Oxygen Restriction Generates Difficult-To-Culture Pathogens

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Abstract

Induction of a non-culturable state has been demonstrated for many bacteria. In a clinical perspective, the lack of growth due to these non-culturable bacteria can have major consequences for the diagnosis and treatment of patients. Here we show how anoxic conditioning (restriction of molecular oxygen, O₂) generates difficult-to-culture (DTC) bacteria during biofilm growth. A significant subpopulation of *Pseudomonas aeruginosa* entered a DTC state after anoxic conditioning, ranging from five to 90 % of the total culturable population, in both planktonic and biofilm models. Anoxic conditioning also generated DTC subpopulations of *Staphylococcus aureus* and *Staphylococcus epidermidis* (89 and 42 % of the total culturable population, respectively). Growth of the DTC populations were achieved by substituting O₂ with 10 mM NO₃⁻ as an alternative electron acceptor for anaerobic respiration or, in the case of *P. aeruginosa*, by adding sodium pyruvate or catalase as scavengers against reactive oxygen species (ROS) during aerobic respiration. An increase in normoxic plating due to addition of catalase suggests the molecule hydrogen peroxide as a possible mechanism for induction of DTC *P. aeruginosa*. Anoxic conditioning also generated a true viable but non-culturable (VBNC) population of *P. aeruginosa* that was not resurrected by substituting O₂ with NO₃⁻ during anaerobic respiration. These results demonstrate that habituation to an anoxic micro-environment could complicate diagnostic culturing of bacteria, especially in the case of chronic infections where oxygen is restricted due to the host immune response.

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60 **Importance**

61 Diagnostics of bacteria from chronic infections by standard culture-based methods is challenging.
 62 Bacteria in a non-culturable state may contribute to the lack of culturing from these infections.
 63 Many stressors are known to induce a non-culturable state, among others the absence of molecular
 64 oxygen, which is evident in chronic infections due to high rates of oxygen consumption by the host
 65 response. In this study, we have shown that *Pseudomonas aeruginosa*, *Staphylococcus aureus* and
 66 *Staphylococcus epidermidis* can enter a non-culturable state after oxygen restriction. Regrowth was
 67 not possible using conventional normoxic plating where oxygen served as electron acceptor.
 68 Instead, regrowth was enabled during anoxic conditions with added nitrate as alternative electron
 69 acceptor. In the case of *P. aeruginosa* we were able to counteract this non-culturable state by adding
 70 scavengers for reactive oxygen species during normoxic incubation. This suggest a link between
 71 reactive oxygen species and a non-culturable state and it also renders a solution to circumvent this
 72 problem in a clinical diagnostic setting. Our findings show that bacteria can habituate to their
 73 environment and that it has to be taken into consideration especially when culturing clinical samples
 74 e.g. from chronic infections.

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75 Introduction

76 Clinical laboratories often culture bacteria with enriched media, developed to support maximal
77 growth of particular pathogens. However, even these bacterial human pathogens can enter a growth-
78 restricted, transient state resulting in loss of culturability, especially following antibiotic treatment
79 (Pasquaroli et al., 2013). Non-culturable bacteria are apparent by microscopic identification in
80 clinical cases presenting sign of infection, but no positive cultures. These cases are especially
81 prevalent during chronic infection, where lack of culturability prevents proper diagnosis and
82 treatment (Høiby et al., 2010; Costerton et al., 2011; Høiby et al., 2015).

83 Two well defined, yet difficult to distinguish, non-culturable states have been described for
84 bacteria: viable but non-culturable (VBNC) and persisters (Oliver, 2010; Wood et al., 2013; Fisher et
85 al., 2017; Kim et al., 2018). Several environmental stresses have been associated with non-
86 culturable states, e.g. oxidative stress, change in temperature or pH, nutrient starvation, change in
87 osmotic concentrations and presence of heavy metals or antibiotics (Oliver, 2010; Ayrappetyan et al.,
88 2015). Oxygen starvation, referred to as anoxic conditioning in this paper, has also shown to induce
89 a non-culturable state in batch cultures of *Pseudomonas aeruginosa*, but supplementation with
90 nitrate (NO_3^-) as an alternative electron acceptor during anoxic plating restored culturability
91 (Binnerup, 1993). The lack of growth during normoxic plating suggests that anoxic conditioning
92 sensitizes a subpopulation of *P. aeruginosa* to O_2 or its toxic derivatives, such as reactive oxygen
93 species (ROS). ROS are continuously created during aerobic respiration by incomplete reduction of
94 O_2 , which is toxic to cells if not scavenged (Fenchel and Finlay, 2008). Presence of ROS has, in
95 some studies, been shown to generate VBNC bacteria (Oliver, 2010; Noor, 2015), but most research
96 has focused on *Escherichia coli* strains or *Vibrio* spp., while the effects of ROS on *P. aeruginosa*
97 and other facultative pathogens are not well characterized.

