrochlorin (Harry A. Dailey *et al* 2017).

When uro'gen III is methylated, it seals its fate in the heme synthesis pathway, steering itself in the direction of siroheme. Whereas uro'gen III suffered decarboxylation steers it toward heme and chlorophyll synthesis (139).

Generally, bacteria synthesize siroheme from uro'gen III through a pathway composed of three steps, similar to the ones described in higher plants: methylation of uro'gen III, oxidation of precorrin-2, and insertion of iron into sirohydrochlorin (141, 142).

In *Escherichia coli* and *Salmonella enterica*, the reaction occurs in three steps accomplished by a single multifunctional enzyme named siroheme synthase CysG (141). It is a protein of 457 amino acid residues, that is composed by a N-terminal region with dehydrogenase and ferrochelatase enzyme activity, and a C-terminal region with S-adenosyl-l-methionine (SAM)-dependent uroporphyrinogen III methyltransferase SUMT activity, that converts uro'gen III into precorrin-2 (142).
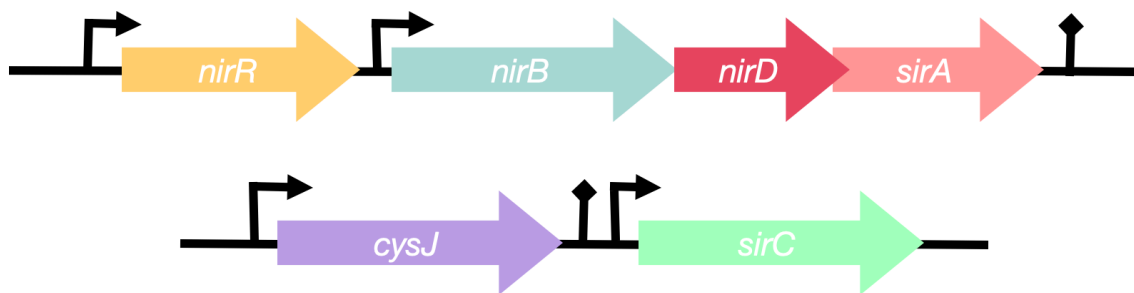
Figure 6: Genomic organization of genes encoding enzymes involved in siroheme biosynthesis in *S. aureus*. Uro'gen III methyltrhasferase is located downstream of the genes encoding nitrite reductase large subunit (NirB) and nitrite reductase small subunit (NirD). While precorrin-2 degydrogenase/sirohydrochlorin ferrochelatase is located downstream of gene enconding a sulfite reductase flavoprotein CysJ.

Aims of the Study

In *Staphylococcus aureus* the expression of genes involved in siroheme biosynthesis and nitrite reduction (NirBD) in hypoxic conditions have not been studied before. Given that *S. aureus* is subjected to a large variation of oxygen concentration in tissues during the course of infection, it is important to characterize the role of these pathways on the adaption of *S. aureus* to these environments.

For this purpose, this work aimed to:

- Establish how the growth is *S. aureus* is affected by different oxygen concentration, including under hypoxic conditions, by using a Bioreactor of the model Biostat A to control temperature and pH.

- Observe how the growth of *S. aureus* is affected in the presence of nitrate.

- Study changes in gene expression of genes involved in siroheme biosynthesis and nitrite respiration pathways (*nirR, nirB, sirA and sirC*) under different conditions.

- Study the growth of *S. aureus* NARSA mutant strains (Δ*nirB*, Δ*nirD*, Δ*nirR* and Δ*sirC*) in anaerobic conditions and in the presence of nitrites and how it affects the survival of this bacteria.

Materials and Methods

Microorganism and inoculum preparation.

The pre-inoculum was always prepared in 20 ml of Tryptic Soy Broth, in a 100 ml Erlenmeyer, adding 700 μL of a stock solution containing JE2 *Staphylococcus aureus* unless otherwise indicated. The strain *S. aureus* USA300 JE2 is a plastid-cured derivative of *S. aureus* USA300 LAC, which is a highly characterized community-associated methicillin-resistant *S. aureus* (MRSA) strain isolated from the Los Angeles County jail.

The inoculum was maintained with an agitation of 150 rpm and at a 37ºC, overnight, in an IKA ® KS 400 ic control.

For one of the experiments, the inoculum was prepared in 20 ml of Synthetic Nose Medium 3 + AA6, in a 100 ml Erlenmeyer, adding with 800 μL of a stock solution containing JE2 *Staphylococcus aureus*.

Growth conditions.

During the optimization stage, growth was performed in a total of four media. Firstly, cells were grown in a defined medium which contained glucose as the primary carbon source, Synthetic Nose Medium (SNM), in flasks. It was made following the instruction on Bernhard Krismer *et al*, 2014, and then modified in three distinct ways.

The three defined conditions for SNM3 was SNM3 with the amino acid concentration multiplied by two (SNM3 + AA6), SNM3 with all the components multiplied by 2 ((SNM3)2) and SNM3 with all the components multiplied by 3 ((SNM3)3).

One modification present in all three conditions was the absence of 2-2'-Bipyridine, since it's a chelation of iron, an important compound in siroheme production.

The strain was grown under aerobic conditions, at 37ºC and 150rpm, in an IKA ® KS 400 ic, it was cultured in a SNM3, in the three evaluated conditions: SNM3 + AA6, (SNM3)2 and (SNM3)3, and OD was measured hourly.

All the following three media were used in the bioreactor Biostat A under controlled conditions of pH, temperature, as well, oxygen. The second medium tested, was a chemically defined medium, Pattee and Neveln (PN) (93). The medium compounds were specified in table 3**.**

The medium preparation was done accordingly to the original recipe, except for presence of noble agar. Glucose was autoclaved at 121ºC for 15 minutes before insertion instead of sterilized by a passage through a 0,22 µm membrane filter like it was performed for the other solutions.

L-Threonine was dissolved in 50 ml of HCl solution (1M) instead of being dissolved in distilled water at pH 7.

Initially PN medium was used with the specified glucose concentration, but growths were also performed in an altered glucose concentration (from 5 g/L or 27mM to a concentration of 10.8 g/L or 60mM), in order to test if glucose was a limiting factor in *S. aureus* growth.

Table 3: Compounds present in Pattee and Neveln Medium (1975)**.**

	Concentration (g/L)		Concentration (mg/L)
Glucose	5 / 10.8	**Aminoacids**	
Salts		L-Glutamic acid	100
K2HPO4	7	L-Serine	30
KH2PO4	2	L-Methionine	3
Na3.Citrate	0.4	L-Tyrosine	50
MgSO4.7H2O	0.05	L-Alanine	60
(NH4)2SO4	1	L-Lysine	50
Vitamins	**Concentration (mg/L)**	L-Threonine	30
Thiamine	1	L-Phenyalanine	40
Niacin	1.2	L-Histidine	20
Biotin	0.005	Glycine	50
Ca- Pantothenate	0.25	L-Tryptophan	10
Purines and Pyrimidines		L-Isoleucine	30
Adenine	5	L-Valine	80
Guanine	5	L-Leucine	90
Citosin	5	L-Aspartic Acid	90
Uracil	5	L-Arginine	50
Timine	0.02	L-Proline	80
		L-Cystine	20

Following these two media, it was tested chemically complex media, firstly Luria-Bertani (LB) and the fourth and final medium was Tryptic Soy Broth (TSB) medium, TSB was the medium chosen for the progression of the work.

