

Effect of Oxygen on the Competition between *Pseudomonas aeruginosa* and
Staphylococcus aureus

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved November 2014 by the
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December 2014

ABSTRACT

The viscous lung mucus of cystic fibrosis (CF) patients is characterized by oxygen gradients, which creates a unique niche for bacterial growth. *Pseudomonas aeruginosa* and *Staphylococcus aureus*, two predominant microorganisms chronically infecting the airways of CF patients, typically localize in hypoxic regions of the mucus. While interspecies interactions between *P. aeruginosa* and *S. aureus* have been reported, little is known about the role of low oxygen in regulating these interactions. Studying interspecies interactions in CF lung disease is important as evidence suggests that microbial community composition governs disease progression. In this study, *P. aeruginosa* lab strain PAO1 and two primary clinical isolates from hypoxic tissues were cultured alone, or in combination, with methicillin resistant *S. aureus* (MRSA) strain N315 under hypoxic or normoxic conditions. Herein, it is shown for the first time that low oxygen conditions relevant to the CF lung affect the competitive behavior between *P. aeruginosa* and *S. aureus*. Specifically, *S. aureus* was able to better survive competition in hypoxic versus normoxic conditions. Competition data from different oxygen concentrations were consistent using PAO1 and clinical isolates even though differences in the level of competition were observed. PAO1 strains carrying mutations in virulence factors known to contribute to *S. aureus* competition (pyocyanin/*phzS*, elastase/*lasA* and *lasI* quorum sensing/ Δ *lasI*) were used to determine which genes play a role in the differential growth inhibition. The *lasA* and *lasI* mutants competed less effectively with *S. aureus* regardless of the oxygen level present in the culture compared to the isogenic wild type strain. These results are consistent with previous findings that elastase and *lasI* quorum sensing play a role in competitive behavior of *P. aeruginosa* and *S. aureus*. Interestingly, the *phzS* mutant competed less effectively in

hypoxic conditions suggesting that pyocyanin may be important in microaerophilic conditions. This study demonstrates that oxygen plays a role in competition between *P. aeruginosa* and *S. aureus* and contributes to understanding CF environmental factors that may regulate microbial community dynamics important for disease progression with potential for development of therapeutic avenues.

ACKNOWLEDGMENTS

To my family who has been my rock during my whole life, for always being there and for their unconditional love and support. I would not know what I would have done without you.

To Dr. Cheryl Nickerson for the financial support given to my research and for her valuable insight.

To Dr. Aurelie Crabbe, my Research Mentor, for believing in me, for her guidance and for introducing me to this wonderful world of *Pseudomonas aeruginosa*.

To Drs. Josephine Clark-Curtiss, Valerie Stout, Jorge Reyes del Valle and Mark Ott, my Committee Members, for their valuable comments.

To Drs. John Alverdy and Olga Zaborina, for providing the strains used in this study.

To Dr. Juan Xicohtencatl for his advice and support in all these years. Thank you.

To the members of the Nickerson Lab, especially Richard Davis, whom without his help this thesis would have not be completed on time.

To my friends for their support and encouragement throughout this process.

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A BRIEF BACKGROUND ON *Pseudomonas aeruginosa*

The information presented in this background will be used in the chapter “*Pseudomonas aeruginosa* response to spaceflight and low shear modeled microgravity: Implications for astronaut health and the clinic” to be published in *Effect of Spaceflight and Analogue Culture on Human and Microbial Cells: Novel insights into Disease Mechanisms*; Nickerson, C.A., Ott, C.M., Pellis, N., Eds.; Springer-Verlag.

1.1 Occurrence and clinical importance

Pseudomonas aeruginosa is a member of the Gamma Proteobacteria class of bacteria, belonging to the Pseudomonaceae family. Carle Gessard, a chemist and bacteriologist from Paris, discovered this microorganism in 1882 (Gessard, 1882). This facultative, motile, non-lactose fermenting Gram Negative rod is a highly adaptable bacterium that can colonize various environmental niches such as plants, animals, soil and marine environments (Khan, Ahsan, Taylor, & Kogure, 2010; Kimata, Nishino, Suzuki, & Kogure, 2004; Stover et al., 2000). *P. aeruginosa* is an opportunistic pathogen that is responsible for 10-15% of the nosocomial infections worldwide (Blanc, Petignat, Janin, Bille, & Francioli, 1998), causing infections in the skin, eyes, urethra, ear and the respiratory tract of immunocompromised patients. In particular, *P. aeruginosa* causes severe infections in burn wound and cystic fibrosis (CF) patients. *P. aeruginosa* infections are difficult to treat because of their natural resistance and their ability to acquire resistance to several groups of antimicrobial agents. This microorganism has many of the known intrinsic and acquire antibiotic resistance mechanisms (Pechere & Kohler, 1999), which results in a multi-drug resistant phenotype (McGowan, 2006).

1.2 Virulence factors

1.2.1 Quorum sensing

P. aeruginosa regulates many virulence factors using interbacterial communication, called Quorum Sensing (QS). QS is a signaling mechanism that bacteria use to regulate gene expression in a population density-dependent manner. In this system, bacteria produce and secrete small signaling molecules called autoinducers; when these molecules reach a concentration threshold, they diffuse back into the cell and coordinate a response that will allow the bacteria to promote their survival as a group (Stevens, Schuster, & Rumbaugh, 2012). *P. aeruginosa* uses QS to regulate the production of multiple virulence factors such as motility, biofilm formation, efflux pump expression, elastase, catalase, pyocyanin, rhamnolipid production, lectins, iron chelators, and exotoxin A (Williams & Camara, 2009) (Gambello, Kaye, & Iglewski, 1993; Hassett et al., 1999; Ochsner & Reiser, 1995; Toder, Ferrell, Nezezon, Rust, & Iglewski, 1994) (Brint & Ohman, 1995; Latifi et al., 1995; Pearson, Pesci, & Iglewski, 1997; Winzer et al., 2000).

P. aeruginosa possesses three quorum-sensing systems: Las, Rhl and PQS with their corresponding autoinducers *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C₁₂-HSL), *N*-butanoyl-L-homoserine lactone (C₄-HSL) and 2-heptyl-3-hydroxy-4-quinolone, respectively (Gambello & Iglewski, 1991; Passador, Cook, Gambello, Rust, & Iglewski, 1993; Tashiro, Yawata, Toyofuku, Uchiyama, & Nomura, 2013).

The Las system comprises the LasI synthase protein and the LasR transcriptional regulator. LasI produces the acyl homoserine lactone (AHL) autoinducer molecule 3-oxo-C₁₂-HSL (Gambello & Iglewski, 1991; Pearson et al., 1994). In order for LasR to become an active

transcription factor, it binds to 3-oxo-C₁₂-HSL, which allows this multimer complex to bind to DNA and regulate gene transcription (Kiratisin, Tucker, & Passador, 2002). The Rhl system consists of the RhlI synthase which produces the AHL C₄-HSL and the transcriptional regulator RhlR, which upon binding its cognate autoinducer is activated to regulate gene expression (Ochsner, Koch, Fiechter, & Reiser, 1994; Pearson, Passador, Iglewski, & Greenberg, 1995). For the *Pseudomonas* quinolone signal (PQS) system, the PQS autoinducer signal is recognized by the cognate receptor PqsR and regulates the production of PQS along with other virulence factors such as pyocyanin, rhamnolipids and elastase (Gallagher, McKnight, Kuznetsova, Pesci, & Manoil, 2002).

1.2.2 Biofilm formation and exopolysaccharides

Biofilms are multicellular bacterial communities associated with a surface, and encapsulated in an extracellular polymeric matrix (EPS) made up of nucleic acids, proteins and polysaccharides that facilitate the interaction between cells and between cells and a surface (Flemming & Wingender, 2010). Biofilm formation can form on abiotic (non-biological) and biotic surfaces (biological *e.g.* wounds, surgical implants, mucus in CF lung) (Donlan & Costerton, 2002), respectively and confers resistance against antimicrobial agents and immune mechanisms of defense, and disinfectants (Harmsen, Yang, Pamp, & Tolker-Nielsen, 2010). Briefly, biofilm occurs in a process that is initiated by surface attachment of planktonic bacteria forming a monolayer and multiplication; aggregation leading to microcolony formation and differentiation into structured, mature, antibiotic-resistant communities (Costerton, Stewart, & Greenberg, 1999; Lopez,

Vlamakis, & Kolter, 2010; O'Toole & Kolter, 1998; Stapper et al., 2004). In the CF lung mucus, *P. aeruginosa* forms a non-conventional type of biofilm, in which bacteria are self-attached and are adhering to mucus compounds, and not the epithelial cell surface (Alhede et al., 2011; Lam, Chan, Lam, & Costerton, 1980). The following virulence factors are involved in *P. aeruginosa* biofilm formation: QS, type IV pili, flagella, free DNA, alginate, Pel and Psl polysaccharides and rhamnolipids (Boles, Thoendel, & Singh, 2005; Klausen et al., 2003; Singh et al., 2000; Stapper et al., 2004). The Psl-encoded exopolysaccharide is rich in mannose and galactose, and is involved in attachment, biofilm maintenance, and self-aggregation (Friedman & Kolter, 2004; Jackson, Starkey, Kremer, Parsek, & Wozniak, 2004) (Jackson et al., 2004; Matsukawa & Greenberg, 2004) (Hausler et al., 2003; Ma, Jackson, Landry, Parsek, & Wozniak, 2006; Matsukawa & Greenberg, 2004; Sarkisova, Patrauchan, Berglund, Nivens, & Franklin, 2005; Smith et al., 2006). The Pel exopolysaccharide is rich in glucose, and is involved in the formation of pellicles in *P. aeruginosa*, i.e. microbial aggregations at the interface between air and liquid (Yamamoto, Arai, Ishii, & Igarashi, 2011, 2012; Yamamoto, Haruta, Kato, Ishii, & Igarashi, 2010). In the lungs of patients with CF, *P. aeruginosa* can convert from a non-mucoid to a mucoid phenotype through overproduction of the exopolysaccharide alginate, indicating a chronic infection (Pressler et al., 2011). Alginate is a polymer of β -D-mannuronic acid and α -L-guluronic acid (Evans & Linker, 1973) that protects *P. aeruginosa* from phagocytosis and antibodies, giving the bacteria a survival advantage. Mucoidity conversion follows when biofilms are exposed to polymorphonuclear leucocytes (Mathee et al., 1999), antibiotics

(Govan & Fyfe, 1978), hydrogen peroxide (Mathee et al., 1999) and nutrient starvation (Speert et al., 1990; Terry, Pina, & Mattingly, 1991).

The key player in the regulatory pathway leading to alginate production is the alternative sigma factor AlgU (also known as AlgT). AlgU is regulated by the *algUmucABCD* operon (DeVries & Ohman, 1994; Martin, Holloway, & Deretic, 1993). In the absence of a specific stimulus or in non-mucoid strains, the anti-sigma factor MucA binds AlgU, causing a decrease in the expression of promoters targeted by AlgU. MucB, which is associated to the periplasmic domain of MucA, is a negative regulator for the synthesis of alginate. In addition, AlgR connects alginate production with antibiotic resistance and QS by negatively regulating *algT/U* expression (Balasubramanian et al., 2011).

1.2.3 Exoproteases

P. aeruginosa produces exoproteases including elastases, alkaline protease and phospholipase C (Plc). The elastolytic zinc metalloprotease elastase B (LasB) is a major virulence factor in *P. aeruginosa* (McIver, Kessler, & Ohman, 2004; Morihara, 1964). LasB, also known as pseudolysin, is believed to cause tissue damage in the host by hydrolysis of extracellular matrix constituents and by attacking intercellular tight junctions (Azghani, 1996; de Bentzmann et al., 2000). It has also been shown to degrade elements of the immune system such as TNF- α , IFN- γ , IL-2, IL-8 and surfactant protein A (SP-A) (Azghani, 1996) (Horvat, Clabaugh, Duval-Jobe, & Parmely, 1989) (Leidal, Munson, Johnson, & Denning, 2003) (Parmely, Gale, Clabaugh, Horvat, & Zhou, 1990) (Theander et al., 1988) and antibacterial peptides (Schad et al., 1987). In addition to LasB, the LasA

protease (also called staphylolysin) is a 20-kDa endopeptidase secreted by *P. aeruginosa* that has the ability to lyse *Staphylococcus aureus* (E. Kessler, 1995; E. Kessler, Safrin, Olson, & Ohman, 1993) (E. a. D. E. O. Kessler, 1998). LasA is able to hydrolyze the pentaglycine bridge required for peptidoglycan stabilization in the cell wall of staphylococci, but has a low level of elastolytic activity and rather is thought to enhance the elastolytic activity of LasB (E. Kessler, Safrin, Abrams, Rosenbloom, & Ohman, 1997).