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98 Evidence of anoxic zones and anaerobic bacterial activity in chronic infections suggests that
 99 colonizing bacteria, such as *P. aeruginosa*, experience anoxic conditioning (Hassett et al.,
 100 2002; Worlitzsch et al., 2002; Kolpen et al., 2014). High rates of O₂ consumption by
 101 polymorphonuclear leukocytes generate local, anoxic zones and likely play a major role in O₂
 102 depletion during chronic infection (Høiby et al., 2015). In addition, other cells of the host may also
 103 consume O₂, increasing the likelihood of bacteria experiencing anoxia (Worlitzsch et al., 2002).
 104 Many chronic infections are thought to contain bacteria in the biofilm mode of growth (Costerton et
 105 al., 2003) and endogenous O₂ depletion inside the biofilm (Sønderholm et al., 2018), along with
 106 intense O₂ consumption by the host immune response (Worlitzsch et al., 2002; Kolpen et al.,
 107 2010; Trunk et al., 2010; Kragh et al., 2014; James et al., 2016; Jensen et al., 2017), may therefore
 108 increase the number of VBNC bacteria.
 109
 110 This study aimed to determine whether anoxic conditioning generates VBNC cells of *P. aeruginosa*
 111 during biofilm growth. Furthermore, we investigated whether ROS are involved in the loss of
 112 culturability.

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113 Materials and Method

114 Bacterial strains

115 *Pseudomonas aeruginosa* (PAO1, ATCC 15692), a catalase A deficient *Pseudomonas aeruginosa*
116 strain ($\Delta katA$ PAO1) (Hassett et al., 1999), *Staphylococcus epidermidis* ATCC 14990,
117 *Staphylococcus aureus* NCTC 8325-4 (methicillin susceptible) (Frees et al., 2004), *Staphylococcus*
118 *aureus* USA300 JE2 (MRSA) (Fey et al., 2013), *Escherichia coli* CFT073 (Welch et al., 2002) and
119 a clinical strain of *Enterococcus faecalis* from the Department of Clinical Microbiology,
120 Copenhagen University Hospital – Rigshospitalet, Denmark were used in this study.

121 Agar plates and media

122 Primarily lysogeny broth (LB) agar plates were applied in this study. Lysogeny broth (pH 7.5)
123 consisted of 5 g/L yeast extract (Oxoid, Roskilde, Denmark), 10 g/L tryptone (Oxoid), 10 g/L NaCl
124 (Merck, USA). All plates contained 2 % agarose. Plates used for anoxic growth were supplemented
125 with 10 mM KNO₃ (Sigma-Aldrich, USA) to serve as alternative electron acceptor during anoxic
126 plating, referred to as NO₃⁻ throughout the paper. All agar plates and media in this study were
127 supplied by the Panum Institute Substrate Department (Copenhagen, DK).

128 Anoxic growth

129 Experiments investigating growth under anoxic conditions were performed in an anaerobic chamber
130 (Concept 400 Anaerobic Workstation, Ruskinn Technology Ltd, UK). The gas atmosphere
131 consisted of N₂/H₂/CO₂ (ratio - 80:10:10). Anoxic chamber environment was confirmed with an
132 oxygen sensor (HQ40d multi, HACH Company, USA). All media and chemical solutions used in
133 anaerobic experiments were equilibrated in the anaerobic chamber 3 days prior to experiment. In
134 the case of solutions requiring refrigeration, a minimal volume was applied (< 1 mL), sealed with
135 Parafilm M, and thoroughly shaken upon entry into anaerobic chamber for quick gas equilibration.

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Direct viable count with LIVE/DEAD staining

LIVE/DEAD staining was applied to estimate the proportion of viable and non-viable cells in 24-hour-old batch cultures and in 16-day-old anoxically conditioned batch cultures of *P. aeruginosa*. The dyes consisted of two fluorescent nucleic acid stains, the green fluorescent stain (live cells) SYTO9 (Invitrogen, USA) and the red fluorescent stain (dead cells) propidium iodide (PI, Sigma-Aldrich, USA). SYTO9 penetrates both intact and damaged membranes while PI only stains damaged cells, thereby creating an opportunity to discriminate between live and dead cells (Li et al., 2014). Bacterial suspensions were vortexed thoroughly (1 min) and sonicated (5 min degas + 5 minutes sonication) before staining. To stain the cells, one μL of PI and SYTO9 was added to 1 mL of bacterial suspension and incubated for 15 minutes at room temperature. Suspensions of each biological replicate were then filtered through a 0.2 μm black Whatman, Nuclepore Trach-Etch Membrane (Sigma-Aldrich, Denmark) and visualized with confocal laser scanning microscopy using a Zeiss LSM 710 with a 63 $\times$ /1.4 (numerical aperture) objective (Zeiss, Germany). Fifteen random fields (135 μm x 135 μm) were examined for each filter (n=3). Enumeration of live (green) and dead (red) bacteria were done with the IMARIS software package (Bitplane AG, Schwitterland). Briefly, individual particles in each field were identified and counted as bacteria. Particles that were stained with both PI and SYTO9 were considered non-viable and thus counted as dead cells. Conversion of enumerated viable and dead cells to bacterial counts per milliliter was performed as described (Boulos et al., 1999) to compare them with CFU/mL. Briefly, the numbers of particles per mL were estimated using the following equation $\frac{\text{particles}}{\text{mL}} = A * (\frac{B}{C})/D$. Where A is the amount of particles per field, B is the surface of filtration (mm), C is the area of the microscopic field and D the volume of the sample filtered. Normoxic and anoxic CFU/mL was carried out simultaneously to estimate the proportion of VBNC cells. CFU/mL was determined in the same way as described for liquid batch cultures.