Batch fermentations

Batch fermentation was performed in order to analyze the growth of *Staphylococcus aureus* in a controled environment. These types of experiments were performed in a Sartorius Biostat A bioreactor (Germany) equipped with a 1-L vessel. It is a Bioreactor equipped with sensors and actuators to measure and control some parameters namely, pH, temperature and oxygen concentration, in real-time via a digital control unit.

The base-line fermentation medium:

1) Was modified Pattee and Neveln medium, with the specifications previously describe. The salt solution was added to the vessel and supplemented with antifoam SE-15 (Sigma) solution at a concentration of 1g/mL and the distilled water necessary to make a final volume of 1L, before being autoclaved at 121 °C for 30 min. The remaining stock solution were added in a sterile environment seconds before the insertion of the inoculum, in the bioreactor;

2) Was LB medium supplemented with antifoam SE-15 (Sigma) solution at a concentration of 1g/mL;

3) Was TSB medium supplemented with antifoam SE-15 (Sigma) solution at a concentration of 1g/mL.

4) Was TSB medium supplemented with antifoam SE-15 (Sigma) solution at a concentration of 1g/mL and, freshly prepared daily, Sodium Nitrate ($NaSO_3$) at a 2 mM concentration.

The pre-inoculum was used to inoculate fresh TSB medium in the bioreactor at the OD, 600 nm, = 0.1.

The OD at 600nm was measured from the TSB inoculum, and calculations were made each time, to discover the volume of TSB inoculum that was needed to insert in the bioreactor in order to obtain a 0.1 OD at 600nm in the 0 hours point.

A connecter equipped with a syringe allowed the collection of culture samples. The bioreactor has also some other connectors for the addition of solutions - Base, Acid and Antifoam. The digital control unit of the bioreactor was connected with the interface.

The interface allows real-time supervision of the evolution of the different variable measures, modification of the desired set point imposed on different operating parameters,

experimental data storage, etc. Basic control loops were applied to some key parameters during the duration of the cultures to maintain them at optimal values for the cell growth:

- The temperature of the culture medium was maintained at 37ºC by action on the temperature of the water circulating in the double wall and by the sleeve around the bioreactor. The temperature was defined at this set point in order to mimic the human body environment, which as a basal temperature of 37ºC;

- The pH of the culture medium was maintained at 7 with the addition of a basic solution of NaOH (10 M) and acidic solution of HCl (1 M). This pH was also chosen based on human physiological condition.

- To mimic different tissue in the human body the pO_2 was observed at 0%, 2%, 5%, 10% and 21%.
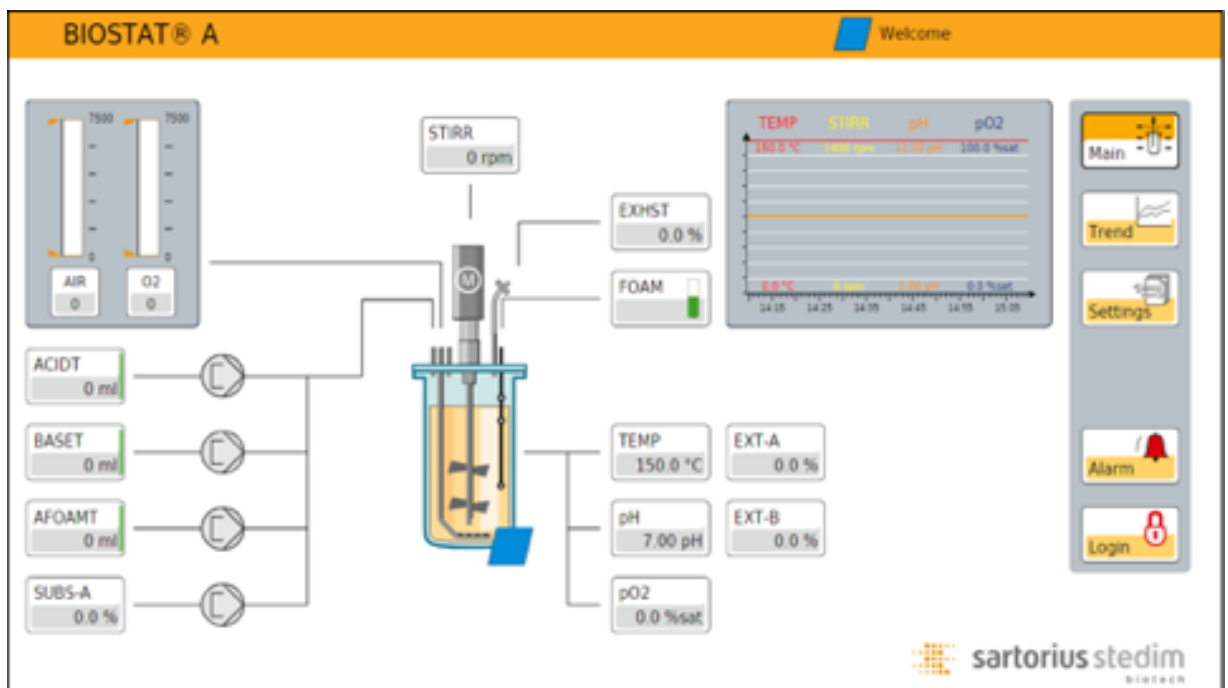
Figure 7: Bioreactor Biostat A Interface. Sartorius stedim.

RNA extraction and qRT-PCR

To access the influence of oxygen concentration and nitrate in the variation in expression of *S. aureus* genes *nirR, nirB, nirD* and *sirA,* qRT-PCR assays were performed.

In total, two RNA extraction were performed from two distinct biological samples, that were analyzed, each, in two qRT-PCR independent assays in duplicate.

RNA was extracted from samples in order to obtain cells, samples were collected from bioreactor growth in three distinct points of the curve: two hours, three and half hours and the six-hour mark, that corresponds two the early log (EL), middle log (ML) and late log (LL), respectively, for each of the conditions tested.

Total RNA extraction was achieved using the High Pure RNA isolation Kit (Roche). Cells are initially thawed on ice and resuspended in 180 uL of 10 mM Tris-HCl pH 8.0 with EDTA, where the concentration of cells were equal by the formula OD x mL = 3. Lyses of the cell samples were obtained by incubation with lysozyme 2 mg/mL and lysostaphin 0.1 mg/ml at 37ºC for 30 minutes, followed by the addition of Lysis solution, supplemented with 1% B-mercapto-ethanol) and isopropanol at 70%.

The lysates were transferred to a High Pure Filter Tube and manufacturer's protocol was followed. Contaminating DNA was removed by using Turbo DNA-*freeI* ™ Kit (Ambion), following the manufacturer's instructions. The absence of chromosomal DNA was confirmed by PCR, using oligonucleotides for 16S gene. RNA concentration and purity were evaluated in a Nanodrop ND-1000 UV-visible spectrophotometer (Thermo Fisher Scientific) and by agarose gel electrophoresis.

To synthesize complementary DNA (cDNA), 3 μg of the total RNA extracted were reverse transcribed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche).

The qRT-PCR assays were conducted in a LightCycler® 480 (Roche), using ≈ 3ug of cDNA, 0,5 uM of gene-specific oligonucleotides for the genes *nirR, nirB, nirD* and *sirA*, PCR-grade water and the LightCycler® 480 SYBR Green Maxter Mix (Roche), which contains FastStart Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I Dye and $MgCl_2$.

