Oxygen-mediated regulation of biofilm development is controlled by the alternative sigma factor σ^B in *Staphylococcus epidermidis*

Running title: Oxygen-mediated regulation of biofilm in *S. epidermidis.*

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Abstract

Using a modified rotating-disk reactor to sparge oxygen to *Staphylococcus epidermidis* cultures, we report that oxygen negatively regulates biofilm development by influencing the activity of σ^B. Under anaerobic conditions increased σ^B activity activates *icaADBC*, which encodes enzymes responsible for polysaccharide intercellular adhesin synthesis, by repressing transcription of the negative regulator *icaR*.

Coagulase-negative staphylococci and *Staphylococcus aureus* are the most common cause of device-related infections (DVIs) and are known to cause 50-70% of intravenous catheter related infections (1). Biofilm development by *S. epidermidis* and *S. aureus* on the surfaces of implanted devices can give rise to persistent and difficult to treat infections. In both *S. aureus* and *S. epidermidis*, polysaccharide intercellular adhesin (PIA) is an important component of the staphylococcal biofilm (10). Synthesis of PIA requires enzymes encoded by the intercellular adhesion (*ica*) operon, *icaADBC* (15, 17). A number of studies have indicated that the *ica* locus may be a useful marker for distinguishing between significant and contaminating isolates (6, 7, 14, 21). In *S. epidermidis* strains carrying the *ica* locus, up-regulation of *ica* operon expression and PIA production are also required for biofilm formation. Environmental triggers such as ethanol and salt stress, excess glucose and subinhibitory antibiotic concentrations can also activate biofilm development (2, 3, 4, 11, 18, 19). A number of important *ica* operon regulators have now been identified. The *icaR* gene, which is located adjacent to the *ica* operon encodes a transcriptional repressor of the *ica* locus (3, 11). Expression of *icaR* is in turn repressed indirectly by the alternative, stress-responsive sigma factor, σ^B (13). Thus environmental conditions that activate σ^B result in repression of the *icaR* gene and de-regulation of the *icaADBC* operon (2, 12, 16, 20).

Biofilm development by *S. epidermidis* 1457 (12) and its isogenic mutant M15, which contains a Tn917 transposon insertion in the *rsbU* gene of the *sigB* locus (16), were investigated under anaerobic conditions. Biofilm development on polycarbonate coupons was measured in a modified rotating-disk reactor (RDR, Biosurface Technologies Corp., MT, USA), in which a sparger extending from the lid to below the liquid level was

employed to enable precise control of dissolved oxygen concentrations. Profiles of oxygen utilization of 1457 within the RDR were performed using oxygen sensor spots (PreSens GmbH, Regensburg, Germany) for the extremes of oxygen concentrations tested (Fig. 1). These profiles show that the rate of oxygen utilization by the cells is higher than the supply. It is important to note at this stage however, that though the profiles look identical from 7 h onwards (not shown), the cells would be in completely different conditions. Cells sparged with 0% oxygen are being forced to grow anaerobically, whereas cells at 21% are consuming the amount of oxygen supplied.

Biofilm development in the reactor was examined at oxygen concentrations of 0%, 7%, 14% or 21% with a gas flow rate of 0.5 L min^{-1} in all cases. Each run initially involved a 24 h batch phase, followed by a 24 h continuous phase in which quarter-strength Brain Heart Infusion media (Oxoid) was fed at 90 ml h^{-1} using a peristaltic pump (Watson Marlow, UK). Diluted concentrations of BHI are commonly used by others to culture biofilms (2, 9, 25), and our previous work (not shown) revealed quarter strength to be the optimum nutrient concentration for biofilm growth by this strain in our studies. Total RNA was extracted from biofilm cells as described previously (2) with the following modifications. Immediately on termination of a RDR run, the rotating-disk was aseptically removed and washed. Biofilms were scraped from the coupons and the viton surface of the disk and resuspended in RNA*later* (Ambion, TX) to maintain the biofilm mRNA expression profile. Prior to RNA purification, RNA*later* was removed and the biofilm dispersed by treatment with 100 µl 0.2M sodium metaperiodate (Sigma-Aldrich, Germany) for 5 mins as described previously (18). RT-PCR analysis, using the OneStep RT-PCR kit (Qiagen, UK), of *ica* operon transcript levels in RNA extracted