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160 **Bead-embedded inoculum of *P. aeruginosa***

161 Preparation of alginate beads with *P. aeruginosa* was carried out according to a method described
 162 by Snderholm *et al.* (Snderholm et al., 2017). Subsequently, beads were divided (10 beads per
 163 vial) into vials (Oximate Vial, PerkinElmer Inc., USA) containing 15 mL LB medium. Vials were
 164 sealed with Parafilm M (oxygen permeable) and incubated at 37C on an orbital shaker at 180 rpm.
 165 Anoxic and normoxic CFU counts were determined from beads and from the surrounding
 166 suspension from the same vials on each sampling day (day 3, 6, 8, 12, 19 and 21). Two beads were
 167 sampled per biological replicate (n=4). Before determination of CFU, the beads were washed twice
 168 with 0.9 % NaCl to remove non-attached cells and transferred to 1.5 mL microcentrifuge tubes
 169 (Sigma-Aldrich, USA). One-hundred microliters of 0.1 M sodium carbonate (Na₂CO₃) followed by
 170 100 L of 0.04 M citric acid was added to the tubes to dissolve the beads. The suspensions were
 171 then sonicated (5 min degas + 5 minutes sonication; Bransonic ultrasonic cleaner 2510, Emerson
 172 Electric, USA) before ten-fold dilution series were performed in 0.9 % NaCl. CFU was determined
 173 by plating three, 10 L-drops per dilution per replicate. Anoxic CFU determinations were
 174 performed inside an anaerobic chamber on LB plates supplemented with 10 mM NO₃⁻. The same
 175 dilution series were applied to normoxic CFU determination outside the anaerobic chamber on LB
 176 plates with 10 mM NO₃⁻. Plates were incubated 2 days before counting CFU.

177 **Filter biofilms with *P. aeruginosa***

178 This protocol was adapted to grow reproducible biofilms under anoxic and normoxic conditions.
 179 The method has previously been described by Bjarnsholt *et al.* (2015). The filter biofilms were kept
 180 on the same LB plates throughout the experiment. *P. aeruginosa* was propagated from frozen stock
 181 and grown overnight in 20 mL LB medium at 37C on an orbital shaker at 180 rpm. Cultures were
 182 adjusted to an optical density of 0.05 (OD₆₀₀; UV spectrophotometer UV-1800 UV-VIS, Shimadzu
 183 corporation, JP) and 10 L was transferred to the cellulose nitrate membrane filters (25 mm in

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diameter, GE Healthcare Life Sciences, UK). Plates were incubated under normoxic and anoxic conditions and kept in plastic bags with wet paper to avoid dehydration. Two filters were sampled per biological replicate (n=4) on each sampling day (day 1, 3, 7, 15 and 17). Filters were removed, placed in 10 mL tubes containing 5 mL 0.9 % NaCl, vortexed thoroughly (one min.) and sonicated (5 min degas + 5 minutes sonication). CFU/mL was determined as previously described.

Liquid batch cultures of *P. aeruginosa*

P. aeruginosa was propagated from frozen stock and grown overnight in 20 mL LB media at 37°C and orbitally shaken at 180 rpm. Cultures were adjusted to an optical density (OD₆₀₀) of 0.1 in glass vials (Oximate Vial) with a final volume of 20 mL LB. Vials were left to incubate at 37°C and orbitally shaken at 180 rpm. To create a normoxic environment, half of the vials were incubated with Parafilm M on top (normoxic conditioning), while the rest were incubated with a lid on top creating an anoxic environment (anoxic conditioning). Anoxic and normoxic plating was carried out from normoxic and anoxic conditioned liquid batch cultures (referred to as batch cultures throughout the paper) on each sampling day (day 1, 3, 5, 9, 11, 14, 16, 18, 21 and 28). Normoxic plating was carried out on LB plates with and without 10 mM NO₃⁻ to test whether presence of NO₃⁻ affected the number of CFU. Two mL (2 x 1 mL = 2 technical replicates) were sampled per biological replicate (n=4) on each sampling day. CFU/mL was determined as previously described.

Colonies of *P. aeruginosa*

P. aeruginosa was propagated from frozen stock and grown overnight in 20 mL LB media at 37°C on an orbital shaker at 180 rpm. The cultures were then streaked onto LB plates supplemented with 10 mM NO₃⁻. Plates were incubated under normoxic and anoxic conditions and kept in plastic bags with wet paper to avoid dehydration. A one-μl loop was used to sample colony material from each biological replicate (n=3) on each sampling day (day 6, 9, 13, 15 and 20). Colonies were transferred to 1.5mL microcentrifuge tubes (Sigma-Aldrich, Denmark) with 0.5 mL 0.9 % NaCl, vortexed

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thoroughly (1 min) and sonicated (5 min degas + 5 minutes sonication). CFU/mL was determined as previously described.