The expression levels of *S. aureus nirR, nirB, nirD* and *sirA* genes were normalized relative to the housekeeping gene 16S. The fold change in gene expression were calculated using the comparative CT method (143).

Mutant's Growth

A culture of Δ*nirB*, Δ*nirD*, Δ*sirC* and Δ*nirR S. aureus* mutant strains, were diluted in TSB for an OD_{600} of 0.1 and in a complete anaerobic atmosphere, purged of the media

with N_2 for 15 minutes, and in TSB. The medium was also supplemented with 2mM of Sodium Nitrate ($NaNO_2$) freshly prepared daily.

The growth was maintained with an agitation of 150 rpm and at a 37ºC in a IKA ® KS 400 ic control for a 8-hour period. OD600 was measured hourly and the collected sample was used in assays of nitrite-reductase activity.

Nitrite reductase activity: Griess Method

The Griess Solution is composed of 1 % sulfanilamide, 0.1% Naphthylene diamine dihydrochloride (NEDD) and 2 % H_3PO_4 and dissolved in demineralized water (144).

The consumption of nitrite was measured in resting cells that were cultivated anaerobically in the presence of nitrite (2 mM), they were harvested every hour, and equal parts of Greiss solution and sample were added to three wells in a 96 well plate, and OD was measured at 540 nm. Through a calibration curve, it was possible to ascertain the concentration of nitrites in the medium during growth.

1. *Staphylococcus aureus* shows growth variation when exposed to different host environments.

During the course of infection, *S. aureus* finds different oxygen tensions depending on the infected tissue. In order to find a suitable media to study the effect of oxygen in *S. aureus* growth, several preliminary tests were done with different media. For a better control of nutrient supplementation in the medium of growth of *S. aureus*, it was analyzed the possibility of using a chemical defined medium, where the carbon source was glucose.
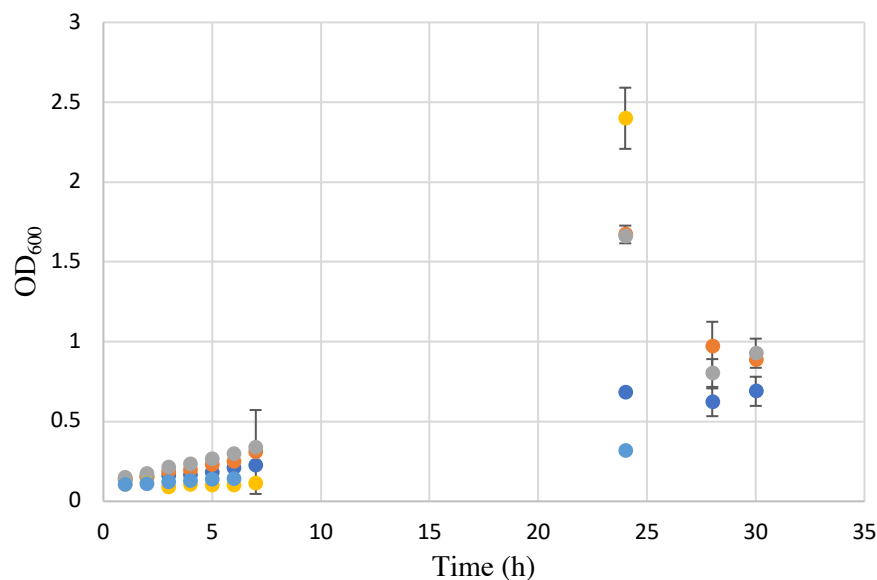
Figure 8: Aerobic growth of *S. aureus* JE2 in the chemical defined medium, SNM at 37ºC and 150 rpm, in flasks. SNM3 supplemented with the original amino acid concentrations multiplied by 6 instead of 3, as specified by the original medium, SNM3+AA6 (●); SNM3 with all its components multiplied once more by a factor of 2, (SNM3)2 (●); SNM3 with all its complements multiplied once more by a factor of 3, (SNM3)3 (●), all inoculated with a TSB pre-inoculum and SNM3+AA6, with pré-inoculum being prepared in the same medium, SNM3 + AA6; SNM3 with all its components multiplied once more by a factor of 3 (●); (SNM3)3, inoculated with bacteria grown in a pre-inoculum from a (SNM3)3 medium (●).

Synthetic Nose Medium, also known as, SNM, was described by Krismer B. *et al* (2014), is a medium that mimicked the environment where *S. aureus* is commensal, the nasopharynx, and where the carbon source is glucose. As described in the article, SNM was a lacking medium that suffered some changes, which resulted in the creation of

SNM3, which had the same composition as the original, but the concentration of certain compounds was tripled. In order to achieve a high OD_{600}/growth and cell production, we tested three variations of this medium: SNM3 + AA6, (SNM3)2 and (SNM3)3; which were inoculated from a pre-inoculum grown in TSB grown overnight, and then tested the two other conditions: SNM3 + AA6 and (SNM3)3, inoculated with pre-inoculum grown in the respective medium.

   When using a pre-inoculum grown in TSB, the conditions of SNM3 + AA6 and (SNM3)3 showed a high optical density peak at 24 hours followed by a subsequence decrease of OD_{600} in the order of ≈ 0.8. This decrease indicates that after 24 hours *S. aureus* enters a senescence phase, or a late stationary phase, this can be caused by the exhaust of resources of the medium (**figure 8**).

   (SNM3)2 on the other hand showed the less growth it is not possible to know if at the stationary state and at the 24 hours it is already at the senescence state (**figure 8**).

   As such, it was tested the possibility of using a pre-inoculum collected from the 23-hour mark of a growth in SNM3+AA6 and (SNM3)3 and inoculate, respectively, which media, reducing this way the adjusting time, *S. aureus* might have when transferred from TSB to the SNM3 variants. In this test it was possible to observed that the OD_{600} of SNM3+AA6 reached a greater OD_{600}, of ≈ 2.4, while in (SNM3)3 it plateaus in an OD_{600} ≈ 0.3. In SNM3+AA6 it is possible to see a long lag phase between the 0 hour and the 7th hour, while in SNM3 it did not grow significantly, which led us to test other media (**figure 8**).

   After SNM we proceeded to test to grown *S. aureus* in the bioreactor under a temperature of 37ºC and a pH of 7 in two different media: Pattee and Neveln Medium, PN, a chemically defined medium with glucose as the carbon source, and Luria-Bertani Broth, LB, a chemical complex medium.