from biofilm cells involved a reverse transcription (RT) step at 55°C for 30 min followed by 31 amplification cycles of 90°C 20 s, 50°C 20 s and 72°C 20 s. Similar reaction conditions were used to measure *ica* operon expression in RNA extracted from planktonic cells but only 26 amplification cycles were required. RT-PCR analysis of *asp23* transcript levels, which is a known target gene of σ^B (8) involved an RT step at 55°C for 30 mins followed by 12 amplification cycles of 90°C 20 s, 50°C 20 s and 72°C 20 s. 16S rRNA yields were compared by agarose gel electrophoresis and the constitutively expressed *gyrB* gene (2) was used as an internal standard in all RT-PCR experiments.

Oxygen availability in the infection environments of implanted medical devices, which typically involve biofilms, are likely to vary and may in some instances be anoxic (22). By tightly controlling oxygen concentrations our data revealed enhanced *S. epidermidis* biofilm formation under anaerobic conditions, with a statistical difference evident between biofilms grown at 0% and 21% oxygen ($p < 0.05$) (Fig. 2). Examination of planktonic cells grown at 0% oxygen and 21% oxygen within the reactor (data not shown) revealed that differences in biofilm colony forming unit counts could be directly attributed to enhanced biofilm development. RT-PCR analysis revealed a substantial increase in *icaA* expression under anaerobic compared to aerobic (21% oxygen) conditions (5), in both biofilm (Fig. 3) and planktonic cells (Fig. 4). It is important to note that *ica* transcripts are still detected at low levels in samples grown at 21% oxygen, and that *icaADBC* transposon mutant abolishes biofilm in 1457 (12). Concomitant with the activation of the *ica* operon, expression of the *icaR* gene was also substantially higher in cells grown at 21% oxygen explaining, at least in part, why more biofilm was

consistently formed under anaerobic conditions. These data suggest that activation of the *ica* locus under anaerobic conditions is the result of *icaR* repression. To investigate the possible mechanism of *icaR* repression by oxygen, we examined the impact of oxygen on the activity of the alternative sigma factor σ^B by measuring transcription of *asp23*. Significantly *asp23* expression was dramatically activated under anaerobic conditions, indicating that σ^B activity is increased in the absence of oxygen (Fig. 4). Under anaerobic conditions in the modified RDR biomass yields of the *rsbU* mutant, *S. epidermidis* M15 (13), were significantly lower than the wild type strain (Fig. 5). These data strongly suggest that activation of σ^B activity under anaerobic conditions increases *icaADBC* expression, and accordingly biofilm formation, by repressing transcription of the *icaR* gene. Under aerobic conditions, biomass yields of M15 were similar to the wild type strain. These results, which contrast with previously published data (13), may be explained by differences in the growth environment between the modified RDR and 96-well plates. For example, localized anoxic regions are more likely to occur in the latter, where the specific oxygen transfer rate can be expected to be lower than in the RDR. Importantly ethanol also activates the *ica* operon and biofilm development by repressing *icaR* transcription, but in a σ^B-independent manner (2, 12, 13). Using the RDR system we confirmed that biofilm formation by M15 was restored to wild type levels under anaerobic conditions in BHI media supplemented with 4% ethanol (Fig. 5). These data support the existence of two separate pathways for *ica* locus activation in *S. epidermidis* and further reveal that anaerobic activation of *ica* operon expression and biofilm is dependent on the σ^B regulatory pathway. Under high oxygen conditions, it appears that that σ^B is less important for biofilm, as the wild type (1457) and the mutant exhibit