Reactive oxygen species (ROS)

To elucidate whether growth of anoxically conditioned bacteria was restricted by creation of ROS during aerobic respiration, sodium pyruvate and catalase (Sigma-Aldrich, USA) was tested as ROS scavenger in LB plates with 10 mM NO_3^- . Sodium pyruvate (0.3 %) was tested during anoxic and normoxic plating for 24-hour-old batch cultures and anoxically conditioned 16-day-old batch cultures of *P. aeruginosa*. Catalase (~50,000 units of catalase per L medium) was tested during anoxic and normoxic plating for anoxically conditioned 16-day-old batch cultures of *P. aeruginosa*. Furthermore, CFU/mL was also determined from 16-day-old anoxically conditioned batch cultures of *P. aeruginosa* and a catalase A deficient *P. aeruginosa* (ΔkatA PAO1) to test the influence of ROS. Lastly, creation of ROS was measured along with optical density (OD) at day 1, 8 and 16 for normoxically and anoxically conditioned batch cultures of *P. aeruginosa*. Creation of ROS was measured by adding 5 μM 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA; Sigma) to the bacterial suspension. This concentration has previously been used to detect ROS for *P. aeruginosa* (Jensen et al., 2014). Two hundred microliter of anoxically conditioned cultures (OD_{600} adjusted to 0.1 in fresh LB medium) was added to each well of black 96-well microtiter plates with transparent flat bottom (16503, Thermo Fisher Scientific, Rochester, NY). OD and ROS was measured over 15 hours at 27°C with a VICTOR Multilabel Plate Reader (Perkin Elmer, MA). All experiments consisted of 3-4 biological replicates.

Filter biofilms with other pathogens

To determine if other bacteria behaved in the same way as *P. aeruginosa*, we investigated the effect of anoxic conditioning on a selected group of pathogens (*Staphylococcus epidermidis* ATCC 14990, *Staphylococcus aureus* NCTC 8325-4 (methicillin susceptible), *Staphylococcus aureus* USA300

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JE2 (MRSA), *Escherichia coli* CFT073 and a clinical strain of *Enterococcus faecalis*). The filter biofilm method was used as described earlier. For the methicillin susceptible strain, CFU was determined in the same way as described for the filters with *P. aeruginosa*. For the remaining strains, CFU was determined at day 1 and 9 on LB plates supplemented with 10 mM $\text{NO}_3^- \pm \text{O}_2$. Furthermore, CFU was determined on LB plates supplemented with 10 mM NO_3^- and 0.3 % sodium pyruvate to evaluate the effect of ROS. A minimum of three biological replicates was performed and CFU was carried out in the same way as described earlier.

Statistics

To evaluate the difference in bacterial growth between incubation conditions (normoxic and anoxic conditioning), a linear regression was used with the difference between the logarithmically transformed values for normoxic and anoxic plating as outcome and with day (categorical) and interaction between day and plating condition (binary) as explanatory variables in SAS Genmod Procedure. The p-value of the interaction term was used as the p-value for the difference in bacterial growth. Log difference was calculated as $(\log_{10}[\text{CFU/mL}]_{\text{anoxic}} - \log_{10}[\text{CFU/mL}]_{\text{normoxic}})$. The mean and standard error of the mean (SEM) were calculated for recovering bacteria and plotted using GraphPad Prism 6.1 (GraphPad Software, La Jolla, USA). From the ratio between anoxic (CFU - O_2) and normoxic (CFU + O_2) colony counts it was possible to calculate the fraction of DTC bacteria [% DTC bacteria = $\frac{(\text{CFU} - \text{O}_2) - (\text{CFU} + \text{O}_2)}{(\text{CFU} - \text{O}_2)} \times 100$]. Data that were not part of the long-term experiments were instead analyzed with a 1-way ANOVA followed by Tukey's multiple comparison tests or an unpaired t-test. A p-value ≤ 0.05 was considered statistically significant. The tests were performed with either Prism 6.1 (GraphPad Software, La Jolla, USA) or SAS v.9.4 (SAS Institute Inc., Cary, NC, USA).

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256 Results

257 Direct viable counts with LIVE/DEAD staining revealed a VBNC *P. aeruginosa* population.

258 Direct viable counts were carried out for 16-day-old, anoxically conditioned batch cultures of *P.*
 259 *aeruginosa* to investigate if anoxic conditioning could generate a VBNC population. Findings were
 260 compared to normoxic and anoxic plating (plating methods) performed on LB plates supplemented
 261 with 10 mM NO₃⁻ (Fig. 1B). Anoxic plating yielded a significantly ($p = 0.023$) higher count of
 262 colony forming units per milliliter (CFU/mL) than normoxic plating ($0.85 \log \text{ values} \pm 0.04 \text{ SD}$).
 263 When comparing bacterial plate counts with direct viable counting, significantly more viable cells
 264 were measured compared to normoxic ($2.31 \log \text{ values} \pm 0.47 \text{ SD}$, $p < 0.0001$) and anoxic plating
 265 ($1.46 \log \text{ values} \pm 0.5 \text{ SD}$, $p = 0.0009$). The cells represented by this difference were considered
 266 VBNC. Direct viable counts were also performed on 24-hour-old batch cultures to investigate
 267 whether findings of VBNC *P. aeruginosa* were restricted to anoxically conditioned cells. No
 268 difference in bacterial counts was observed (Fig. 1A).