   In the PN medium, *S. aureus* is capable of growth, achieving OD_{600} of 2.6 and 2.2 for the 21% and 5% oxygen, respectively, but the growth at a higher oxygen concentration did not show a great variation from the lowest concentration, which led us to believe that the concentration of one or more compounds of PN medium were a limiting factor, since

this medium is not nutritionally optimized for a specific microorganism, namely, *S. aureus* (**figure 9**).
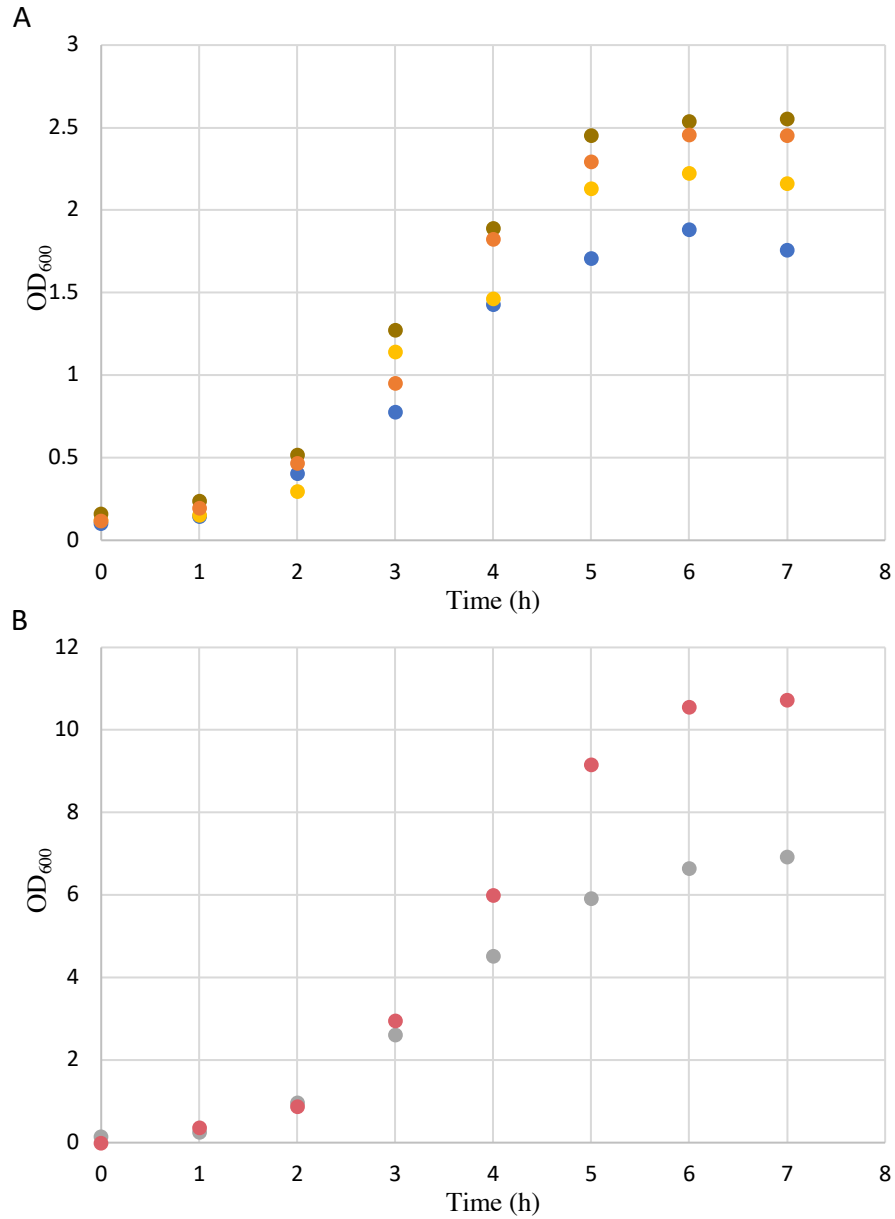
Figure 9: Growth of *S. aureus* JE2 in different media in a Bioreactor, with a 37ºC, pH 7, controlled by NaOH and HCl solutions. A) Pattee Neveln (PN) at 5% (•) and 21% (•) oxygen, with a glucose concentration of 27 mM, and 5% (•) and 21% (•) oxygen, with a glucose concentration of 60 mM. b) Luria-Bertani (LB) medium at the at 5% (•) and 21% (•) oxygen,

Since most of the times bacterial growth is limited is a result of an insufficient availability of carbon source, we increased the glucose concentration from 27 mM to 60

mM. The change in glucose concentration resulted in an OD_{600} of 2.5 for the 21% O_2, while at 5% O_2 the OD_{600} was 1.8 (**figure 9**). Not varying significantly from the growth performed at a lower glucose concentration, which proved that glucose was not the limiting factor.

Since growth in chemical defined media was limiting the growth of *S. aureus* JE2, we started by performing growth of this strain in the bioreactor at the predefined conditions (37ºC and pH 7) in Luria-Bertani (LB) broth, for the O_2 concentrations of 5% and 21%.

In LB, a complex and rich medium, *S. aureus* was capable of achieving the OD_{600} of 10.7 and 6.9 for the 21% and 5% oxygen, respectively, much higher values than the ones obtained for the previously used media (**figure 9**).

It was decided to proceed with the use of TSB medium, a typical growth medium used in *S. aureus* studies, given its use in studies of gene expression for this type of bacteria and given that showed similar optical densities as LB.

2. Growth of *Staphylococcus aureus* in the complex medium Tryptic Soy Broth is affected by oxygen concentrations.

We ascertained Tryptic Soy Broth (TSB) as one of the most used media in growth of *S. aureus* for works that had the finality of microarrays and gene analysis.

As stated before, throughout the human body the concentration of oxygen varies with which tissue, and in order to test the ability and mechanism used for survival by the pathogen in study, it was preformed growth under the controlled conditions of pH (7), medium (TSB) and temperature (37ºC), changing the supplementation of oxygen (0%, 2%, 5%, 10% and 21%) in the Bioreactor Biostat A.
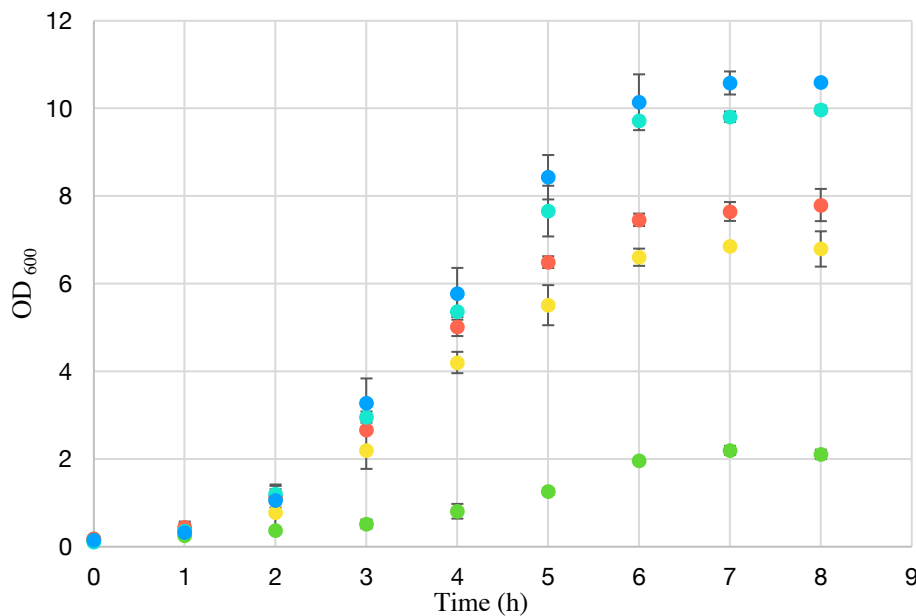
Figure 10: Growth of *S. aureus* JE2 in TSB medium at 37ºC, pH 7, controlled by NaOH and HCl solutions, and at 0% (●), 2% (●), 5% (●), 10% (●) and 21% (●) oxygen.