1.2.4 Rhamnolipids

P. aeruginosa produces rhamnolipids, bacterial glycolipidic biosurfactants (Abdel-Mawgoud, Lepine, & Deziel, 2010) that act as heat-stable extracellular hemolysins (Johnson & Boese-Marrazzo, 1980), and lyse polymorphonuclear leukocytes (PMNs) (Jensen et al., 2007) and monocyte-derived macrophages (McClure & Schiller, 1992), causing necrotic cell death. Rhamnolipids have also been detected in sputum from CF patients chronically infected with *P. aeruginosa* (Read et al., 1992).

The synthesis of rhamnolipids is carried out by two glycosyl transfer reactions, catalyzed by a specific rhamnosyltransferase (Burger, Glaser, & Burton, 1963). The first rhamnosyltransferase is encoded by the *rhlAB* operon, and is in charge of the formation of mono-rhamnolipids; the second rhamnosyltransferase, encoded by the *rlhC* gene, converts mono-rhamnolipids into di-rhamnolipids. The expression of these genes is regulated by the *rhl* QS and PQS systems (Deziel, Lepine, Milot, & Villemur, 2003). Antimicrobial activity of rhamnolipids have been observed against Gram-negative bacteria such as *Serratia*, *Klebsiella* and *Enterobacter*, Gram-positive species including *Mycobacterium*, *Staphylococcus*, *Bacillus* and *Rhodococcus* (Abdel-Mawgoud et al., 2010) and also

against fungi but not yeast (Abdel-Mawgoud et al., 2010; Haba et al., 2003). Another important function of rhamnolipids is the promotion of swarming motility, defined as a rapid and coordinated multicellular movement of bacteria across a semi-solid surface (Caiazza, Shanks, & O'Toole, 2005).

1.2.5 Phenazines

Phenazine compounds are secondary metabolites that are known as redox-active and pigmented antibiotics. In *P. aeruginosa*, these compounds include pyocyanin, phenazine-1-carboxylic acid (PCA), 1-hydroxyphenazine (1-OH-PHZ) and phenazine-1-carboxiamide (PCN), which are regulated by QS. Pyocyanin is a blue pigment produced and secreted in stationary phase and upregulates genes that have been demonstrated to be controlled by QS (Dietrich, Teal, Price-Whelan, & Newman, 2008). Because pyocyanin can cross biological membranes easily, it serves as a mobile electron carrier for *P. aeruginosa*. In the soluble phase of CF sputum, pyocyanin has been detected at concentrations of up to 27.3 mg/ml (130 mM) (Wilson et al., 1988). Hunter et al. revealed that the concentration in sputum from CF correlated with the decline in lung function and in the degree of severity of the disease fluctuating from 7.7 mM in unobstructed airways to 46.8 mM in severely obstructed airways (Paroutis, Touret, & Grinstein, 2004).

1.2.6 Lectins

The ability of pathogenic microorganisms to adhere to host tissues is critical for the onset of infection. Adhesion is frequently mediated by surface glycoconjugates which are recognized by specific receptors (Karlsson, 2001). In *P. aeruginosa*, this adhesion is mediated by numerous adhesins such as lectins. LecA and LecB, two soluble lectins that bind to galactose and fucose respectively, were described in the cytoplasm of this bacterium (Glick & Garber, 1983); at the same time, large amounts of these lectins were also found in the outer membrane, suggesting that the lectins could play a role in adhesion (Glick & Garber, 1983; Tielker et al., 2005). Lectins are regulated by QS and the alternative sigma factor RpoS (Winzer et al., 2000). LecA has been shown to: a) have cytotoxicity on respiratory epithelial cells through a decrease in their growth rate (Bajolet-Laudinat et al., 1994); b) induce a permeability defect in the intestinal epithelium, causing an increase in absorption of exotoxin A (Laughlin et al., 2000). LecB was shown to be involved in protease IV activity and pilus biogenesis (Sonawane, Jyot, & Ramphal, 2006).

1.2.7 Hfq

Hfq is a highly conserved RNA chaperone that binds to small non-coding regulatory RNAs thereby facilitating their association with mRNAs, the result of which plays a diverse role in post-transcriptional regulation of prokaryotic gene expression, virulence, and physiology in response to stress (reviewed by (Majdalani, Vanderpool, & Gottesman, 2005; Valentin-Hansen, Eriksen, & Udesen, 2004)). In *P. aeruginosa*, Hfq has been reported to regulate virulence and stress response (Sonnleitner et al., 2003) (Sonnleitner, Schuster, Sorger-Domenigg, Greenberg, & Blasi, 2006). Specifically, Hfq mutants of *P.*

aeruginosa exhibited a 10 to 50-fold decrease in virulence in a murine model of infection (Sonnleitner et al., 2003). Moreover, Hfq regulates the production of key virulence factors, including alginate, catalase, pyocyanin, elastase, and the *las* and *rhl* QS systems (Chugani et al., 2001; Gallagher & Manoil, 2001; Passador et al., 1993; Sonnleitner et al., 2003; Suh et al., 1999; Usher et al., 2002).

A BRIEF BACKGROUND ON *Staphylococcus aureus*

Staphylococcus aureus is a Gram positive bacteria belonging to the Staphylococcaceae family and the genus *Staphylococcus*. Staphylococci are facultative anaerobes, catalase positive and oxidase negative and can grow at a temperature range of 15 to 45 °C and at concentrations as high as 15% NaCl. *S. aureus* are nonmotile and nonsporeforming microorganisms.

S. aureus is a commensal and a pathogen. The anterior nares are the major site of colonization in humans. Around 20-30% of individuals are persistent carriers of *S. aureus* and 30% are intermittent carriers (Wertheim et al., 2005).

This bacterium is one of the main causes of hospital- and community-acquired infections (Diekema et al., 2001). Nosocomial *S. aureus* infections affect the bloodstream, soft tissues, skin and lower respiratory tracts. It also causes serious infections such as endocarditis and osteomyelitis (Schito, 2006). In addition to the infections just listed, this bacterium is often responsible for toxin-mediated diseases such as toxic shock syndrome, staphylococcal foodborne disease and scalded skin syndrome. Furthermore, besides its ability to cause life-threatening infections, *S. aureus* has remarkable potential to develop antimicrobial resistance, which contributes to its clinical significance.

S. aureus is equipped with a great variety of virulence factors participating in pathogenesis. *S. aureus* carries surface proteins named “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMs) that mediate attachment to host tissues and begin colonization leading to an infection. The following are the well-known and studied

MSCRAMMs: fibronectin binding proteins A and B, collagen binding protein Can, clumping factors A and B and protein A (Plata, Rosato, & Wegrzyn, 2009).

Previous reports have shown that 60% of the *S. aureus* strains were able to produce biofilm, relevant to the implanted biomedical device-related *S. aureus* infections on the pathogen's ability to attach to the surface of abiotic as well as abiotic surfaces (Arciola, Baldassarri, & Montanaro, 2001).

Another important virulence factor is the ability of *S. aureus* to secrete toxins that disrupt membranes on host cells such as alpha-hemolysin, beta-hemolysin, gamma-hemolysin, leucocidin and PVL (Kaneko & Kamio, 2004) and the superantigen toxin (Kotzin, Leung, Kappler, & Marrack, 1993).

Many of the *S. aureus* virulence genes are controlled by quorum sensing. This regulation is, in large part, due to the accessory gene regulator (*agr*) two component system (Novick & Geisinger, 2008).

Methicillin-resistant *S. aureus* (MRSA) was recognized as a major pathogen in the 1980s with the emergence of new strains, frequently resistant to multiple different classes of antibiotics. MRSA has been isolated with increasing frequency from CF units with prevalence rates ranging from 0% to about 23% (Burns et al., 1998).

INTRODUCTION

Cystic fibrosis (CF) is a life-shortening inherited disease that results from a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a protein involved in the transport of electrolytes across epithelial and other cell membranes (Winstanley & Fothergill, 2009). This defect results in the presence of viscous mucus and failure in airway mucociliary clearance, leading to life-threatening bacterial infections. The most common and important CF airway pathogen is *Pseudomonas aeruginosa*, which affects up to three-fourths of adults (Lipuma, 2010; Lyczak, Cannon, & Pier, 2002) and causes chronic pulmonary infections.

P. aeruginosa is a clinically important Gram-negative bacterium that has developed an array of virulence mechanisms and secretes a wide variety of extracellular virulence factors such as proteases elastase and LasA (Kamath, Kapatral, & Chakrabarty, 1998; Rumbaugh, Griswold, Iglewski, & Hamood, 1999; Tommassen, Filloux, Bally, Murgier, & Lazdunski, 1992), exotoxin A (Wick, Hamood, & Iglewski, 1990), rhamnolipids (Kownatzki, Tummler, & Doring, 1987), pyocyanin, and siderophores (Crosa, 1989).

The microbial flora of the respiratory tract in patients with CF represents a complex and diverse niche in which many microbial species coexist (Harrison, 2007). The majority of studies carried out on pathogens associated with CF have focused on three bacterial species: *P. aeruginosa*, *Burholderia cepacia* and *Haemophilus influenzae*. In addition, patients with CF have a higher frequency of *P. aeruginosa*-*S. aureus* co-infections than any other patient groups and healthy individuals (Koch, 2002). These two microbial species

are the most predominant pathogens found in adult patients with CF (CysticFibrosisCanada, 2010, 2011; Kerem, 2006).

Synthesis of the above mentioned extracellular molecules, most of them controlled by QS, are known to reduce the growth of *S. aureus*, including LasA, AHL, alkyl quinolone *N*-oxides (HQNO) and pyocyanin (Goerke & Wolz, 2010; Hoffman et al., 2006; Machan, Taylor, Pitt, Cole, & Wilson, 1992; Tashiro et al., 2013).

Collectively, the above-mentioned factors affect the competition between *P. aeruginosa* and *S. aureus* and determine not only the outcome of the disease but also pose major challenges for clinical management of these patients.

One of the hallmarks of *P. aeruginosa* infections impacting CF patients is the establishment of biofilms in the highly viscous mucus layer in the lung. The CF lung mucus has been reported to contain regions with a steep oxygen gradient that ranges from aerobic to anaerobic, due to abnormal oxygen consumption of the epithelial cells and bacterial metabolism (Sanderson, Wescombe, Kirov, Champion, & Reid, 2008; Worlitzsch et al., 2002). Microaerophilic conditions are encountered in the CF lung environment and are the preferred growth condition for *P. aeruginosa* (Costerton et al., 1999; de Beer, Stoodley, Roe, & Lewandowski, 1994; Xu, Stewart, Xia, Huang, & McFeters, 1998). It has been shown that low oxygen plays an important role in the production of alginate and the appearance of mucoid variants of *P. aeruginosa* (Krieg, Bass, & Mattingly, 1986; Leitao & Sa-Correia, 1997; Xu et al., 1998). In a previous paper looking at a mixture of mucoid and non-mucoid *P. aeruginosa* strains, results showed that aerobic culture conditions favorably selected for the growth of mucoid strains; in contrast, sensitivity to oxygen was

observed in non-mucoid *P. aeruginosa* strains, which is a phenotype typically observed in CF patients who are chronically infected with this bacterium (Krieg et al., 1986).

The following findings support the hypothesis that *P. aeruginosa* is located in low oxygen regions of CF lungs: **i)** evidence of *P. aeruginosa* growth as microcolonies in the airway intraluminal rather than the epithelial surface compartments (Baltimore, Christie, & Smith, 1989; Jeffery & Brain, 1988; Simel et al., 1984; Worlitzsch et al., 2002); **ii)** thickened mucus and high oxygen consumption by CF epithelium that generates steep oxygen gradients within the adherent mucus; **iii)** bacteria in the thickened mucus can penetrate into hypoxic zones; **iv)** *P. aeruginosa* can grow in hypoxic/anaerobic CF mucus using available electron acceptors; **v)** increased alginate production by PAO1 strains in response to hypoxia, and **vi)** capacity of *P. aeruginosa* to proliferate in hypoxic mucus generating anaerobic conditions in patients with persistent CF airway infection (Worlitzsch et al., 2002).

We hypothesize that low oxygen levels in the CF lung mucus affect the competition between *P. aeruginosa* and *S. aureus*. Therefore, we studied growth and survival of both species in microaerophilic versus aerobic conditions when cultured alone and together, and aimed at gaining insight into the virulence factors that contribute to this competition under aerobic and microaerophilic conditions.

Understanding how virulence factors regulated by QS behave under the oxygen conditions tested will help in the development of new therapeutic products to control this disease.