270 Anoxic conditioning generated oxygen intolerant subpopulations of *P. aeruginosa* in biofilm 271 and planktonic models.

272 To examine if biofilm growth affected the subsequent normoxic and anoxic plating, *P. aeruginosa*
 273 was grown in an alginate-bead biofilm model (Sønderholm et al., 2017). Plate counts were
 274 performed on LB plates supplemented with 10 mM NO₃⁻. Plates were incubated under normoxic
 275 and anoxic conditions and plate counts were compared from the beads (biofilm) and the
 276 surrounding media (planktonic) of the same vials over a period of 21 days (Fig. 2A and 2B,
 277 respectively). The log difference was significantly ($p = 0.003$) higher for biofilm than planktonic
 278 cells (Fig. 2C). The cells represented by this difference were considered to be in a difficult-to-

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279 culture (DTC) state and ranged from five to 54 % of the entire population from day six to 19 (Fig.
280 S1).

281 *P. aeruginosa* was also grown anoxically or normoxically on a filter biofilm model using LB plates
282 supplemented with 10 mM NO₃⁻ over a period of 17 days. CFU/mL was then determined using
283 anoxic and normoxic plating, as above (Fig. 3A and 3B, respectively). The log difference between
284 plating methods was significantly ($p = 0.01$) higher for anoxically conditioned biofilms than
285 normoxically conditioned biofilms (Fig. 3C). The fraction of DTC *P. aeruginosa* ranged from six to
286 23 % from day three to 17 (Fig. S1).

287 Similar results were obtained for anoxically and normoxically conditioned planktonic batch cultures
288 of *P. aeruginosa* over a period of 28 days (Fig. 4A and 4B, respectively). The log difference
289 between plating methods was significantly ($p < 0.0001$) higher for anoxically conditioned batch
290 cultures (Fig. 3C). The fraction of DTC bacteria ranged from 60 to 90 % from day nine to 21 (Fig.
291 S1).

292 The effect of anoxic conditioning was further investigated on colonies of *P. aeruginosa* grown on
293 LB plates supplemented with 10 mM NO₃⁻ under anoxic and normoxic conditions over a period of
294 20 days (Fig. 5A and 5B, respectively). The log difference between plating methods was
295 significantly higher ($p < 0.0001$) for anoxically conditioned colonies (Fig. 5C). The fraction of DTC
296 *P. aeruginosa* ranged from 12 to 46 % from day six to 20 (Fig. S1).

297

298 **10 mM NO₃⁻ in LB plates did not affect CFU/mL in batch cultures of *P. aeruginosa*.**

299 To ensure that presence of NO₃⁻ did not affect the number of CFU generated on LB plates, CFU/mL
300 was determined anoxically (LB plates + 10 mM NO₃⁻) and normoxically (LB plates ± 10 mM NO₃⁻)
301 from 24-hour-old batch cultures of *P. aeruginosa*. No difference was observed between type of
302 plating ($p = 0.93$): anoxic log CFU/mL + NO₃⁻ = 9.96 (± 0.22 SD), normoxic log CFU/mL + NO₃⁻ =

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9.91 ($\pm$ 0.10 SD) and normoxic log CFU/mL - NO_3^- = 9.91 ($\pm$ 0.14 SD). Furthermore, there was no effect of NO_3^- supplementation on normoxic plating in the prolonged experiment with 28-day-old batch cultures (Fig. 4A and 4B). It was not possible to detect growth of *P. aeruginosa* on LB plates under anoxic conditions without NO_3^- , but growth was observed when these plates subsequently were placed under normoxic conditions.

308

Oxidative stress restricted the growth of anoxically conditioned *P. aeruginosa* when re-grown in a normoxic environment.

Bacteria from 16-day-old anoxically and normoxically conditioned batch cultures of *P. aeruginosa* were plated on 10 mM NO_3^- LB plates $\pm$ sodium pyruvate and $\pm$ catalase to investigate whether formation of ROS contributed to the DTC state induced by anoxic conditioning. Presence of sodium pyruvate as a ROS scavenger (Mizunoe et al., 1999) significantly ($p = 0.04$) increased normoxic CFU/mL compared to plating without sodium pyruvate (Fig. 6B). This was not the case in 24-hour-old batch cultures of *P. aeruginosa* ($p = 0.62$), indicating that the effect was restricted to anoxically conditioned cells (Fig. 6A). Presence of catalase also significantly ($p = 0.03$) improved normoxic plate counts compared to normoxic plating without catalase (Fig. 6B). These results show that hydrogen peroxide is involved in the induction of a DTC state created by anoxic conditioning. To add proof to this hypothesis, a similar experiment was carried out with a catalase deficient *P. aeruginosa* mutant ($\Delta katA$ PAO1), which is more susceptible to oxidative stress (Hassett et al., 1999; Jensen et al., 2014). The log difference between plating methods for the $\Delta katA$ mutant and reference strain was significantly ($p = 0.00002$, unpaired t-test) different. These log difference values were 3.02 ± 0.05 SD and 1.61 ± 0.07 SD for $\Delta katA$ PAO1 and the reference strain, respectively (Fig. 6C). Moreover, measurement of ROS and optical density (OD) was carried out for *P. aeruginosa* at day 1, 8 and 16 from anoxically and normoxically conditioned batch cultures.