In this assay it is simple to observe the effect of different concentrations of oxygen in *S. aureus*' growth. *S. aureus* formed a plateau at the $OD_{600} \approx 10.5$ for the 21%, atmospheric condition, and 10%, high concentration in human blood, oxygen concentration. While 5%, high oxygen concentration in organs, and 2%, hypoxic condition, showed another plateau of a value of OD_{600} of approximately ≈ 7. Lastly, for the complete anaerobic condition, 0%, an $OD_{600} \approx 2.2$ (**figure 10**).

3. Growth of *Staphylococcus aureus* improves when nitrate is used electron acceptor

In order to ascertain the concentration of sodium nitrate that should be used in this study, we performed growth in flasks with different concentrations of sodium nitrate, namely, 2 mM, 5 mM, 10 mM and finally 20 mM, under anaerobic conditions.

After deciding 2 mM of sodium nitrate concentration, as so growths were performed with this concentration in the bioreactor in the conditions specified before but only for the oxygen concentration of 0%.
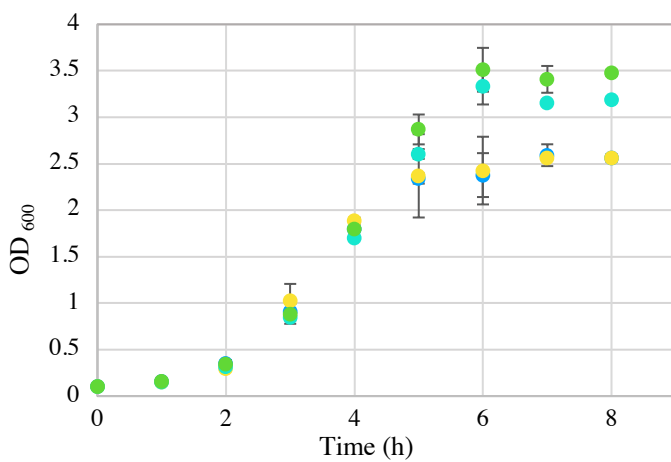
Figure 11: Growth in flasks in a strict anaerobic atmosphere of *S. aureus* JE2 in TSB with different nitrate concentrations. Growth performed at 37ºC supplemented with sodium nitrate in the concentration of 2 mM (●), 5 mM (●), 10 mM (●) and 20 mM (●).
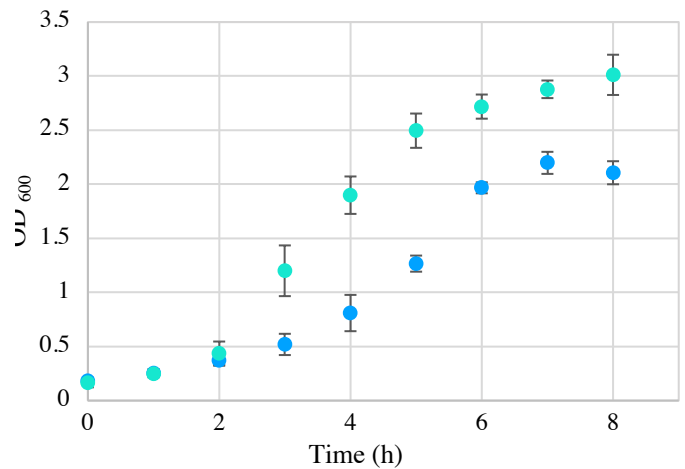
Figure 12: Growth of *S. aureus* JE2 in TSB medium supplemented or not with 2 mM of sodium nitrate. Growth performed at 37ºC, pH 7, controlled by NaOH and HCl solutions, in complete anaerobic atmosphere (0% oxygen) and with the conditions of supplemented (●) and not supplemented (●) with sodium nitrate.

It's possible to observe that there is little difference between the 2 mM and the 5 mM condition a considerable difference between these two conditions and the conditions on 10 mM and 20 mM of sodium nitrate (**figure 11**).

In the condition where the media was supplemented with 2 mM of nitrate there was an increase, since it reached an OD_{600} of ≈ 3 and the non-supplemented condition reached ≈ 2.1, which suggests that the supplementation of nitrate improves growth conditions for *S. aureus* under anaerobic conditions (**figure 12**).

4. *Staphylococcus aureus*' Gene Expression is differently affected by physiological conditions

4.1. *nirR*, *nirB*, *sirA* e *sirC* are differently expressed depending on the growth phase and oxygen concentration

To survive under anaerobic conditions, *S. aureus* switches to a fermentative metabolism, or, when in the presence of the respective electron donors (nitrate), uses the anaerobic respiration pathway. The *nirR*, *nirB*, *sirA* e *sirC* are genes that putatively encode, respectively, a transcriptional regulator, the large subunit of the enzyme nitrite reductase, and two enzymes that are involved in siroheme biosynthesis, namely uroporphyrin-III C-methyltransferase and precorrin-2 dehydrogenase.

To assess the effect of oxygen concentration on the expression levels of these four genes, *S. aureus* were grown in TSB medium in the bioreactor biostat A with different controlled oxygen amounts. Total RNA was extracted from these cells in early log (EL) stage (2h), mid-log (ML) (3.5 h) and late log (LL) (6 h), purified and reverse transcribed to cDNA, which served as template for qRT-PCR. The quantification of the expression levels was normalized using the 16S gene, which is a constitutively expressed gene.

It was then evaluated the fold change for the ML and the LL, relative to the EL phases.

The data shows that the expression of *nirR*, *nirB* and *sirA* genes do not change significantly when the cells are grown with 21%, 10% and 5% of O_2 (**figure 13: A/B/C**).

In the 2%of O_2 we can observe a fold of ≈ 4.5 for the genes in the *nir* operon *nirR*, *nirB* and *sirA*, from the 2-hour mark to the 3.5-hour mark and then a significant decrease in gene expression when evaluate the 6-hour mark fold, reducing to ≈ 0.3-fold relative to the early log phase (**figure 16:D**).