similar biofilm phenotypes. In *S. aureus,* the staphylococcal respiratory response regulator SrrA directly activates *ica* transcription under anaerobic conditions and does not modulate *icaR* expression (23). A potential role for SrrA in *S. epidermidis* has yet to be investigated, but amino acid sequence alignments suggest that no SrrA homologue exists in *S. epidermidis*. These findings may suggest that σ^B is less important for oxygen-dependent biofilm regulation in *S. aureus* and are consistent with previous studies indicating that σ^B plays a more important role in *S. epidermidis* biofilm regulation than in *S. aureus* (5, 12, 13, 24).

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Figure legends:

1. Dissolved oxygen profiles for *S. epidermidis* 1457 planktonic cultures grown in a modified RDR sparged with different oxygen concentrations using a baffle inserted directly into the media. Profiles are a mean of two independent runs.
2. Biofilm development by *S. epidermidis* 1457 on polycarbonate coupons in a modified rotating disk reactor after 48 h growth in quarter-strength BHI media at 21%, 14%, 7% and 0% dissolved oxygen concentrations. Data represent the number of colony forming units per cm^2. Error bars represent standard error from three independent experiments.
3. Comparative measurement of *icaA*, *icaR* and *gyrB* (control) transcription by RT-PCR in biofilm biomass of *S. epidermidis* 1457 grown in a modified rotating disk reactor in quarter-strength BHI media at 21%, 14%, 7% and 0% sparged oxygen concentrations. Comparative intensities of 16S rRNA bands after agarose gel electrophoresis are also shown. These experiments were performed three times and representative results are shown.
4. Comparative measurement of *icaA*, *icaR, asp23* and *gyrB* (control) transcription by RT-PCR in 48 h planktonic cultures of *S. epidermidis* 1457 biofilm grown in a modified rotating disk reactor in quarter-strength BHI media at 21% and 0% sparged oxygen concentrations. Comparative intensities of 16S rRNA bands after agarose gel electrophoresis are also shown. The cells were harvested from the reactor waste. These experiments were performed three times and representative results are shown.

5. Comparison of biofilm development of *S. epidermidis* 1457 and its isogenic *rsbU* mutant M15 on polycarbonate coupons in a modified rotating disk reactor after 48 h continuous growth in quarter-strength BHI media or BHI supplemented with 10% ethanol. CFUs for biofilm grown under aerobic (21% oxygen) and anaerobic (0% oxygen) conditions are shown. Data represent the number of colony forming units per cm^2. Error bars represent standard error from three independent experiments.