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These results showed creation of ROS during normoxic growth. The longer PAO1 was anoxically conditioned; the lower was the following normoxic growth, as measured by OD. This effect was less pronounced for normoxically-conditioned cells (Fig.7 A-C).

Anoxic conditioning affects *Staphylococcus aureus* and *Staphylococcus epidermidis*, but not *Escherichia coli* and *Enterococcus faecalis*

The effect of anoxic conditioning was then tested on a selected group of pathogens to determine if this phenomenon was restricted to *P. aeruginosa*. *S. aureus* (methicillin susceptible) was tested as described in the *P. aeruginosa* filter biofilm setup. Plate counts for anoxically and normoxically conditioned filters was determined (Fig. 8A and 8B, respectively). The log difference between plating method was significantly higher ($p < 0.001$) when plate counts were performed from anoxically conditioned filter biofilms than from normoxically conditioned filter biofilms (Fig. 8C). The fraction of DTC *S. aureus* ranged from three to 89 % from day one to day 17 (Fig. S1). Since both *P. aeruginosa* and *S. aureus* demonstrated effects of anoxic conditioning, a smaller experiment was initiated to test other pathogens. The effect was determined on LB plates supplemented with 10 mM NO_3^- $\pm$ sodium pyruvate to investigate if ROS were involved in the lack of growth. Both *S. aureus* (MRSA) and *S. epidermidis* showed effects of anoxic conditioning, resulting in an significant ($p = 0.01$ and $p = 0.03$, respectively) increase in CFU/mL during anoxic plating compared to normoxic plating (Fig. S2). The fraction of DTC *S. aureus* (MRSA) and *S. epidermidis* was 36 and 42 % at day 9, respectively. There was no observed effect of sodium pyruvate during normoxic plating for these two strains. In the case of *E. coli* and *E. faecalis*, there was no observed effect of anoxic conditioning (Fig. S3).

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351 Discussion

352 DTC and VBNC *P. aeruginosa*

353 In the current study, we demonstrate that plate counting with *P. aeruginosa* is significantly affected
 354 by anoxic conditioning when cultured in the presence of atmospheric oxygen levels. A DTC state
 355 was created after only a few days of anoxic conditioning in both biofilm and planktonic models.
 356 DTC *P. aeruginosa* was observed in the beads from the bead biofilm model, but not in the
 357 surrounding suspension, despite that the cultures had full access to atmospheric oxygen. This
 358 indicates that oxygen restriction within a biofilm generates a DTC subpopulation. The DTC state,
 359 detected as increased growth during anoxic plating with NO_3^- , has previously been generated in
 360 planktonic *P. aeruginosa* by energy starvation after cultivation without O_2 as electron acceptor for
 361 aerobic respiration (Binnerup, 1993). *In vitro* biofilms of *P. aeruginosa* may contain internal anoxic
 362 zones (Walters et al., 2003; Kolpen et al., 2016), so we hypothesized that anoxia inside the biofilm
 363 contributes to the induction of DTC *P. aeruginosa*. Accordingly, DTC *P. aeruginosa* was only
 364 observed in the filter biofilm model and the colony model when the model was anoxically
 365 conditioned. Interestingly, we found that 99.68 % of the population was VBNC with the
 366 LIVE/DEAD assay after anoxic conditioning. LIVE/DEAD staining is based upon membrane
 367 permeability and is only an approximation of true viability. Cells that were stained both red and
 368 green (resulting in yellow) were counted as dead cells but may still be viable.
 369 Approximately 50 % of the population in the alginate beads was DTC even though they were kept
 370 in normoxically-conditioned vials. In comparison, the fraction of DTC cells was approximately 90
 371 % in the anoxically conditioned batch cultures. The difference (90 % vs. 50 %) may be explained
 372 by the fact that a majority of the bacteria in the beads are peripherally located where they have
 373 increased access to oxygen compared to the center of the beads (Sønderholm et al., 2017). In
 374 comparison, bacteria from the anoxically conditioned batch cultures were fully deprived of O_2 . In

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the present study, the effect of anoxic conditioning was restricted to an intermediate period. This is probably due to the static methodological setup. Dynamic processes, such as entry of nutrients and removal of waste, occur during infection and are not modeled here (Brown et al., 2008). Nevertheless, this is not the first time a “resuscitation window” has been described for non-culturable bacteria. Similar studies have shown that VBNC bacteria can only be detected in an intermediate period of culturing (Pinto et al., 2015).