S. aureus cells grown under completely anaerobic conditions shows an extremely high fold change of ≈ 10 for the *nirB* and *sirA* gene and a further significant increase from the middle to the late log, reaching values of 12.2 for *nirR*, 20 for *nirB and sirA* and 10 for *sirC* (**figure 13:F**).
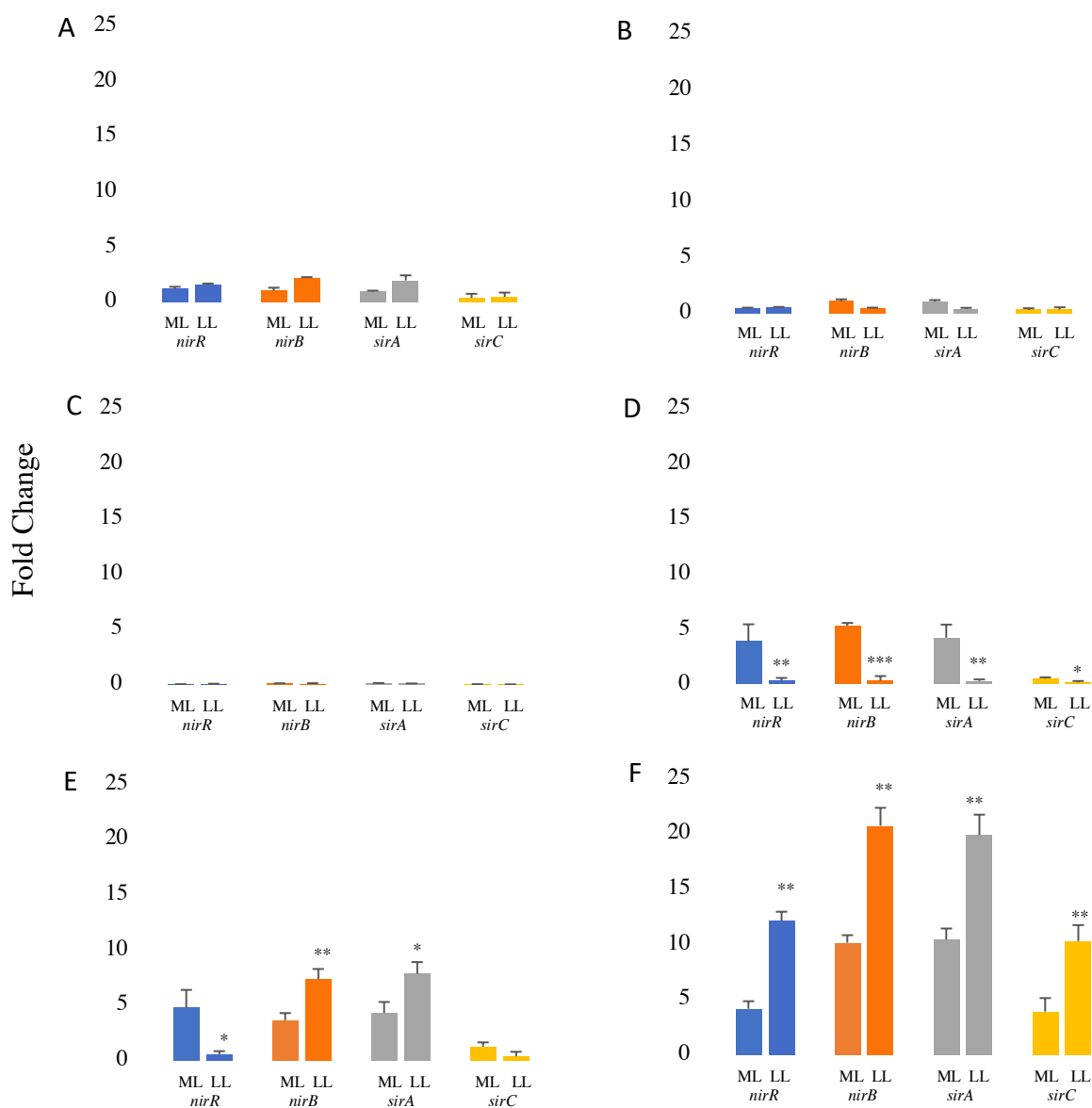
Figure 13: Effects of oxygen concentration on the transcription levels of *S. aureus* JE2 *nirR*, *nirB*, *sirA* and *sirC* genes. The genes *nirR* (■), *nirB* (■), *sirA* (■) and *sirC* (■) are represent the bars. All growths were performed in TSB in the bioreactor Biostat A 1L-Vessel, with pH controlled, by the system, at 7 and temperature maintained, by the system, at 37ºC in order to maintain human physiological conditions. A) Growth performed at a controlled oxygen concentration of 21% (atmospheric conditions), completely aerobic; B) Growth performed at a controlled oxygen concentration of 10%; C) Growth performed at a controlled oxygen concentration of 5%; D) Growth performed at a controlled oxygen concentration of 2%, hypoxic condition; E) Growth performed at a controlled oxygen concentration of 0%, supplemented with $NaSO_3$ (sodium nitrate) at the beginning of growth at a 2 mM concentration. F) Growth performed at a controlled oxygen concentration of 0%, anaerobic condition. Asterisks represent statistically significant data, relative to the control; $*P < 0.05$, $**P < 0.01$ and $***P < 0.0001$.

4.2. *nirR*, *nirB*, *sirA* e *sirC* are differently expressed depending on the growth phase and the presence of nitrate.
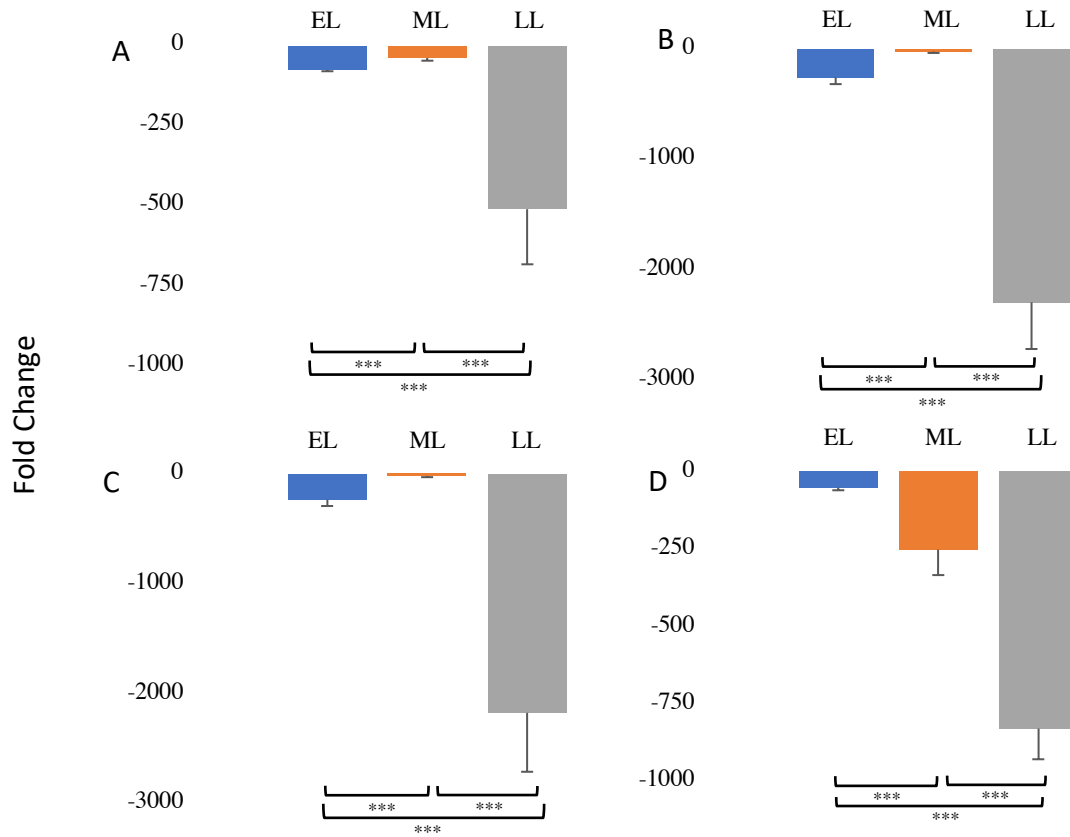
Figure 14: Effects of Nitrate supplementation on the transcription levels of *S. aureus* JE2 *nirR*, *nirB*, *sirA* and *sirC* genes. Comparison, of the effects of the presence of nitrate during the growth at the early, middle and late log(EL; ML; LL) in a anaerobic condition in the bioreactor biostat A with temperature and pH regulated to human physiological conditions, 7 and 37ºC, respectively. A) Variation on *nirR* gene expression throughout the growth in comparison to the expression on non-supplemented anaerobic growth. B) Variation on *nirB* gene expression throughout the growth. C) Variation on *sirA* gene expression throughout the growth. D) Variation on *sirC* gene expression throughout growth. ***$P < 0.0001$.