MATERIALS AND METHODS

Bacterial isolates. *P. aeruginosa* PAO1 clinical isolate was the prototypical reference strain used in the present study. Other clinical isolates of *P. aeruginosa* and *S. aureus* were obtained from different sites of isolation and their characteristics are listed in Table 1.

The *lasI* mutant was derived from the PAO1 strain (source: University of Brussels) used in this study. Briefly, an in-frame deletion mutant was constructed by allelic exchange (Datsenko & Wanner, 2000). Flanking regions were amplified, cloned into a suicide vector and introduced in *E. coli* S17-1 λ pir. Recombinant clones were used as donor strain to conjugate *P. aeruginosa* PAO1 strain by tri-parental mating with a helper strain harboring plasmid pRK2013. Transconjugants were selected on LB with chloramphenicol and then counter-selected in LB without NaCl supplemented with sucrose (the mutant was constructed in the laboratory of Prof. Pierre Cornelis at the University of Brussels, and the data is not yet published).

P. aeruginosa strain PAO1 (termed MPAO1, source: University of Washington) and the isogenic *phzS* and *lasA* mutants (Table 1) were obtained from the University of Washington transposon mutant library. Transposon (derived from Tn5) insertions were generated by mating *P. aeruginosa* PAO1 with *E. coli* strain SM10pir/pCM639 (IS*phoA*/hah insertions) (Jacobs et al., 2003).

Bacterial strains were grown aerobically (21% O₂) and in microaerophilic conditions (2% O₂) in Lennox L broth base (LB broth, Lennox; Fisher Scientific) at 37 °C, with shaking. Even though LB is not the ideal medium to use to mimic the CF microenvironment, this medium has been extensively used in *P. aeruginosa* research and it is suitable to compare

the results obtained from the study to already published literature. The 2 % concentration of oxygen used in this study was used as a reference for an oxygen environment which is relevant for the microaerophilic niches in the CF lung.

Competition assays for *P. aeruginosa* and *S. aureus* in single and co-culture.

Competition assays were performed in LB at 37 °C in either aerobic (21% O₂) or microaerophilic conditions (2% O₂) with shaking. Co-cultures were inoculated with pure cultures of *P. aeruginosa* and *S. aureus* grown overnight (15 h, aerated, 37 °C, shaking), diluted to an OD of 0.01, and combined in a 1:1 ratio. Bacterial cultures were inoculated and then grown for 24 h as described above. Samples were serially diluted in sterile phosphate-buffered saline (PBS), plated onto LB agar for pure cultures, for co-cultures in LB agar plus 50 µg Triclosan (selective media for *P. aeruginosa*) and Mannitol Salt Phenol Red Agar (Sigma Aldrich) (selective media for the isolation of pathogenic *Staphylococci*) to differentiate the two bacterial species. The plates were incubated overnight at 37 °C and colony forming units (CFU) were enumerated. In initial studies with PAO1, growth of this *P. aeruginosa* strain and also of *S. aureus* was followed over time and at 24 h no *S. aureus* could be recovered compared to earlier tested time points. Thus, this seems to be a suitable time point for comparative studies in competition at different oxygen levels.

Results are expressed as the average log CFU and each experiment was repeated at least twice. Significant differences were considered >2-fold and p<0.05.

Single culture and co-culture growth curves. Growth curves were performed in LB at 37 °C, in duplicate in aerobic (21% O₂) and microaerophilic conditions (2% O₂) with

shaking. Co-cultures were inoculated by pure cultures of *P. aeruginosa* and *Staphylococcus aureus* grown overnight, diluted to an OD₆₀₀ of 0.01, and combined in a 1:1 ratio. At different time points (0, 2, 4, 6, 8, 10 and 24 h), samples were serially diluted in sterile phosphate-buffered saline (PBS), plated on LB agar for pure cultures or for co-cultures on the selective media described above to differentiate the two bacterial species. The agar plates were incubated overnight at 37 °C and colony forming units (CFU) were enumerated. Results are expressed as the average log CFU and each experiment was repeated at least twice.

Pyocyanin extraction and quantification. For pyocyanin quantification of single and co-cultures (made as described above), the cultures were centrifuged after 24 h (8,000 rpm, 5 min, 25 °C), the bacterial pellet was removed and the supernatant filtered-sterilized. Next, 3 ml of chloroform were added; the solutions were shaken vigorously and the chloroform phase collected and transferred to a new glass tube. Further purification of pyocyanin consisted of an extraction with 1 ml of 0.2 N HCl followed by spectrophotometric assay at 550 nm.

Bioassay for the production of 3-oxo-C₁₂-HSL. The detection of extracellular N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C₁₂-HSL) was performed using the *E. coli* strain harboring reporter plasmid pUCP22NotI-*PlasB*::*gfp*(ASV)*Plac*::*lasR* (Hentzer et al., 2002) which expresses GFP in response to 3-oxo-C₁₂-HSL. The LB plates were overlaid with a thin layer of LB containing 0.6% agar (w/v) and 100 µl of an overnight culture of the

indicator strain. The central wells in the LB agar ($\varnothing=6$ mm) were filled with approximately 70 μ l of culture supernatants. Subsequently, the plates were incubated for 24 h at 37 °C. The diameter of the green color on the agar is indicative of the concentration of 3-oxo-C₁₂-HSL in the supernatants.

Elastase secretion. Extracellular elastase was assessed by means of a Congo Red colorimetric assay (Aendekerk, Ghysels, Cornelis, & Baysse, 2002; Crabbe et al., 2008). The cultures were centrifuged (10,000 g; 4 min) and filtered. One hundred microlitres of cell-free culture supernatant were added to glass test tubes containing 10 mg of elastin Congo Red (Sigma-Aldrich, USA) in 900 μ l of 0.1 M Tris HCl (pH 7.2). After 6 h of incubation at 37°C, the tubes were centrifuged and the optical density at 495 nm was measured with an Ultrospec 3000 spectrophotometer (Pharmacia biotech, New York, USA).

Statistical analyses. All experiments were performed at least in duplicate. The statistical significance ($\alpha= 0.05$, $\alpha= 0.01$ and $\alpha= 0.001$) was calculated with Microsoft Office Excel 2013 using a Student's *t*-test on the biological replicates of each experimental condition.

Table 1. Bacterial strains used in this study

Strain	Characteristics	Reference
<i>P. aeruginosa</i>		
PAO1	Prototypic reference strain; isolated from a burn wound in Melbourne Australia (1954). ATCC 15692.	(Crabbe et al., 2008)
OZ6	Isolated from a Burn Intensive Care Unit (ICU) patient, tracheal aspirate. Also referred to as MDR6.	(Zaborina et al., 2006)
ICU3-4-1	Isolated from stool of critically ill patient	(Romanowski et al., 2012)
MPAO1	Parent strain	(Jacobs et al., 2003)
PW8155 (<i>phzS</i>)	<i>phzS</i> -B10::IS <i>phoA</i> /hah	(Jacobs et al., 2003)
PW4283 (<i>lasA</i>)	<i>lasA</i> -B10::IS <i>phoA</i> /hah	(Jacobs et al., 2003)
Δ <i>lasI</i>	Deletion mutant	Not yet published
<i>S. aureus</i>		
N315	Isolated in 1982 from the pharyngeal smear of a Japanese patient with a surgical wound; methicillin resistant <i>S. aureus</i> (MRSA)	(Kuroda et al., 2001)
<i>E. coli</i>		
Indicator strain	Harbors plasmid pUCP22NotI- <i>PlasB</i> :: <i>gfp</i> (ASV) <i>Plac</i> :: <i>lasR</i>	(Hentzer et al., 2002)

RESULTS

Competition between *P. aeruginosa* and *S. aureus* under aerobic conditions.

We assessed the competitive growth of a *P. aeruginosa* strain collection (PAO1, a lung and intestinal clinical isolates, MPAO1, *phzS*, *lasA*, and *lasI*) and *S. aureus* N315 under aerobic (21% O₂) conditions. In addition, our collection of *P. aeruginosa* strains were cultured under the same conditions as previously described, as single cultures. Colony forming unit (CFU) quantification among the *P. aeruginosa* strains in single versus co-culture did not show significant differences (Figure 1A), except for *lasI* strains which showed a considerable reduction (~85 fold) when co-cultured with *S. aureus*. This result may suggest that the *las* QS system is needed for *P. aeruginosa* survival in aerobic conditions when co-cultured with *S. aureus*, especially when considered in the context of the CF lung, where a range of oxygen gradients are encountered by the polymicrobial community.

The number of CFU of the *S. aureus* strain N315 as single versus co-culture was determined as well (Figure 1B). The growth of *S. aureus* N315 was significantly and strongly inhibited ($p < 0.01$) by the presence of PAO1, the ICU3-4-1 intestinal isolate and MPAO1.

The *phzS* was also significantly inhibited ($p < 0.01$) (Figure 1B). While not as strongly inhibited by the *lasA* mutant, there was still a significant reduction in viable *S. aureus* when co-cultured with this mutant *P. aeruginosa* strain (Figure 1B). No significant differences ($p > 0.05$) among *S. aureus* strain N315 co-cultured with *P. aeruginosa* strain *lasI* were observed (Figure 1B), suggesting that production of 3-oxo-C₁₂-HSL might be an important

virulence factor in the competition of *P. aeruginosa* and *S. aureus* under aerobic conditions.

Taken together these data indicate that *P. aeruginosa* strains isolated from different sources can outcompete *S. aureus* N315 in planktonic cultures and that this competition was presumably not mediated by pyocyanin but by elastase and the production of 3-oxo-C₁₂-HSL.

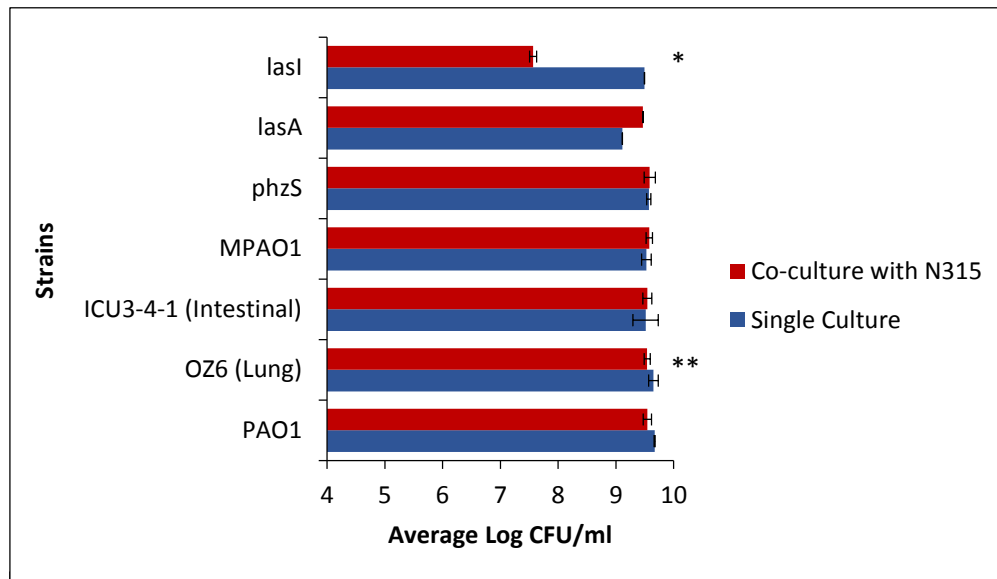
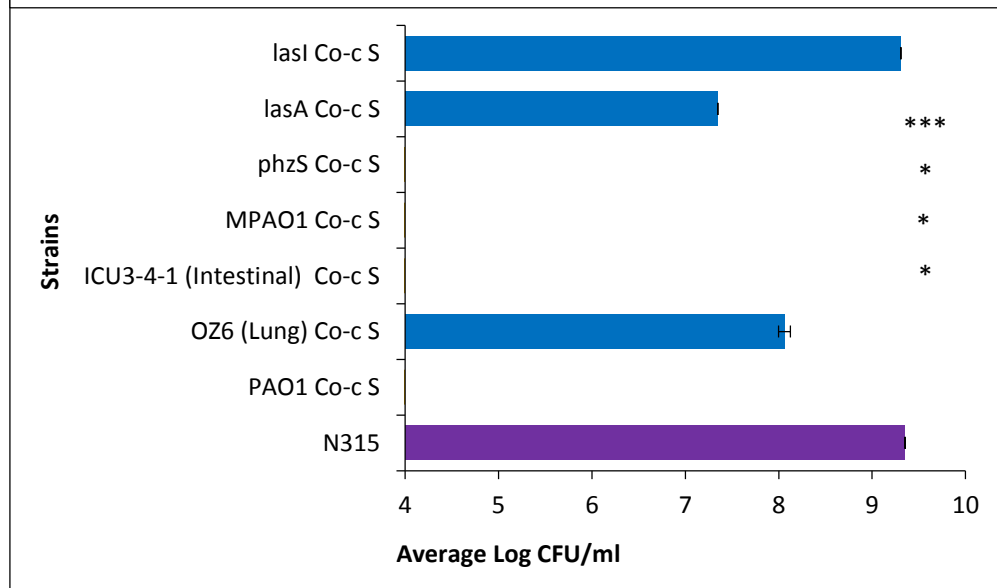
A.**B.**

Figure 1. Competition assays for *P. aeruginosa* and *S. aureus* in aerobic conditions. *P. aeruginosa* (Fig. 1A) strains and *S. aureus* (N315) (Fig. 1B) were grown for 24 h in LB broth in single and co-culture after inoculation at equal ratio from a 15 h overnight culture. Cell concentration at 24 h was monitored by colony count after plating on selective media for both species. Results are expressed as the mean of values from at least obtained from at least two independent experiments. Error bars indicate the standard deviations. Statistically significant differences in Student's t test are indicated by the following symbols: *** $p < 0.001$; ** $p < 0.05$; * $p < 0.01$. Only significant differences were considered for values >2 -fold change and $p < 0.05$. Abbreviations: N315 (*S. aureus* N315), Co-c S (co-cultured with *S. aureus*).