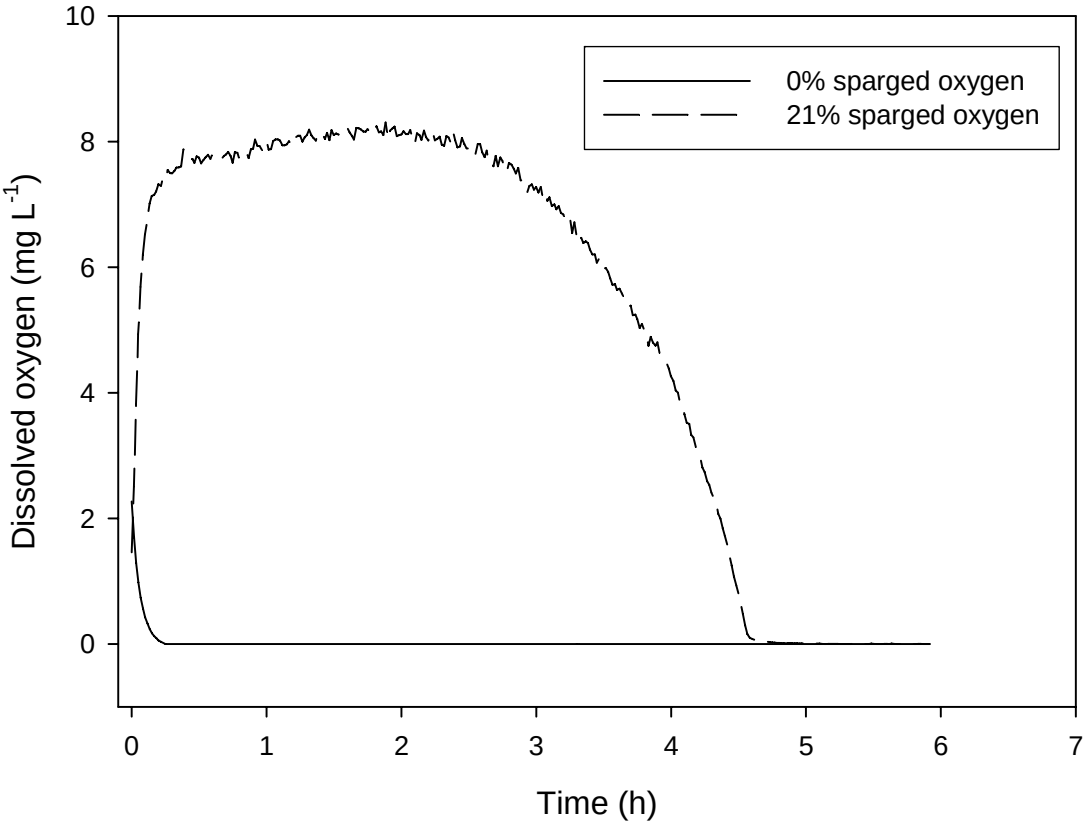
0% sparged oxygen
21% sparged oxygen
Dissolved oxygen (mg L-1)
Time (h)
0
2
4
6
8
10
0
1
2
3
4
5
6
7

Fig. 1

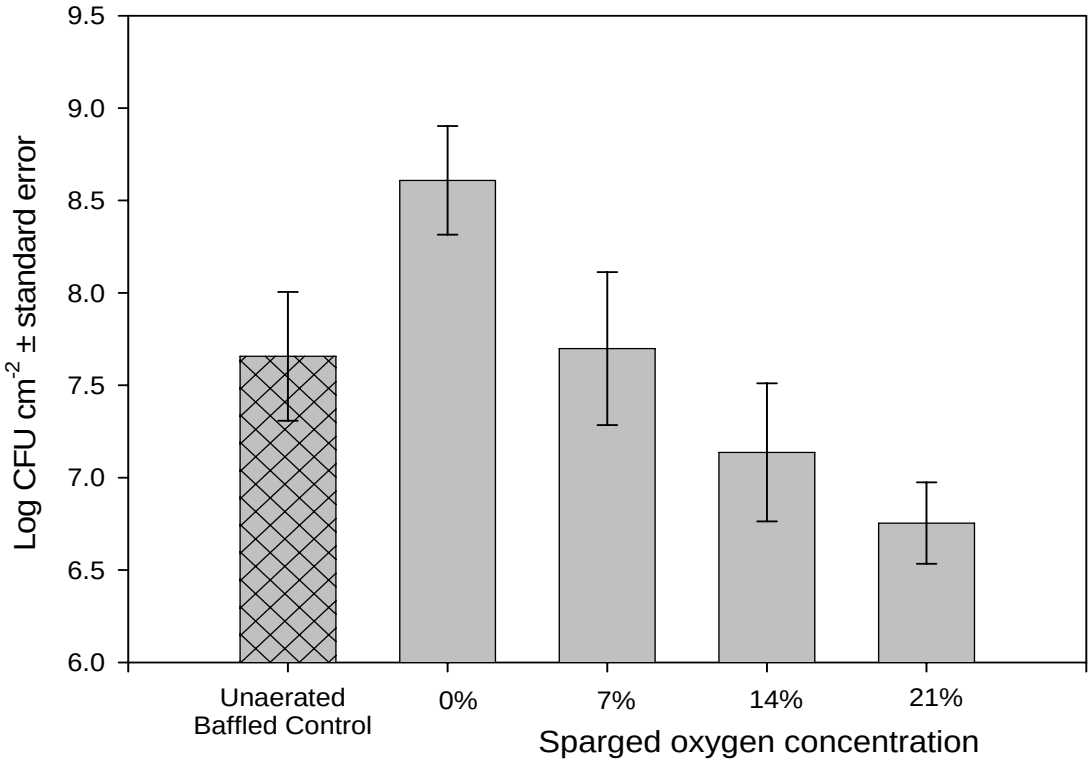
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
Log CFU cm-2 ± standard error
Unaerated Baffled Control
0%
7%
14%
21%
Sparged oxygen concentration