To assess the effect of nitrate in the medium on the expression levels of *nirR* , *nirB*, *sirA* and *sirC*, cells of *S. aureus* were grown in TSB medium supplemented with sodium nitrate and harvested in the EL, ML and LL growth phases. The relative fold change was calculated by comparing cells grown in anaerobic conditions supplemented with 2 mM sodium nitrate with cells grown in the same conditions without sodium nitrate for each growth phase.

In the anaerobic condition supplemented with sodium nitrate, all genes presented a lower fold change when compared with the non-supplemented growth. It can also be observed that at the LL compared to ML, the gene expression changes significantly in all genes, decreasing for *nirR* from 5-fold to a 0.6-fold, and increasing for *nirB* and *sirA* from ≈5-fold to a ≈ 8-fold, *sirC* showed little fold change and non-significantly variation form the exponential to the LL phase compared to the ML, maintaining its fold at an average of ≈ 1 (**figure 13:E**).

Three of the four studied genes, namely *nirR*, *nirB* and *sirA* showed the same pattern of expression. When compared to the non-supplemented growth it is possible to see that all the genes in the *nir* operon, show similar behavior, even though changes in *nirR* are always lower (**figure 14**). At the EL, all of the three genes have immensely lower expression levels in the supplemented growth, this is possible to see, because the fold change is negative and for *nirB* and *sirA*, this fold change is ≈ -250, while *nirR* ≈ -100.

At the middle log phase, the expression of these three genes is equal in both conditions. Suffering a large decrease for the LL in the non-supplemented growth, of ≈ -3000 for *nirB* and *sirA* and ≈ -1000 for *nirR*.

sirC genes shows a different pattern from the genes in the *nir* operon, throughout the growth the supplemented condition showed a decreasing expression level when compared to the non-supplemented (**figure 14:D**).

5. Deletion of genes in the *nir* operon results in the growth impairment of *S. aureus* in an anaerobic environment supplemented with nitrites

Several *S. aureus* NARSA mutants were selected, namely, ΔnirB, ΔnirD, ΔnirR and ΔsirC and they were grown in TSB supplemented with sodium nitrite at 2 mM, under anaerobic conditions, in 50ml flasks. The growth of *S. aureus* mutants and wild type (WT) strains was monitored by measuring the OD_{600}, and the Greiss method was used to monitor the nitrite consumption during growth.
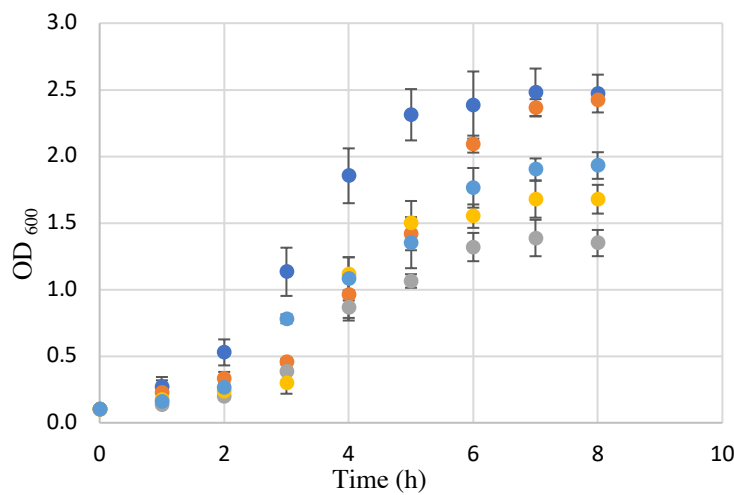
Figure 15: Growth of *S. aureus* NARSA mutants *ΔnirB*, *ΔnirD*, *ΔnirR* and *ΔsirC* and WT strain JE2, in anaerobic condition, supplemented with 2 mM of sodium nitrite. Growth measured hourly through OD_{600} in TSB, of the strains JE2 (●); *ΔnirB* (●); *ΔnirD* (●); *ΔnirR* (●); *and ΔsirC* (●).

Figure 16: Nitrite concentration during growth of *S. aureus* JE2 and mutants growth *ΔnirB*, *ΔnirD*, *ΔnirR* and *ΔsirC*. Concentration of nitrite in the medium during *S. aureus* growth, obtained by the Greiss method, and expressed in mM. **.** JE2 (●); *ΔnirB* (●); *ΔnirD* (●); *ΔnirR* (●); *and ΔsirC* (●).
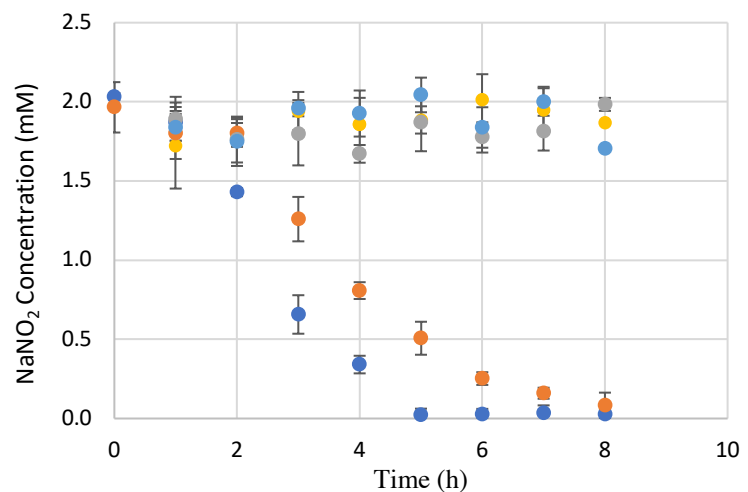
Figure 17 shows that the JE2 wild type strain and the Δ*sirC* strain reached the same OD$_{600}$ as the WT after 8 hours of growth while Δ*nirB*, Δ*nirD* and Δ*nirR* showed a different pattern of growth. Δ*sirC* showed a slightly slower replication rate when compared to the WT while the other mutation, Δ*nirB*, Δ*nirD* and Δ*nirR*, showed much lower OD$_{600}$.

The nitrite consumption also varies among strains (**figure 16**). *ΔsirC* is capable of consuming all of the nitrite present in the medium, even if at a slower rate than the wild type. Along the entire growth period Δ*nirB*, Δ*nirD* and Δ*nirR* showed no consumption of nitrites maintaining a steady value of 2 mM present in the medium.

Discussion

Staphylococcus aureus is a major constituent of the human nasal microbiota. However, it is also an important nosocomial pathogen, being highly associated with community acquired and health cared associated infections. Given the high pathogenicity and ability to infect different tissues *S. aureus* is capable of switching between different metabolic pathways in order to survive.

S. aureus has to adapt to fluctuations in oxygen concentration within the human body, varying from a 12.5% to almost completely anaerobic.

In order to adapt when oxygen is absent, *S. aureus* performs fermentation or anaerobic respiration. In the latter, siroheme is used as the co-factor of nitrite reductase, the enzyme responsible for the conversion of nitrite to ammonia.

Therefore, we thought to investigate the role of the genes involved in siroheme biosynthesis pathway and the nitrite reductase enzyme in the adaptation of *S. aureus* to different oxygen concentrations encountered in the human host, as well as, the effects of the presence of nitrate and nitrite.