Competition assays for *P. aeruginosa* and *S. aureus* under microaerophilic conditions.

Next, we explored the effect of competition between the two bacterial species through co-culture assays under microaerophilic (2% O₂) conditions. The same strains were used in this study as for the aerobic competition assays.

Quantitative analysis of the number of CFU in the *P. aeruginosa* strain collection showed significant differences ($p < 0.05$) when the *P. aeruginosa* lung isolate and the *lasI* strains were incubated with *S. aureus* N315 (Figure 2A). In microaerophilic as well as in aerobic conditions, a significant difference ($p < 0.05$) in these two *P. aeruginosa* isolates when co-cultured with *S. aureus*; a reduction in the number of *Pseudomonas* recovered both in the OZ6 lung isolate and the *lasI* mutants of 5.3-fold and 4.4-fold respectively, was observed. However, no significant differences in the number of CFU were observed in the following combinations: PAO1-*S. aureus* N315, ICU3-4-1 (intestinal)-N315, MPAO1-N315, *phzS*-N315, and *lasA*-N315 (Figure 2A).

Figure 2B shows the recovery of *S. aureus* N315 co-cultured with the *P. aeruginosa* strains collection and *S. aureus* N315.

The bacterial co-culture assays among the *S. aureus* strain N315 with six *P. aeruginosa* strains showed significant differences (Figure 2B). A significant reduction ($p < 0.001$) in the number of CFU in *S. aureus* strain N315 was observed when cultured with *P. aeruginosa* strains PAO1, ICU3-4-1 (intestinal isolate), MPAO1, *phzS*, *lasA*, and *lasI*. However, we observed what appeared to be a very slight but significant growth induction ($p < 0.05$) of *S. aureus* strain N315 in the presence of the *P. aeruginosa* OZ6 (lung) isolate (<2-fold).

These results indicate that there is a trend in *S. aureus* to be more resistant to inhibition by *P. aeruginosa* strains in microaerophilic conditions.

Statistical analysis performed on competition results showed that there are significant differences between aerobic and microaerophilic conditions for all the strains [PAO1, ICU3-4-1 intestinal isolate and MPAO1: $p < 0.001$; OZ6 lung isolate, and the *phzS*, *lasA* and *lasI* mutants: $p < 0.01$], indicating that oxygen is playing a role in competition between *P. aeruginosa* and *S. aureus*.

Growth curves were also performed in aerobic and microaerophilic conditions and showed that all the pseudomonal strains grow well under both oxygen conditions. Growth phase of the PAO1, OZ6 lung isolate, ICU3-4-1 intestinal isolate and the MPAO1 strain was not affected by oxygen levels at both oxygen concentrations (Figure 3). However, the *lasI* mutant presumably reached the death phase at 21% O₂ unlike the other strains, but not at 2% O₂. This finding might explain the significant reduction in the number of the *P. aeruginosa lasI* mutant when co-cultured with *S. aureus* in the competition assays in aerobic conditions. A similar behavior was observed for the *phzS* and the *lasA* mutants in 21% O₂, but not to the extent of the *lasI* mutant. It is important to state that the death phase is based on one single data point and additional time points will be generated to confirm this finding.

Modeling of the growth curves is needed to determine the duration of the lag phase, the exponential growth rate and the start of the stationary phase as potential differences in growth of *P. aeruginosa* in both oxygen conditions could explain in part, differences in competition.

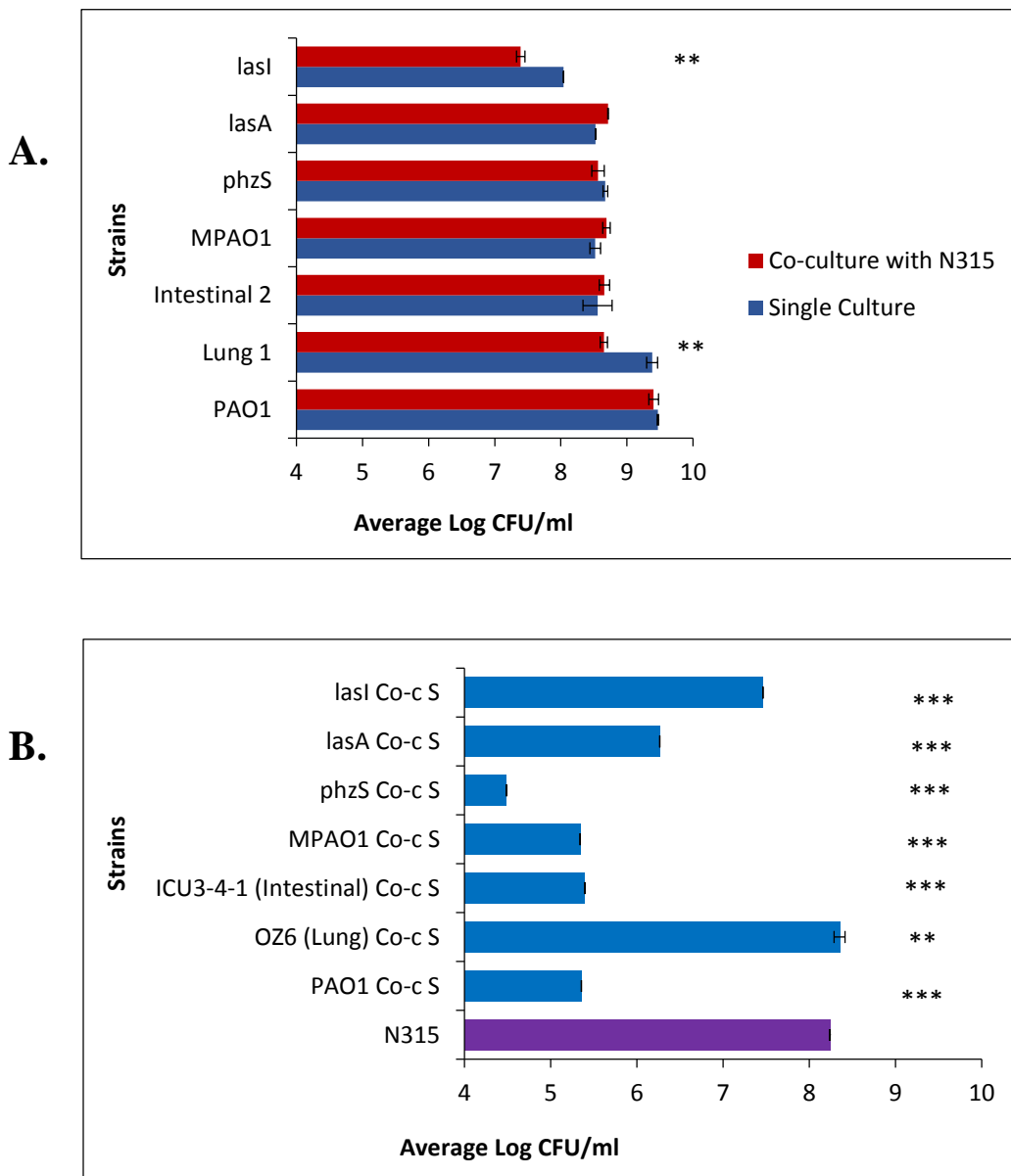
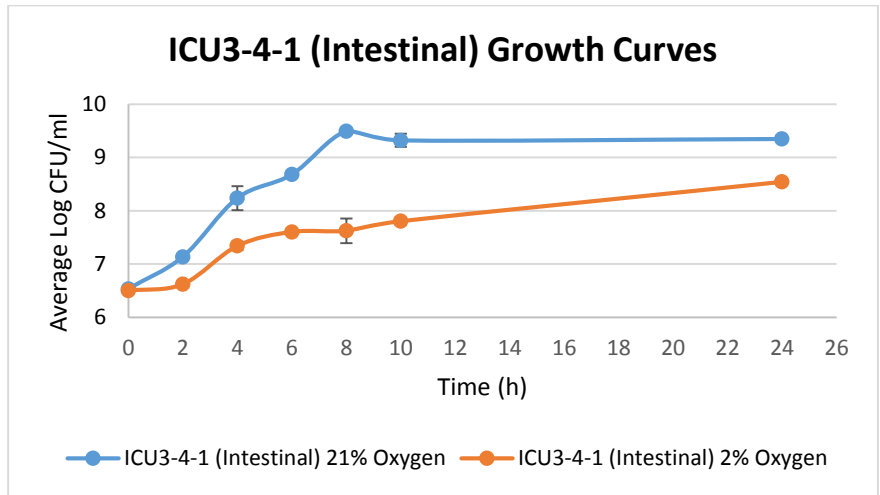
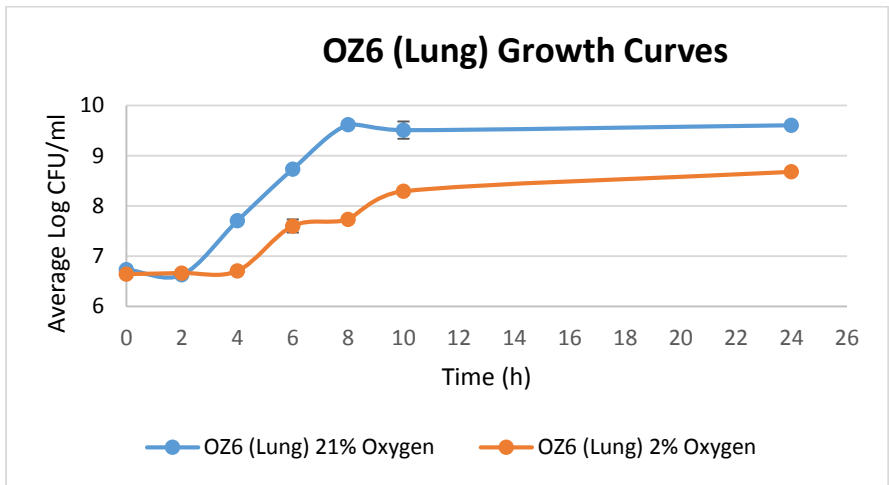
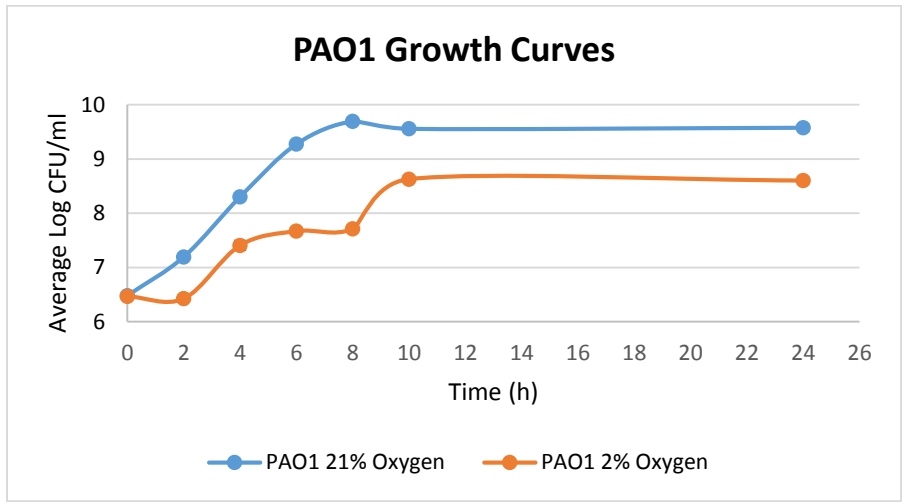
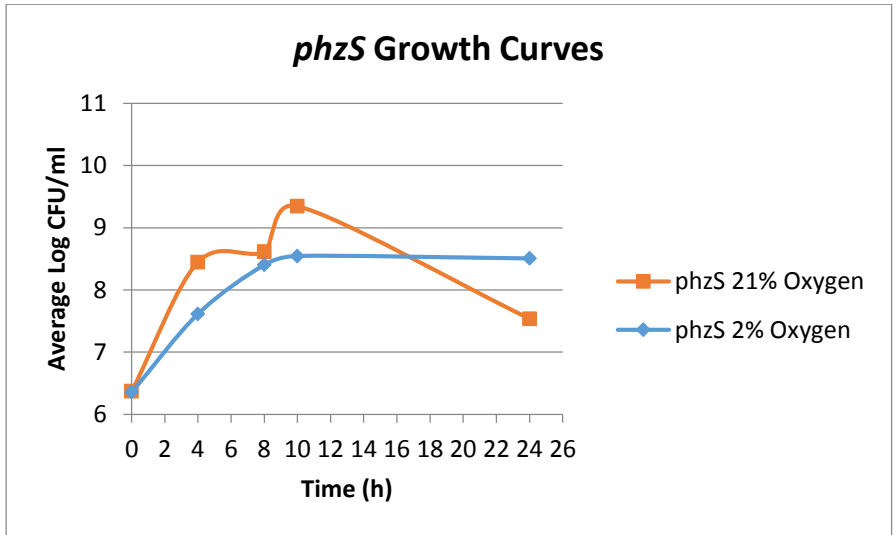
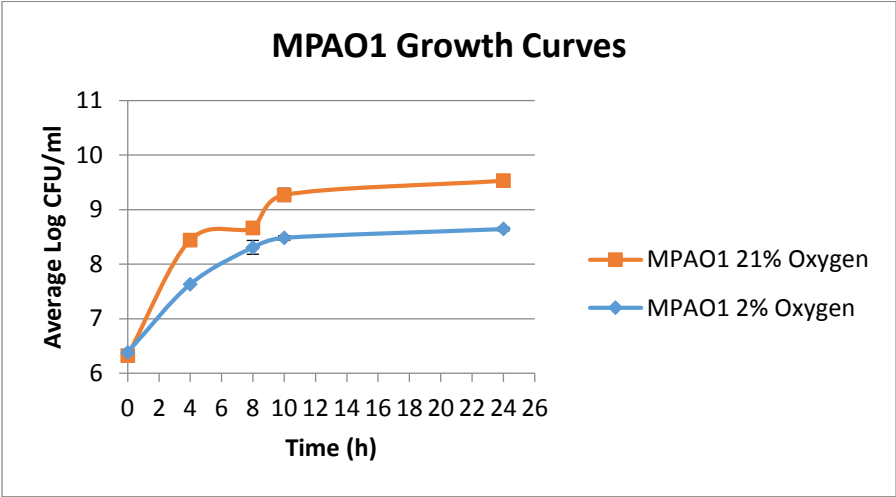