Fig. 2

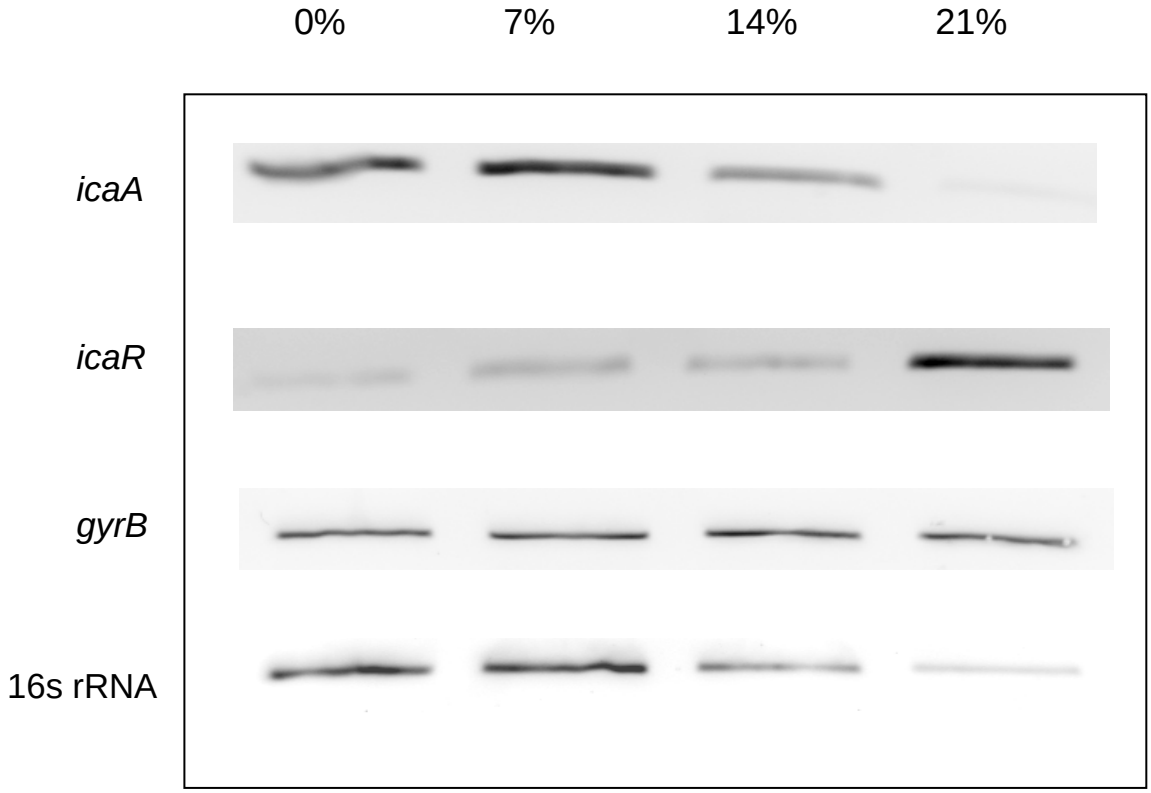