In this work, we showed the formation of two growth plateaus for different oxygen concentration (**figure 10**). The first one corresponds to *S. aureus* cells grown in 21% and 10% oxygen which correspond, respectively, to the oxygen concentrations in the atmosphere, and in arterial blood (38).

The second plateau was verified for the 5% and the 2%, being that 5% is the oxygen concentration associated with the organs with a rich oxygen concentration (41) and 2%. During the growth of *S. aureus* in the several oxygen concentrations, cells were collected in the early log (EL), middle log (ML) and late log (LL) stages of growth. This allowed us to study the expression of *nirR*, *nirB*, *sirA* and *sirC* throughout the growth.

We saw that under oxygenated conditions, more exactly, the 21%, 10% and 5% conditions, these genes were not expressed in any of the growth stages (**figure 12)**. This result was expected, given that these genes are putatively involved in anaerobic pathways such as nitrite utilization. Moreover, it has been shown previously that the expression of this genes, mainly the *nir* operon, is dependent on an oxygen responsive regulator such as NreABC, when *S. aureus* is under anaerobic conditions (145).

Under 2% of oxygen, the hypoxic condition on the human body, it was expected to see a decrease in growth when compared relatively to the 5% of O_2 (**figure 12**). However, that was not verified, and indeed cells grown under 2% of oxygen can support growth as well as cells grown in 5% of oxygen. Contrarily, when we observe the levels of expression of *nirR*, *nirB* and *sirA* genes in the 2% condition we see an upregulation of this genes in the ML stage (**figure 15: C/D**). As stated previously *S. aureus*' ability to upregulate not only anaerobic respiration genes under low-oxygen condition, but also upregulate fermentation metabolic pathways can be the explained by the capability of compensating the lack of oxygen during growth in hypoxic conditions, (145).

In completely anaerobic conditions (0% O_2), *S. aureus* showed a lower OD_{600} value, which is expected since without any oxygen, *S. aureus* reverts to two pathways in order to obtain ATP: fermentation and anaerobic respiration (**figure 12**) (146). In the absence of anaerobic respiration electron donors, it will revert to fermentation, which is a less efficient process, producing much less ATP than aerobic and anaerobic respiration (147).

When grown in completely anaerobic conditions but supplemented with nitrate, *S. aureus* reaches higher cellular density (**figure 12**). It was shown for *Staphylococcus carnosus* that the presence of nitrate or nitrite resulted in the use of glucose in fermentation, but also in anaerobic respiration, which is more energetically efficient (147).

In the literature, it can be found that *Staphylococcus carnosus* expresses genes involved in anaerobic respiration, even in the absence of the nitrate or nitrites. In this work we confirm that, in the non-supplemented 0% condition, genes involved in nitrite respiration (*nirR* and *nirB*) and genes involved in siroheme biosynthesis (*sirA* and *sirC*) all showed upregulation throughout the growth (**figure 15:F**) (148).

The same pattern was not observed in the supplemented anaerobic growth, in this case, *nirB* and *sirA* show an upregulation, even though the fold value is lower in both the ML and LL when compared to the not supplemented (**figure 15:E**).

But in this case *sirC* and *nirR* suffer a upregulation in the ML, for *nirR* is a fold value close to the one of the not supplemented, and then a downregulation in the LL for both of the genes, this is a phenomenon not described in the found literature, since most genes in the *nir* operon in other bacteria show the same level of expression and usually similar pattern as the one observed in the non-supplemented anaerobic growth.

It is possible to see in both of these conditions, that the genes annotated to be on the same operon *nirR*, *nirB* and *sirA* are not expressed in the same way, being that *nirB* and *sirA* show levels of expression always similar to each other, but not the same has the one observed in *nirR* (**figure 15:E/F**). These results may suggest that *nirR* is transcribed independently of *nirB* and *sirA*, and therefore is not part of the operon.

When we compare the expression values of *nirR*, *nirB*, *sirA* and *sirC* between the supplemented and the non-supplemented anaerobic growth it can be seen less expression in the first condition in the EL for all the genes studies, followed by an equal expression in both conditions in ML for the gene *nirR*, *nirB* and *sirA*, while an even bigger downregulation for the *sirC*, and then a global repression of all the studied genes for the LL (**figure 14**).

In a study done in *Rhizobium* it is possible to see, that nitrite accumulate in the cytoplasm of bacteria, while there's still nitrate presence, reaching a complete depletion of nitrate at the 4-hour mark, close to our ML stage. During these first hours (EL) there is no need for genes involved in the nitrite reduction, but for the nitrate reduction, which can result in a repression of this operon. In the ML, after all nitrate has been consumed, nitrite reducing enzymes are necessary which results in an upregulation of these genes similar to the one observed in the in the non-supplemented (149).

In the end, this same study has shown that at the six-hour mark (LL) we see the decrease of expression levels on the supplemented experiment, this may be because all nitrite has been reduced to ammonia, so there is no need for the expression of nitrite reductase or enzymes responsible for its co-factors, which explains the reduced levels of expression in the supplemented growth, when compared to the non-supplemented, but no study has been done in the possible repression effects of nitrate or ammonia *nir* operon.

We have shown that without the genes *nirB* and *nirD*, which encode the large and the small subunit, respectively, of nitrite reductase, *S. aureus* is unable to reduce nitrite to ammonia which results in a steady presence of the original concentration of nitrite in the medium, and a clear effect on bacteria growth (**figure 17/18**). The same was observed for the other gene present in the operon *nirR*, which is annotated as a regulator of this operon (**figure 17**).

In a study done in *Bacillus megaterium*, it was proven that without precorrin-2 dehydrogenase, *sirC*, the precorrin-2 dehydrogenation step can still spontaneously occur.

This would translate in the results observed in this work for *S. aureus*, since consumption of nitrite is still done, even in a lower rate, and the same can be said, when compared to the JE2. This can be explained by this step of siroheme production, occurring naturally without the enzyme, encoded by *sirC*, but without the enzyme the process is slower, which would result in a lower availability of siroheme and without this co-factor a lower efficiency of nitrite reductase (150).

Conclusion

The development of new therapeutic strategies to control community acquired and health-care associated infections, is an important goal, since there is the emerging threat of multi-resistance bacteria. In order to develop these new therapeutic strategies, it is required a deeper understanding, of the mechanisms of survival and adaptation in the host, and by these means understand its limitations.

In this work, the ability of *S. aureus* to survive in different oxygen concentrations was studied and it was shown that the bacterium has the ability to survive from the most oxygenated to the depleted of oxygen environments. We highlighted some mechanisms of survival, under aerobic, hypoxic and anaerobic states. It was possible to understand the importance of the genes involved in siroheme production and nitrite reduction in this adaptation. And also understand the upregulation and downregulation of the *nir* operon, during the different stages of respiration, showing how the presence of nitrate can downregulate de expression of the studied genes.

It was also shown that in most conditions *nirR*, the regulator of the operon, is transcribed differently than the other genes of the operon, *nirB* and *sirA*, which suggests the presence of another promoter in the middle of the *nirR* and *nirB* genes.

Moreover, it was discovered, that *sirC* doesn't play such impactful role in the nitrite reduction, since its deletion did not greatly affect the growth.

Further studies are necessary to discover the possible promoter between the *nirR* and *nirB* genes, bioinformatic methods should be applied. The role of *nirR* should be studied more profoundly, in order to better understand this gene and how does it regulate and is regulated, since the expression pattern and function is not completely understood.

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