Figure 2. Competition assays for *P. aeruginosa* and *S. aureus* in microaerophilic conditions. *P. aeruginosa* (Fig. 2A) strains and *S. aureus* (N315) (Fig. 2B) were grown for 24 h in flushed and in 2% O₂ in LB broth in single and co-culture after inoculation at equal ratio from a 15 h overnight culture. Cell concentration at 24 h was monitored by colony count after plating on selective media for both species. Results are expressed as the mean of values from at least obtained from at least two independent experiments. Error bars indicate the standard deviations. Statistically significant differences in Student's t test are indicated by the following symbols: ***p<0.001; **p<0.05; *p<0.01. Only significant differences were considered for values >2-fold change and p<0.05. Abbreviations: N315 (*S. aureus* N315), Co-c S (co-cultured with *S. aureus*).





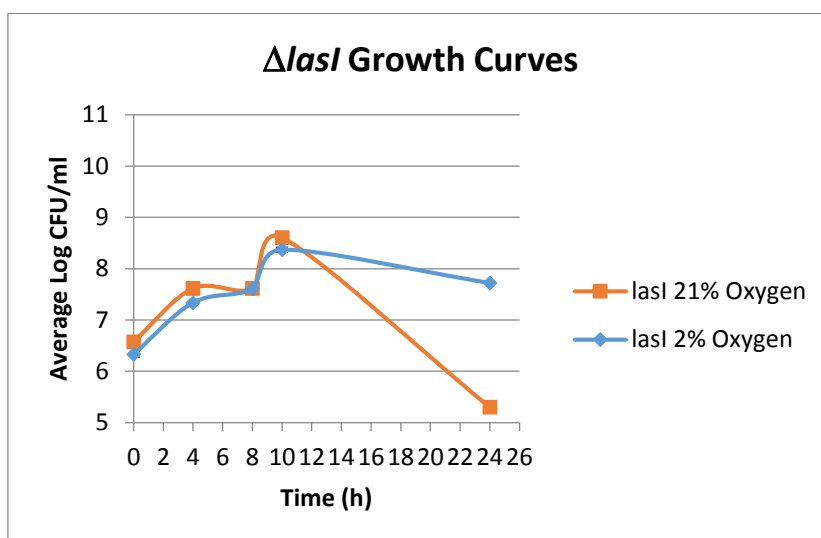
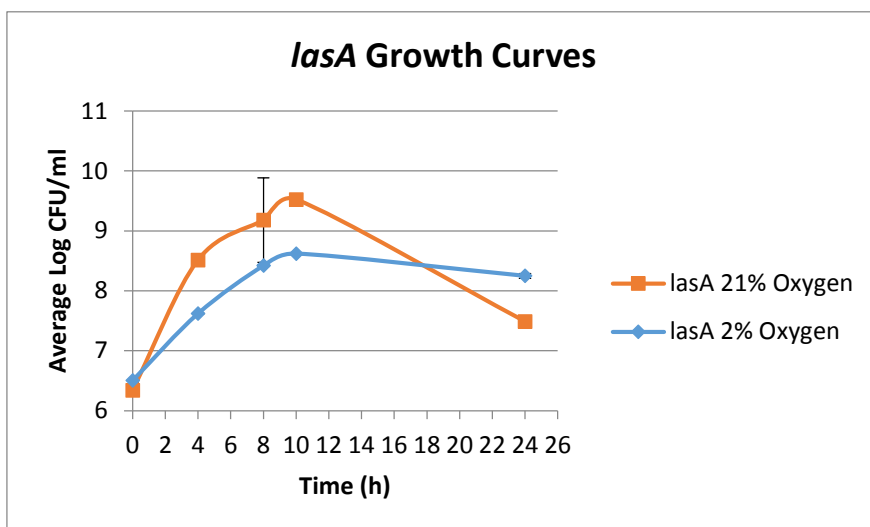


Figure 3. Growth curves of *Pseudomonas* strains in microaerophilic vs. aerobic conditions. Cell concentration at 24 h was monitored by colony count plating at different time points. Results are given in CFU/ml and are represented as a mean of values obtained from at least two independent experiments.

3-oxo-C₁₂-HSL activity in *P. aeruginosa* single and co-cultures under aerobic conditions. In order to understand the effect of the *las* QS system in the competition between *P. aeruginosa* and *S. aureus*, 3-oxo-C₁₂-HSL activity was assessed under aerobic conditions after growth for 24 h. For the single cultures, results showed a weak halo indicating the 3-oxo-C₁₂-HSL activity in the *P. aeruginosa* strain PAO1, the intestinal isolate, MPAO1, *phzS* mutant, and the *lasA* mutant (Figure 4). No halo was visualized in the *P. aeruginosa* lung isolate indicating that this strain did not produce 3-oxo-C₁₂-HSL. *S. aureus* N315 and the *P. aeruginosa lasI* mutant, which were used as controls, were negative for the production of 3-oxo-C₁₂-HSL as expected. Purified 3-oxo-C₁₂-HSL was used as positive control, while LB media was used as negative control.

The type of bioassay performed for QS detection is not sensitive enough to allow visualizing potential differences in QS between both oxygen conditions.

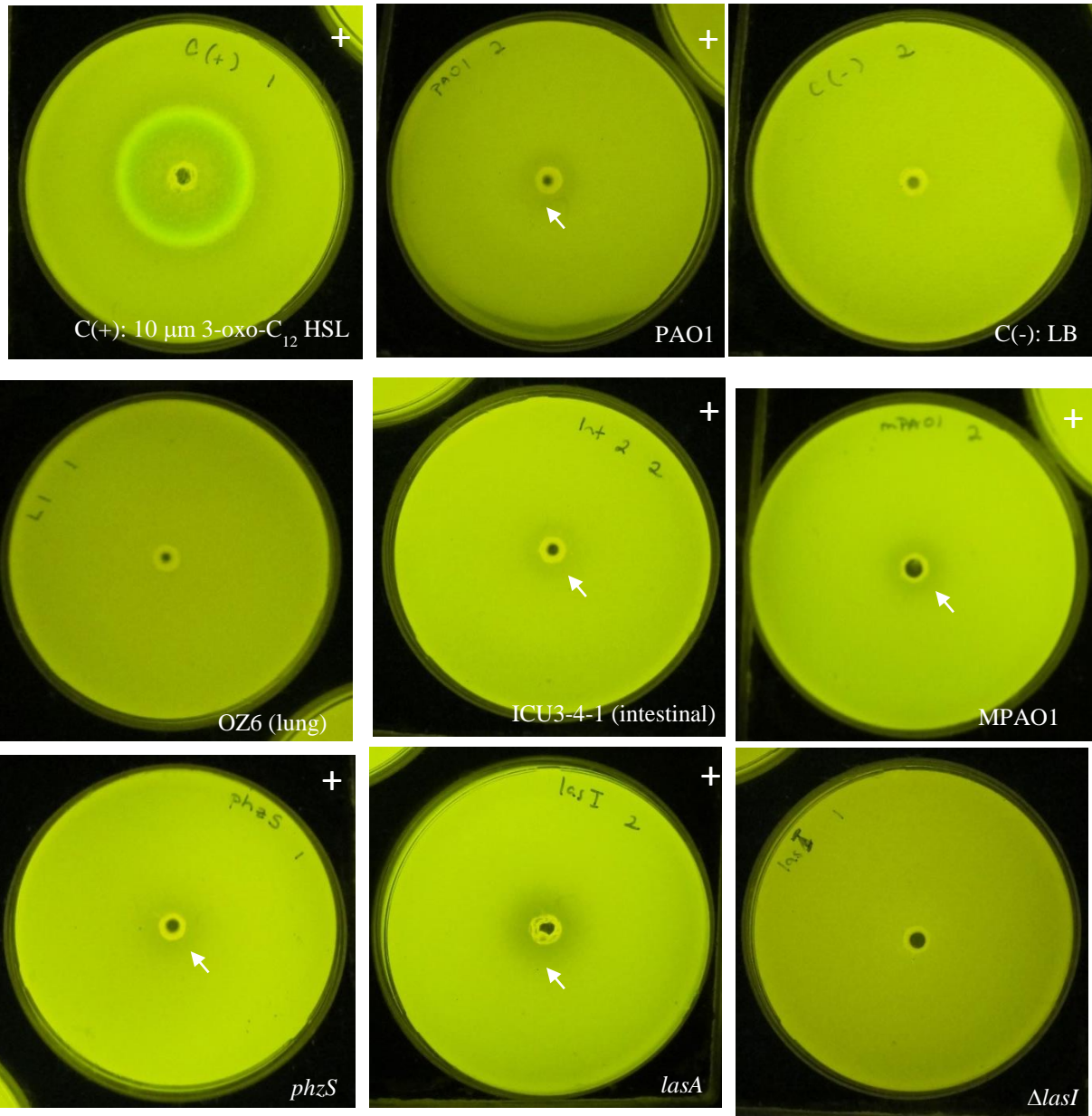


Figure 4. Bioassay for the production of 3-oxo-C₁₂-HSL in *P. aeruginosa* single cultures in aerobic conditions. Detection of 3-oxo-C₁₂-HSL using the *E. coli* strain harboring a reporter plasmid expressing GFP in response to 3-oxo-C₁₂-HSL. The halo indicates the presence of 3-oxo-C₁₂-HSL in the supernatants. The plus (+) sign indicates a positive result for presence of the QS autoinducer. Abbreviations: C(+) [positive control], C(-) [negative control]. The white arrows indicate where the halo starts. We observed no differences in production of 3-oxo-C₁₂-HSL between single and co cultures (Figures 4 and 5).

These data altogether, confirm previous literature on competition of *P. aeruginosa* and *S. aureus* in aerobic conditions, in which the inhibition of *S. aureus* growth was seen in the strains positive for the presence of 3-oxo-C₁₂-HSL.