ROS restricts growth of anoxically conditioned *P. aeruginosa*

The DTC fraction of bacteria in this study was O₂ intolerant given that growth only was achievable when NO₃⁻ served as an alternative electron acceptor during anaerobic respiration. This led us to investigate whether this phenomena was due to oxidizing properties of ROS created by incomplete reduction of O₂ during aerobic respiration (Fenchel and Finlay, 2008). Sodium pyruvate increased counts for anoxically conditioned *P. aeruginosa* during normoxic plating (roughly 43 % of the DTC population) and we believe that it was due to its properties as a ROS scavenger (Mizunoe et al., 1999). Accordingly, 0.3 % sodium pyruvate has been used to resuscitate non-culturable populations of *S. aureus* (Pasquaroli et al., 2013). Catalase also increased counts during normoxic plating (roughly 50 % of the DTC population), indicating that hydrogen peroxide is involved in the induction of a DTC state. The effect of ROS was further confirmed by the reduced growth of a catalase A deficient *P. aeruginosa* mutant ($\Delta katA$ PAO1) after anoxic conditioning. Finally, measurements of ROS along with OD showed that anoxically conditioned batch cultures of *P. aeruginosa* created more ROS when regrown under atmospheric conditions the longer they were deprived from oxygen, resulting in an accumulation of ROS and a reduction in growth represented by lower OD measurements. These results indicate that accumulation of ROS during aerobic respiration has an impact on normoxic plate counting when *P. aeruginosa* has been anoxically conditioned. Furthermore, it seems that hydrogen peroxide is involved in this loss of culturing. It

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has been suggested that VBNC cells cannot be resuscitated by addition of ROS scavengers and that “revived” cells in the presence of ROS scavengers are only injured cells and not VBNC cells (Pinto et al., 2015). It was not possible to determine whether DTC bacteria in this study were in an injured state.

S. epidermidis and *S. aureus* also become DTC after anoxic conditioning

Additional experiments were conducted to elucidate whether anoxic conditioning generated DTC sub-populations in other facultative pathogens. Filter biofilms of methicillin susceptible *S. aureus* showed the same effects of anoxic conditioning as *P. aeruginosa*. These experiments were also repeated for *S. epidermidis*, *S. aureus* (MRSA), *E. coli* and *E. faecalis*. Both *S. epidermidis* and *S. aureus* were significantly ($p = 0.01$ and $p = 0.03$, respectively) affected by anoxic conditioning, whereas *E. coli* and *E. faecalis* were not. The results in this study points toward an intermediate period where anoxic conditioned bacteria are benefitted by anoxic incubation with nitrate as alternative electron acceptor. We cannot rule out that we missed the period where *E. coli* and *E. faecalis* were DTC, given that they were tested at selected days. No effect of sodium pyruvate was found during normoxic plating for any of these tested organisms, suggesting that ROS is not involved in the loss of culturability for these pathogens, but additional ROS scavengers should be tested.

Conclusion

A non-culturable state can be induced by several physiological stresses and our knowledge in this area is expanding, though far from fully resolved. In this study, we demonstrate that anoxic conditioning generates a VBNC subpopulation in *P. aeruginosa* batch-cultures. Furthermore, we demonstrate that anoxic conditioning generates DTC sub-populations of *P. aeruginosa*, *S. aureus* and *S. epidermidis* during biofilm growth. The DTC population was only able to grow under anoxic

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conditions in the presence of NO_3^- as an alternative electron acceptor. In the case of *P. aeruginosa*, this phenomenon was explained by creation of lethal amounts of ROS during aerobic respiration and the bacteria's inability to neutralize it.

Data availability

The data generated in the current study are available from the corresponding author on reasonable request.

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Author contributions

LK performed the majority of the experiments. MK, MS, BFG, SC and KNK also performed experiments. TB, PØJ, KNK AK, and LK conceived and designed experiments. LK wrote the manuscript. MA and PØJ performed statistics. All authors analyzed data. All authors contributed to and corrected the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 – Direct viable counting reveals a viable but non-culturable population of *Pseudomonas aeruginosa* (PAO1) after anoxic conditioning

Bacterial counts per milliliter were determined with plate counting and direct viable counting from 24-hour-old batch cultures (A) and from 16-day-old anoxic conditioned batch cultures (B) of PAO1. Bacteria were stained with LIVE/DEAD staining to estimate the proportion of viable (“live”) and non-viable (“dead”) cells. There was significantly more viable bacterial counts when applying direct viable counting in comparison to normoxic plating ($p < 0.0001$, one-way ANOVA test) and anoxic plating ($p = 0.0009$, one-way ANOVA). Anoxic plating yielded significantly higher bacterial counts ($p = 0.023$, one-way ANOVA test) than normoxic plating. Symbols with error bars indicate the mean $\pm$ SEM ($n = 3$). + NO₃⁻ refers to the addition of 10 mM KNO₃ to LB agar plates.

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Fig. 2 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was generated in the bead biofilm model

Normoxic and anoxic colony forming units per milliliter (CFU/mL) of PAO1 over 21 days from the beads (A) and the surrounding suspension (B). Symbols with error bars indicate the mean $\pm$ SEM (n = 4). + NO₃⁻ refers to the addition of 10 mM KNO₃ to LB agar plates. The log difference (C) represents the difference in mean log CFU/mL between plating methods from the suspension and the beads, respectively. The log difference was significantly higher (p = 0.003, linear regression) in the beads than the surrounding suspension. Symbols with error bars indicate the mean + confidence intervals.