It should be noted that when we compared the positive control (purified 3-oxo-C₁₂-HSL molecule) with the positive strains for the production of this compound, no fluorescence is observed in the latter. This could be due to the fact that in the supernatants different secreted proteins are present that could inhibit the activity of the QS molecules, or the concentration of the QS molecules is too low for visualization of GFP signal with the used methods. However, the presence of halos, confirm the production of this QS chemical signal and are considered as positive.

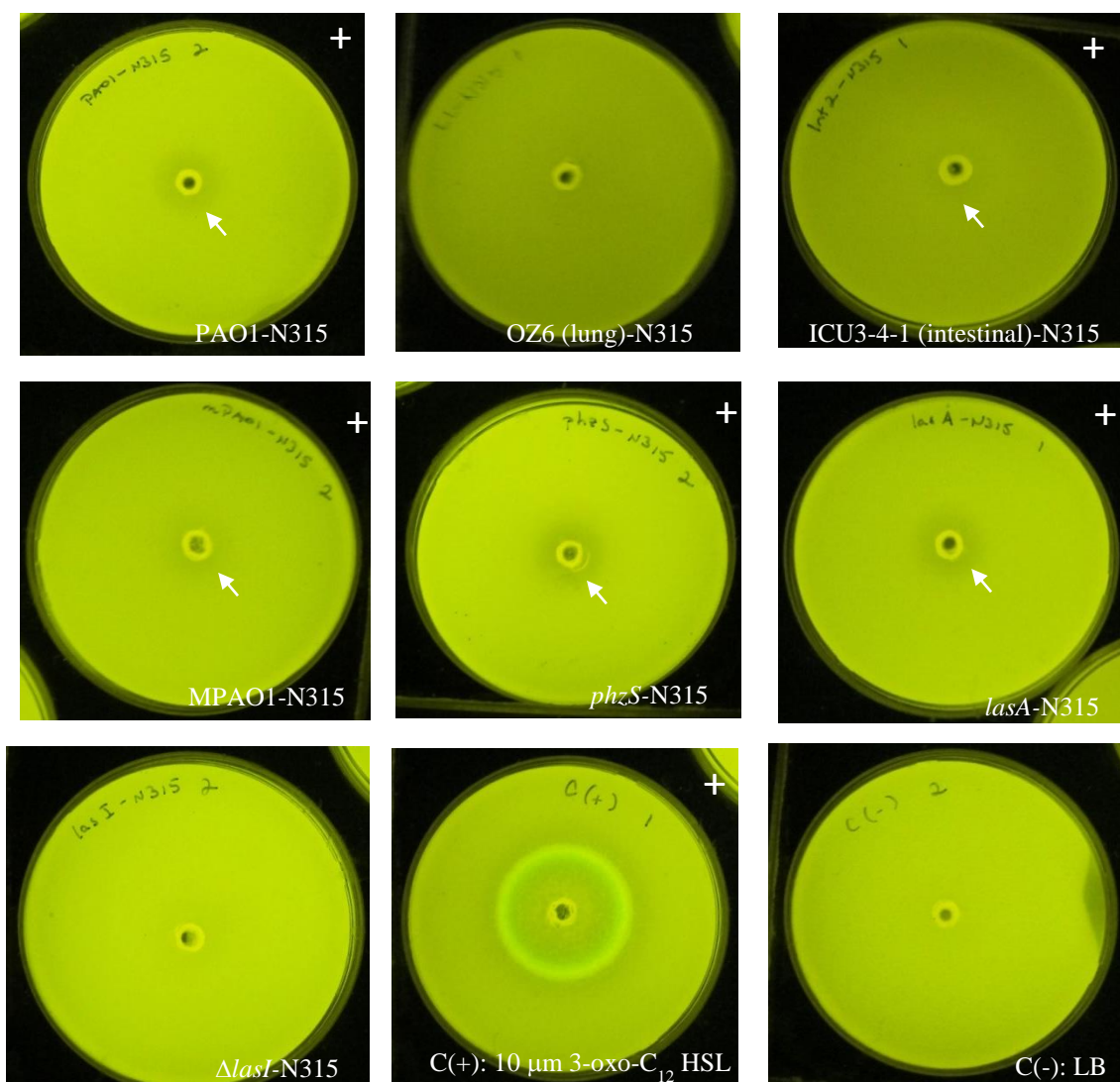


Figure 5. Bioassay for the production of 3-oxo-C₁₂-HSL in *P. aeruginosa* and *S. aureus* co-cultures in aerobic conditions. Detection of 3-oxo-C₁₂-HSL using the *E. coli* strain harboring a reporter plasmid expressing GFP in response to 3-oxo-C₁₂-HSL after 24 h. The halo indicates the presence of 3-oxo-C₁₂-HSL in the supernatants. The plus (+) sign indicates a positive result for the presence of the QS autoinducer. Abbreviations: C(+) [positive control], C(-) [negative control], N315: *S. aureus* N315. The white arrows indicate where the halo starts.

3-oxo-C₁₂-HSL activity in *P. aeruginosa* single and co-cultures under microaerophilic conditions. The 3-oxo-C₁₂-HSL activity for single cultures of *P. aeruginosa* strains was measured under microaerophilic conditions.

Results showed a weak halo indicating the 3-oxo-C₁₂-HSL activity in the *P. aeruginosa* strain PAO1, the Intestinal isolate MPAO1, *phzS*, and *lasA* (Figure 6); the same outcome was observed in single cultures under aerobic conditions indicating that 3-oxo-C₁₂-HSL production in both oxygen concentrations under the conditions tested is not affected. Previous results have shown that when clinical isolates were measured for the production of 3-oxo-C₁₂-HSL, anaerobiosis had an impact on QS activity in a strain dependent manner (Fang et al., 2013); those results are consistent with the results presented here, even when a small percentage of oxygen is present. In addition, no halos could be visualized for the *S. aureus* strain N315, the *P. aeruginosa* lung isolate, and *P. aeruginosa lasI*; therefore, these strains did not show any 3-oxo-C₁₂-HSL activity.

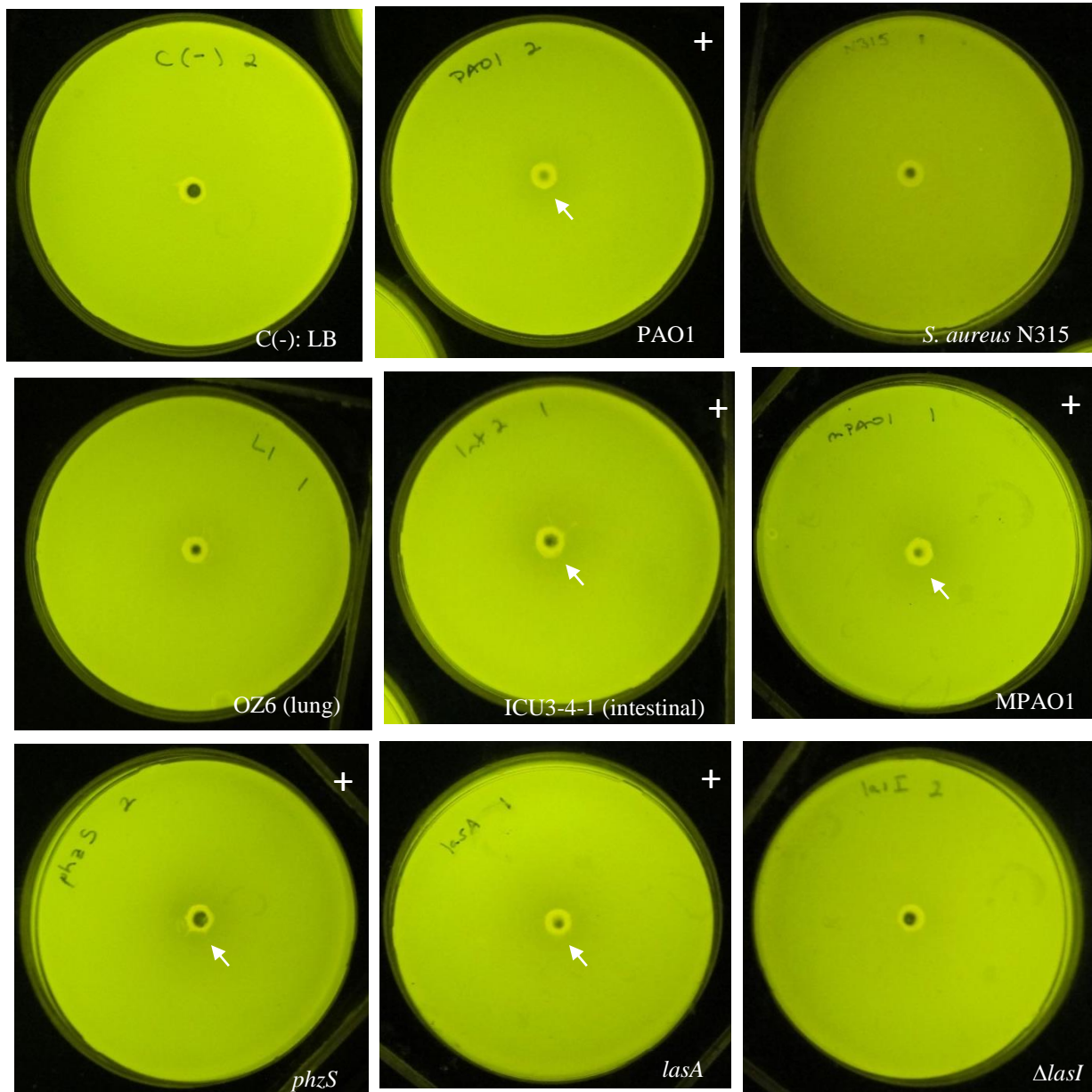


Figure 6. Bioassay for the production of 3-oxo-C₁₂-HSL in *P. aeruginosa* single cultures in microaerophilic conditions. Detection of 3-oxo-C₁₂-HSL using the *E. coli* strain harboring a reporter plasmid expressing GFP in response to 3-oxo-C₁₂-HSL after 24 h. The halo indicates the presence of 3-oxo-C₁₂-HSL in the supernatants. The plus (+) sign in some of the strains indicate a positive result for the presence of the QS autoinducer. Abbreviations: C(-) [negative control]. The white arrows indicate where the halo starts.

Weak halos, indicative of the 3-oxo-C₁₂-HSL activity, were observed in the collected supernatants of the co-cultures in the following combinations: PAO1-N315, MPAO1-N315, *phzS*-N315 and *lasA*-N315 (Figure 7). *S. aureus* growth inhibition is less pronounced in 2% oxygen, but it is still present and the results with the *lasI* mutant under the same condition indicate that this inhibition is QS regulated.

No halos were observed in the co-culture of *S. aureus* N315 with the *P. aeruginosa* lung or intestinal isolates or the *lasI* mutant in microaerophilic growth conditions (Figure 7).

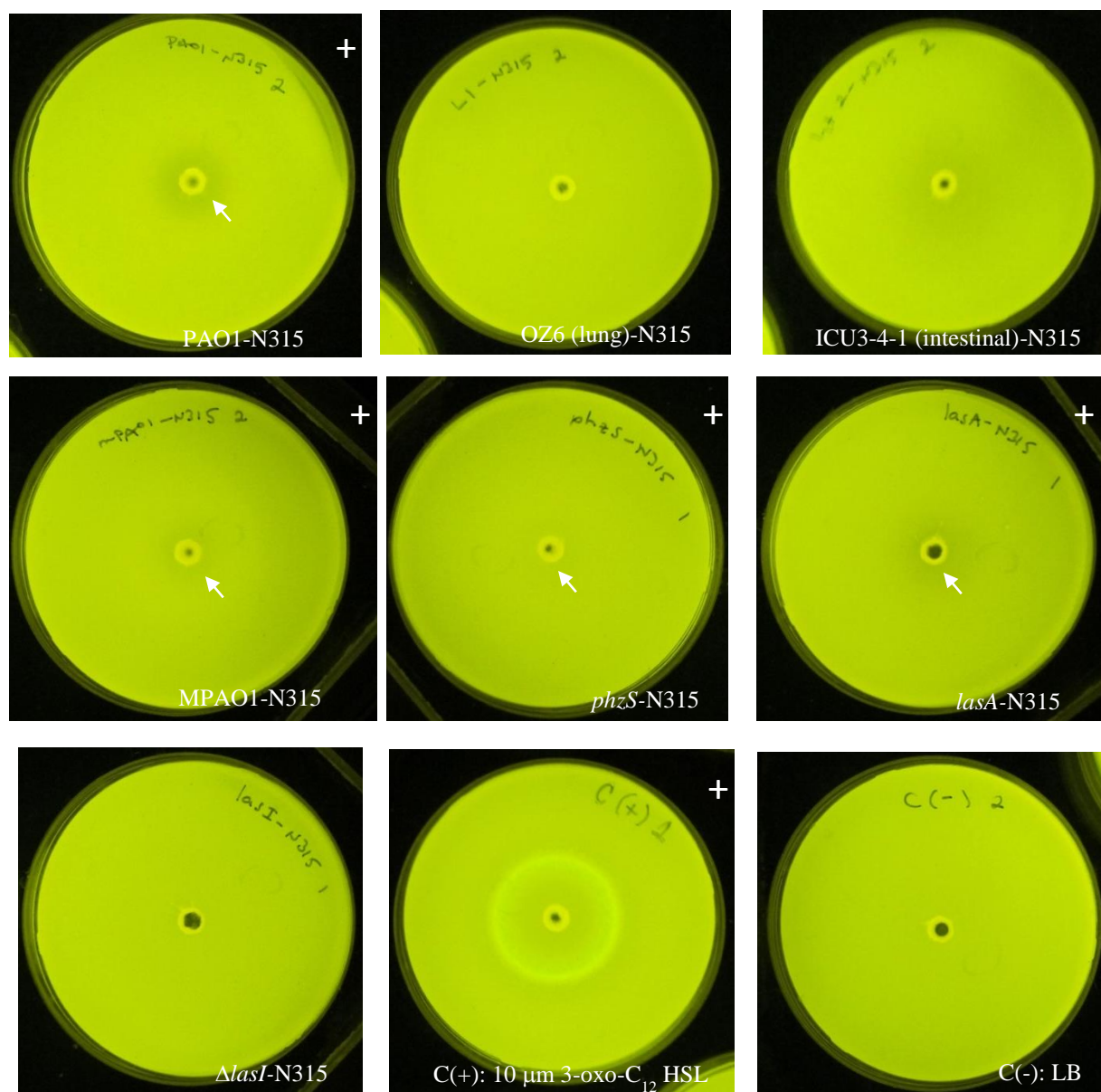


Figure 7. Bioassay for the production of 3-oxo-C₁₂-HSL in *P. aeruginosa* with *S. aureus* co-cultures in microaerophilic conditions. Detection of 3-oxo-C₁₂-HSL using the *E. coli* strain harboring a reporter plasmid expressing GFP in response to 3-oxo-C₁₂-HSL after 24 h. The halo indicates the presence of 3-oxo-C₁₂-HSL in the supernatants. The plus (+) sign in some of the strains indicate a positive result for the presence of the QS autoinducer. Abbreviations: C(+) [positive control], C(-) [negative control], N315: *S. aureus* N315.

Table 2. Summary of QS results from this study

Strains	QS	
	21 %	2 %
PAO1	+	+
OZ6 (Lung)	-	-
ICU3-4-1 (Intestinal)	+	+
MPAO1	+	+
<i>phzS</i>	+	+
<i>lasA</i>	+	+
<i>ΔlasI</i>	-	-
PAO1-N315	+	+
OZ6 (Lung)-N315	-	-
ICU3-4-1 (Intestinal)-N315	+	-
MPAO1-N315	+	+
<i>phzS</i> -N315	+	+
<i>lasA</i> -N315	+	+
<i>ΔlasI</i> -N315	-	-

Abbreviations: + (positive for the presence of halo), - (negative for the presence of halo), QS (Quorum Sensing), N315 (*S. aureus* N315).

Pyocyanin production in *P. aeruginosa* strains in single and co-culture under aerobic conditions. Pyocyanin production by *P. aeruginosa* strains was determined in single and co-culture growth under aerobic conditions (Figure 8). As expected, no pyocyanin production in single culture was observed in the *S. aureus* strain N315. No significant differences ($p>0.05$) were observed in pyocyanin production in the PAO1 strain when cultured in single and co-culture conditions. Likewise, there were no significant differences ($p>0.05$) between the other strains tested in this study. As expected, the *phzS* and *lasI* mutants showed background levels of pyocyanin.

High biological variability was observed for the pyocyanin assay and additional experiments will determine whether there are significant differences between these strains and in different oxygen concentrations.

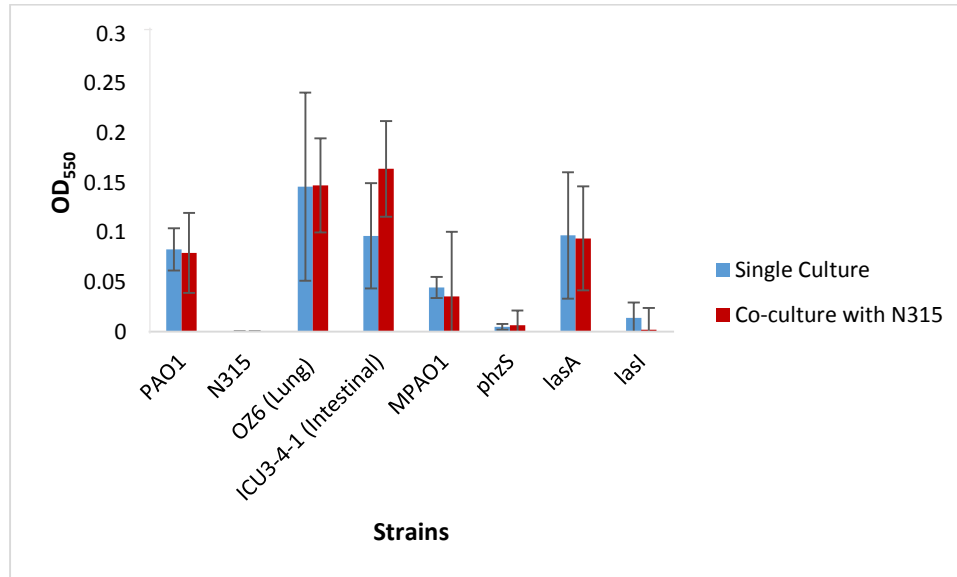


Figure 8. Pyocyanin production in *P. aeruginosa* strains in single and co-culture in aerobic conditions (21% O₂). Pyocyanin absorbance in supernatants of PAO1, lung and intestinal isolates, MPAO1 along with *P. aeruginosa* mutants after 24h. Error bars represent standard deviations from the mean (n=2). Statistically significant differences between single and co-cultures are indicated by the following symbols: ***p<0.001; **p<0.05; *p<0.01.

Pyocyanin production in *P. aeruginosa* strains single and co-culture under Microaerophilic conditions. Pyocyanin production by *P. aeruginosa* strains in single and co-culture in microaerophilic conditions was determined as described before (Figure 9). As expected, no pyocyanin production in single culture was observed in *S. aureus* strain N315. Likewise, no significant differences (p>0.05) in pyocyanin production were found for all the strains when cultured in single versus co-culture conditions.

The results here showed that the pyocyanin production in aerobic and microaerophilic conditions were similar in single and co-culture ($p>0.05$); however, we did observe a trend towards a reduced amount of this compound in microaerophilic conditions. Additional experiments will determine whether this trend can be confirmed, since lower pyocyanin production in microaerophilic conditions could potentially account for the lower level of *S. aureus* growth inhibition. This reduced effectiveness was shown previously and applies for other organisms competing with *P. aeruginosa* (Winstanley & Fothergill, 2009).

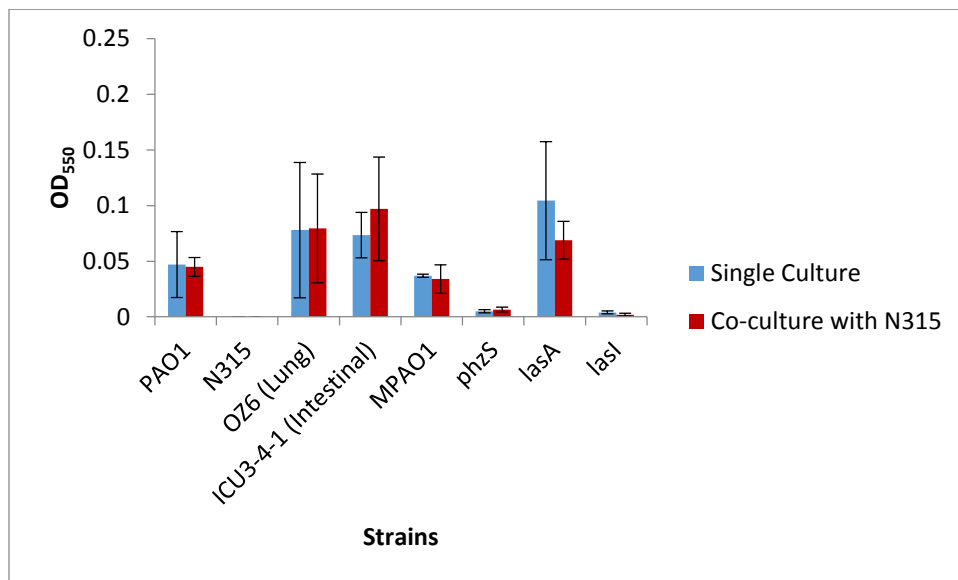


Figure 9. Pyocyanin production in *P. aeruginosa* strains in single and co-culture in Microaerophilic conditions (2% O₂). Pyocyanin absorbance in supernatants of PAO1, lung and intestinal isolates, MPAO1 along with *P. aeruginosa* mutants after 24h (grown in LB). Error bars represent standard deviations from the mean (n=2). Statistically significant differences in Student's t test are indicated by the following symbols: *** $p<0.001$; ** $p<0.05$; * $p<0.01$.

Elastase secretion in aerobic and microaerophilic conditions. In order to better understand the ability of *P. aeruginosa* to express elastase under different oxygen concentrations, we tested the secretion of this compound during aerobic and low oxygen conditions (2% O₂). As expected, elastase was detected in the culture supernatants of all the strains grown in aerobic conditions and surprisingly, in all of the strains grown at 2% O₂ and at the same levels. As expected, the culture supernatant of a mutant in the LasA serine protease still exhibits some elastolytic activity due to the synergistic effect with LasB to degrade elastin. The elastase levels of the mutant in the LasI synthase were the same as those exhibited by *S. aureus* N315 (Fig. 10). No significant differences ($p > 0.05$) were observed in the secretion of elastase between single and co-culture conditions.

Elastase secretion in *P. aeruginosa* strains was expected from previous research studies performed in aerobic environments. It has been reported that under anaerobic conditions elastase activity is significantly repressed for PAO1 (Lee, Yoon, Park, Lee, & Yoon, 2011) and other clinical isolates (Fang et al., 2013). However, elastase secretion at 2% O₂ had not yet been investigated.

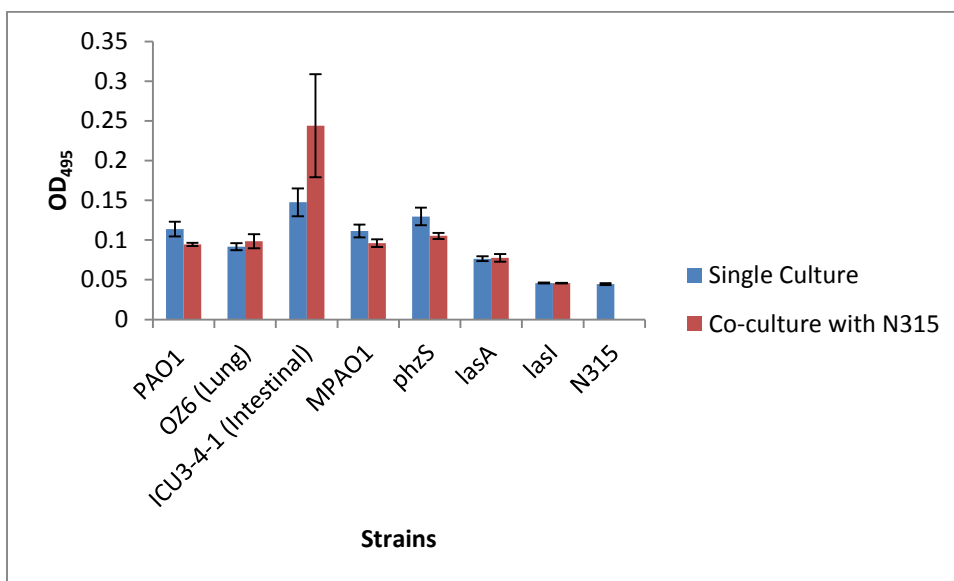
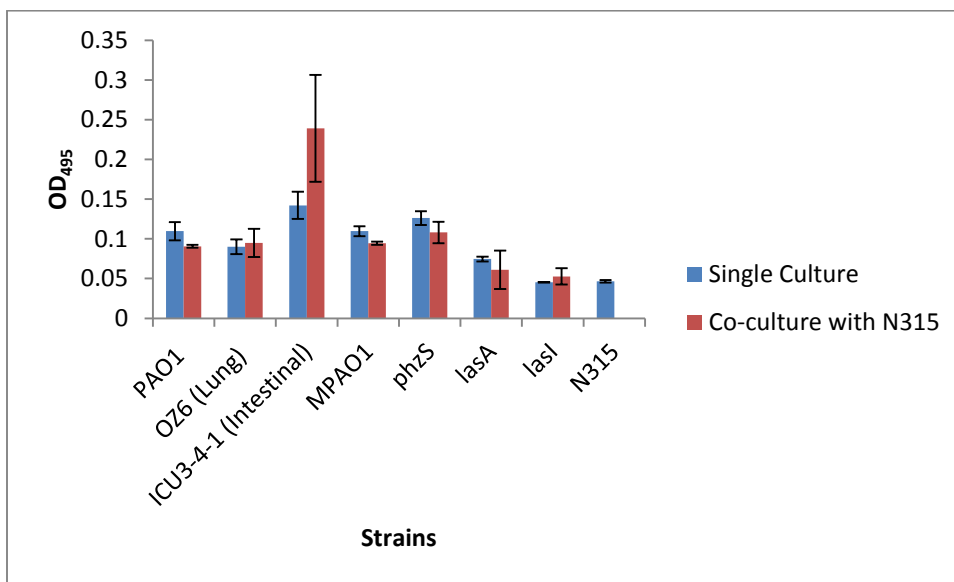
A.**B.**