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Fig. 3 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was generated in the filter biofilm model

Normoxic and anoxic colony forming units per milliliter (CFU/mL) of PAO1 over 17 days from anoxically (A) and normoxically (B) conditioned filters. Symbols with error bars indicate the mean $\pm$ SEM (n = 4). + NO₃⁻ refers to the addition of 10 mM KNO₃ to LB agar plates. The log difference (C) represents the difference in mean log CFU/mL between plating methods from the normoxically and anoxically conditioned filters, respectively. The log difference was significantly higher (p = 0.01, linear regression) in the anoxically conditioned filters than the normoxically conditioned. Symbols with error bars indicate the mean + confidence intervals.

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Fig. 4 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was generated in the planktonic batch cultures

Normoxic and anoxic colony forming units per milliliter (CFU/mL) of PAO1 over 28 days from anoxically (A) and normoxically (B) conditioned batch cultures. Symbols with error bars indicate the mean $\pm$ SEM ($n = 4$). $\pm \text{NO}_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. The log difference (C) represents the difference in mean log CFU/mL between plating methods from the normoxically and anoxically conditioned batch cultures, respectively. The log difference was significantly higher ($p < 0.0001$, linear regression) in the anoxically conditioned batch cultures than the normoxically conditioned. Symbols with error bars indicate the mean + confidence intervals.

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Fig. 5 – An oxygen intolerant subpopulation of *Pseudomonas aeruginosa* (PAO1) was generated in colonies

Normoxic and anoxic colony forming units per milliliter (CFU/mL) of PAO1 over 20 days from anoxically (A) and normoxically (B) conditioned colonies. Symbols with error bars indicate the mean $\pm$ SEM ($n = 3$). $+\text{NO}_3^-$ refers to the addition of 10 mM KNO_3 to LB agar plates. The log difference (C) represents the difference in mean log CFU/mL between plating methods from the normoxically and anoxically conditioned colonies, respectively. The log difference was significantly higher ($p < 0.0001$, linear regression) in the anoxically conditioned colonies than the normoxically conditioned. Symbols with error bars indicate the mean + confidence intervals.

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Fig. 6 – Reactive oxygen species generates a DTC subpopulation of *Pseudomonas aeruginosa* (PAO1)

Normoxic and anoxic colony forming units per milliliter (CFU/mL) were determined for 24-hour-old batch cultures (A) and 16-day-old anoxically conditioned batch cultures (B) of PAO1. Normoxic CFU/mL was determined $\pm$ 0.3% sodium pyruvate (A and B), while addition of catalase was only tested for 16-day-old batch cultures. Symbols with error bars indicate the mean $\pm$ SEM (n = 4). +NO₃⁻ refers to the addition of 10 mM KNO₃ to LB agar plates. There was a significant difference between normoxic plating $\pm$ 0.3 % sodium pyruvate (p = 0.04, one-way ANOVA test) and normoxic plating $\pm$ catalase (p = 0.03, one-way ANOVA test). Normoxic and anoxic determination of CFU/mL were determined for 16-day-old anoxically conditioned batch cultures of PAO1 and Δ *katA* PAO1 (C). Symbols with error bars indicate the mean $\pm$ SEM (n = 3). Significant difference between anoxic and normoxic plating with both PAO1 and Δ *katA* PAO1 (p = 0.0014 and p < 0.0001, respectively, one-way ANOVA test).

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Fig. 7 - Increase in reactive oxygen species affects growth of anoxically conditioned *Pseudomonas aeruginosa* (PAO1) over time

Determination of reactive oxygen species (ROS) and optical density (OD) at day 1 (A), 8 (B) and 16 (C) for anoxically (-O₂) and normoxically (+O₂) conditioned PAO1. ROS was measured as counts per second (CPS) when 2',7'-dichlorodihydrofluorescein diacetate was converted to 2',7'-dichlorofluorescein. Growth was measured with optical density (OD₆₀₀) simultaneously to ROS measurement. Data is generated from 99 continuously measurements presented as a solid line with dots representing $\pm$ SEM (n = 4).

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Fig. 8 - An oxygen intolerant subpopulation of *Staphylococcus aureus* (methicillin susceptible) was generated in the filter biofilm model

Normoxic and anoxic colony forming units per milliliter (CFU/mL) of *Staphylococcus aureus* over 17 days from anoxically (A) and normoxically (B) conditioned filters. Symbols with error bars indicate the mean $\pm$ SEM (n = 4). + NO_3^- refers to the addition of 10 mM KNO_3 to LB agar plates. The log difference (C) represents the difference in mean log CFU/mL between plating methods from the normoxically and anoxically conditioned filters, respectively. The log difference was significantly higher ($p < 0.001$, linear regression) in the anoxically conditioned filters than the normoxically conditioned. Symbols with error bars indicate the mean + confidence intervals.