Figure 10. Elastase Secretion of *P. aeruginosa* strains in single and co-culture in aerobic and microaerophilic conditions. Strains were grown in LB either aerobically (21% O₂) (panel A) or in microaerophilic conditions (2% O₂) (panel B) for 24 h. Statistically significant differences in Student's t test are indicated by the following symbols: ***p<0.001; **p<0.05; *p<0.01.

DISCUSSION

The objective of this study was to investigate the influence of oxygen on the competition between *P. aeruginosa* and *S. aureus*, to enhance our understanding about the interaction between these two pathogens in environmental conditions relevant to the cystic fibrosis lung mucus.

We also evaluated the role of targeted virulence factors that are known to play a role in competition between these species. In our competition assays we observed striking differences between the recovery of *S. aureus* in the presence of *P. aeruginosa* isolates in aerobic and microaerophilic conditions, showing that oxygen is playing a role in competition between these two microorganisms. Specifically, low oxygen levels provided a growth/survival advantage for *S. aureus* in the presence of *P. aeruginosa*, as compared to aerobic conditions. Findings from this study suggested that the *lasA* and *lasI* mutants competed less effectively with *S. aureus* regardless of the oxygen level present in the culture compared to the isogenic wild type strain. These results are consistent with previous findings that elastase and *lasI* quorum sensing play a role in competitive behavior of *P. aeruginosa* and *S. aureus*.

It has been suggested that the CF lung environment is largely anaerobic/microaerophilic due to factors such as 1) the presence of CF airway epithelia that consume 2-3 times more oxygen than the normal airway epithelia, 2) the high numbers of neutrophils which undergo respiratory burst, and 3) growth of a tremendous amount of bacterial and fungal species, leading to depletion of the oxygen that is available (Price-Whelan, Dietrich, & Newman, 2007).

One of the most important and clinically relevant polymicrobial associations in the CF lung is the one that exists between *S. aureus* and *P. aeruginosa* (Peters, Jabra-Rizk, O'May, Costerton, & Shirtliff, 2012). In many cases, *S. aureus* is predominant in childhood infections but colonization by *P. aeruginosa* slowly increases as CF patients grow older. However, *S. aureus* persists and along with *P. aeruginosa*, these two organisms represent the most prevalent species in adult CF patients (CysticFibrosisCanada, 2010, 2011; Kerem, 2006).

P. aeruginosa produces a variety of virulence factors with many being controlled by QS regulatory systems. Among the arsenal of *P. aeruginosa* virulence factors, LasA, alkyl quinolone *N*-oxides (HQNO), hydrogen cyanide and pyocyanin have been reported to contribute to the reduction of *S. aureus* growth (Goerke & Wolz, 2010; Hoffman et al., 2006; Machan et al., 1992) through suppression of aerobic and anaerobic metabolism and growth of *S. aureus* (Biswas, Biswas, Schlag, Bertram, & Gotz, 2009; Hoffman et al., 2006).

QS systems in *P. aeruginosa* have been shown to control the production of factors implicated in virulence. This is true for virulence factors known to play a role in competition with *S. aureus* and studied in this project: pyocyanin production and elastase secretion (Baldan et al., 2014; Hassan & Fridovich, 1980; E. Kessler et al., 1997).

The competition in aerobic and microaerophilic conditions performed in this study showed that in both conditions, the production of the autoinducer 3-oxo-C₁₂-HSL (made by the Las QS system) is important in this competition process when comparing the *lasI* mutant and the QS negative lung isolate to the reference strain PAO1. LasI is an autoinducer synthase

that codes for 3-oxo-C₁₂-HSL. Previous reports have shown the implication of this autoinducer aiding in the inhibition of *S. aureus* growth (Haba et al., 2003; Irie, O'Toole G, & Yuk, 2005).

The three *P. aeruginosa* isolates tested in this study inhibited the growth of *S. aureus* in aerobic conditions (Baldan et al., 2014). These findings are in agreement with previous reports. One example is the demonstration of *P. aeruginosa* inducing lysis of *S. aureus* to use the released iron for its own growth (Mashburn, Jett, Akins, & Whiteley, 2005). The same inhibition was observed in the *P. aeruginosa phzS* strain, responsible for the production of pyocyanin synthesis, indicating that pyocyanin presumably does not play a major role in the competition under the conditions tested. Interestingly, neither the lung isolate (which is negative for the production of 3-oxo-C₁₂-HSL), the *lasA* nor the *lasI* mutant strains co-cultured with *S. aureus* were able to outcompete it effectively, suggesting that the *lasI* QS system plays an important role in the competition of both microbial species. In the case of the lung isolate, we can most likely attribute the lower growth inhibition to the lack of 3-oxo-C₁₂-HSL production. Indeed, CF lung isolates often lack production of 3-oxo-C₁₂-HSL (Bjarnsholt et al., 2010; Heurlier et al., 2005; McKeon, Nguyen, Viteri, Zlosnik, & Sokol, 2011). However, since this 3-oxo-C₁₂-HSL-negative isolate was still able to inhibit *S. aureus* growth, other antimicrobial effectors presumably played a role in the competition as well. By looking at the QS assays in co-culture, most of the *P. aeruginosa* strains tested gave a positive result for the production of the autoinducer, with the exception of the lung isolate in both oxygen conditions. This is consistent with the competition assays where it was shown that the lung isolate in both oxygen conditions

exhibited a reduced ability to reduce the growth of *S. aureus*, which could suggest that 3-oxo-C₁₂-HSL could play an important role in this inhibition. In terms of the progression of CF, most of the infections in the lung by *P. aeruginosa* are chronic infections, therefore, they are most likely to lose their QS as the disease progresses (Bjarnsholt et al., 2010; Heurlier et al., 2005; McKeon et al., 2011). This outcome is linked to loss of regulatory systems such as QS that occur during the adaptive process of the bacteria in the CF lung (D'Argenio et al., 2007) due to mutations in *lasR*; approximately 2/3 of the mutations would result in a loss of LasR function (Wilder, Allada, & Schuster, 2009).

Unfortunately, we do not know from which stage of the disease this clinical strain was isolated. On the other hand, for the strains positive for 3-oxo-C₁₂-HSL and observing the results from the competition assays, there seems to be a correlation between the production of this autoinducer and the inhibition of *S. aureus*.

Interestingly, growth of *S. aureus* in the presence of the lung isolate was induced in 2% O₂. These results altogether suggest that in microaerophilic conditions, *S. aureus* (which is a facultative anaerobe) is more resistant to its growth inhibition than in aerobic conditions.

This is in accordance to the fact that both species co-exist in the same niche in the CF lung and even though *P. aeruginosa* is always the predominant microorganism in the context of CF, there are some studies that suggest that the presence of *S. aureus* enhances the colonization of *P. aeruginosa* (Loening-Baucke, Mischler, & Myers, 1979).

If we translate these results into an *in vivo* setting, it would explain how *S. aureus* is able to survive even in the presence of *P. aeruginosa* in the context of CF.

Pyocyanin has antibiotic activity against other bacteria and fungi, and other organisms such as *Caenorhabditis elegans* (McAlester, O'Gara, & Morrissey, 2008; Price-Whelan, Dietrich, & Newman, 2006). This property gives *P. aeruginosa* an advantage to compete with other bacteria occupying the same niche including with *S. aureus*. The competition in aerobic and microaerophilic conditions performed in this study showed that in both conditions, the production of pyocyanin is not important in this competition process. Even though the experimental data showed considerable variability in the results, the same trends can be seen in both oxygen conditions tested. These results are not consistent with previous papers, where pyocyanin was shown to have inhibitory effects on the growth of *S. aureus* (Fugere et al., 2014; Qin, Yang, Qu, Molin, & Tolker-Nielsen, 2009; Voggu et al., 2006).

Likewise, the results presented here contradict previous studies showing an induction of pyocyanin when PAO1 was co-cultured with *S. aureus* under aerobic conditions (Kluge, Hoffmann, Benndorf, Rapp, & Reichl, 2012). There have also been reports of anti-staphylococcal activity by *P. aeruginosa* present in the sputum of CF patients (Machan et al., 1991). High concentrations of pyocyanin in the sputum of CF patients suggest that this phenazine plays a vital role in pulmonary tissue damage in chronic lung infections (Jayaseelan, Ramaswamy, & Dharmaraj, 2014).

However, looking at the competition results (*phzS* mutant compared to the reference strain PAO1) obtained from this study we can see that pyocyanin does not play an important role in the competition of *P. aeruginosa* and *S. aureus*.

Elastase (encoded by the *lasB* gene) is one of the major virulence factors (Blackwood, Stone, Iglewski, & Pennington, 1983) secreted into the culture media during aerobic

growth by *P. aeruginosa*. The LasA serine protease has been reported to have staphylolytic activity (E. Kessler et al., 1997).

The competition assays in aerobic conditions showed that the final cell concentration of *S. aureus* is inhibited when co-cultured with the PAO1 strain but not inhibited by the *P. aeruginosa lasA* mutant, suggesting that elastase is an important factor in the competition between *P. aeruginosa* and *S. aureus*.

Our results also showed that elastase is secreted into the culture media at the same levels during aerobic and microaerophilic growth in the strains tested. This result does not reflect previous studies that showed that the secretion of elastase in anaerobic conditions was reduced under this condition (Lee et al., 2011); however, we have to take into account the fact that in our study a microaerophilic environment (2% O₂) was employed, and therefore, that low oxygen concentration could account for this observation.

Despite our efforts to investigate the *P. aeruginosa* virulence factors that could be affected by microaerophilic versus aerobic growth and play a role in differential competition (i.e. pyocyanin, elastase A, *las* QS system), it remains unclear what the underlying cause is of the major differences in competitive growth between the oxygen conditions tested. Other virulence factors that deserve further study to understand whether they play a role in differential competition between 2% O₂ and 21% oxygen growth conditions include PQS, which is greatly inhibited during anaerobic growth of *P. aeruginosa* (Schertzer, Brown, & Whiteley, 2010; Toyofuku et al., 2008). However, no data has been reported regarding the outcome of competition under conditions with reduced oxygen tension.

A recently published paper showed that mupirocin, an antibiotic isolated from *P. fluorescens*, has broad antibacterial activity especially against Gram-positive bacteria including *S. aureus*. (Matthijs et al., 2014).

Cyanide, the other factor that could be involved in explaining these differences, is only produced over a narrow spectrum of environmental oxygen tensions (microaerophilic) and synthesis is quickly inactivated under atmospheric oxygen and strictly anaerobic conditions (Blumer & Haas, 2000).

The *S. aureus* clinical strain chosen for this study was not co-isolated with the *P. aeruginosa* strains analyzed - thus further studies are necessary to address the occurrence and clinical significance of the *P. aeruginosa*-*S. aureus* relationship in co-infected CF patients.

Bacterial population behavior in CF is complex and much more research is required to understand the underlying causes of the *P. aeruginosa* dominated chronic lung infections. Studies like those conducted in this work are useful for finding future therapeutic alternatives for CF patients that are co-infected by *P. aeruginosa* and *S. aureus*. Specifically, these studies will aid in the understanding of the CF environmental factors that play a role in microbial community dynamics and could provide new therapeutic avenues.

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