Fig.3

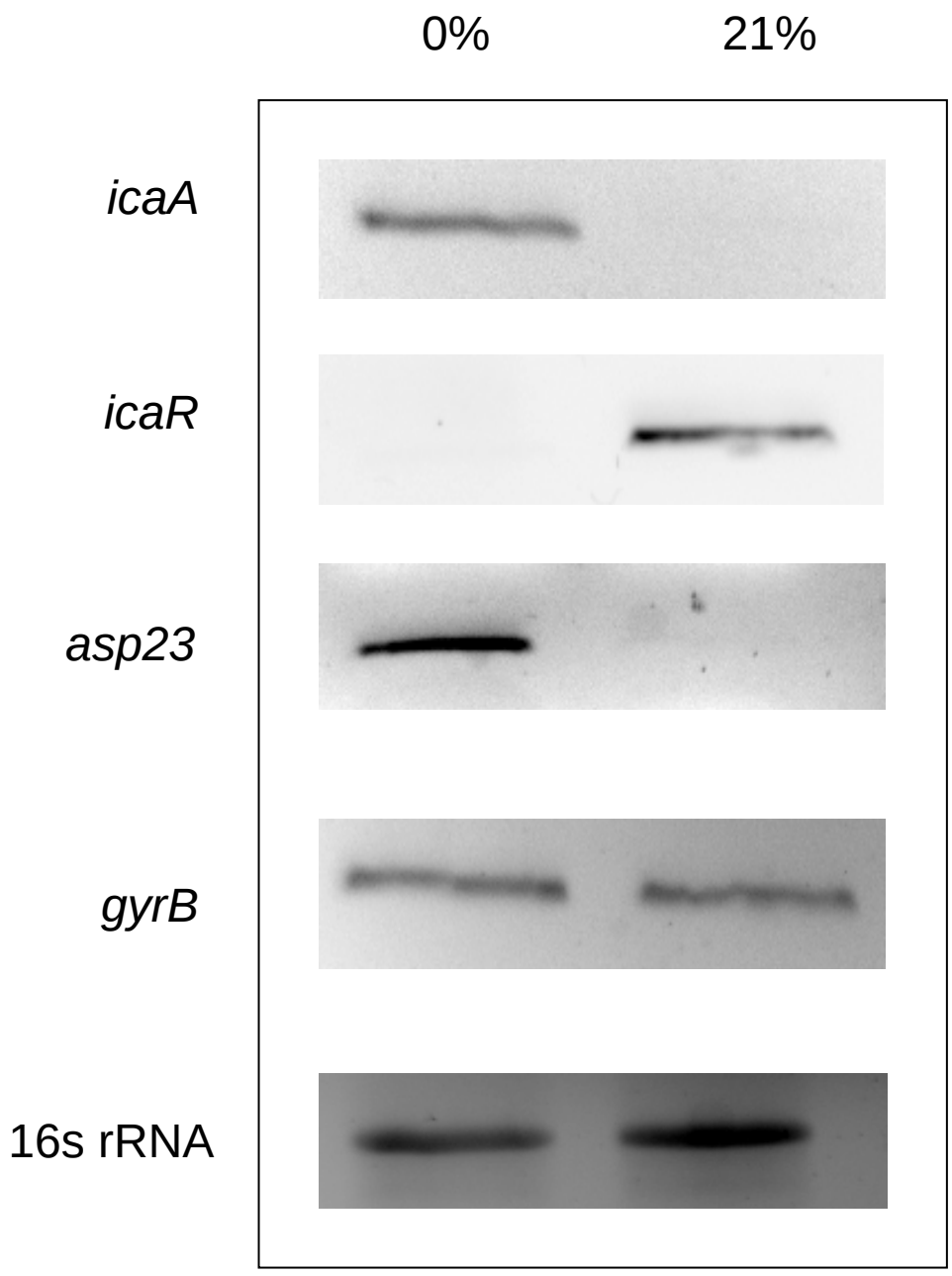

Fig. 4

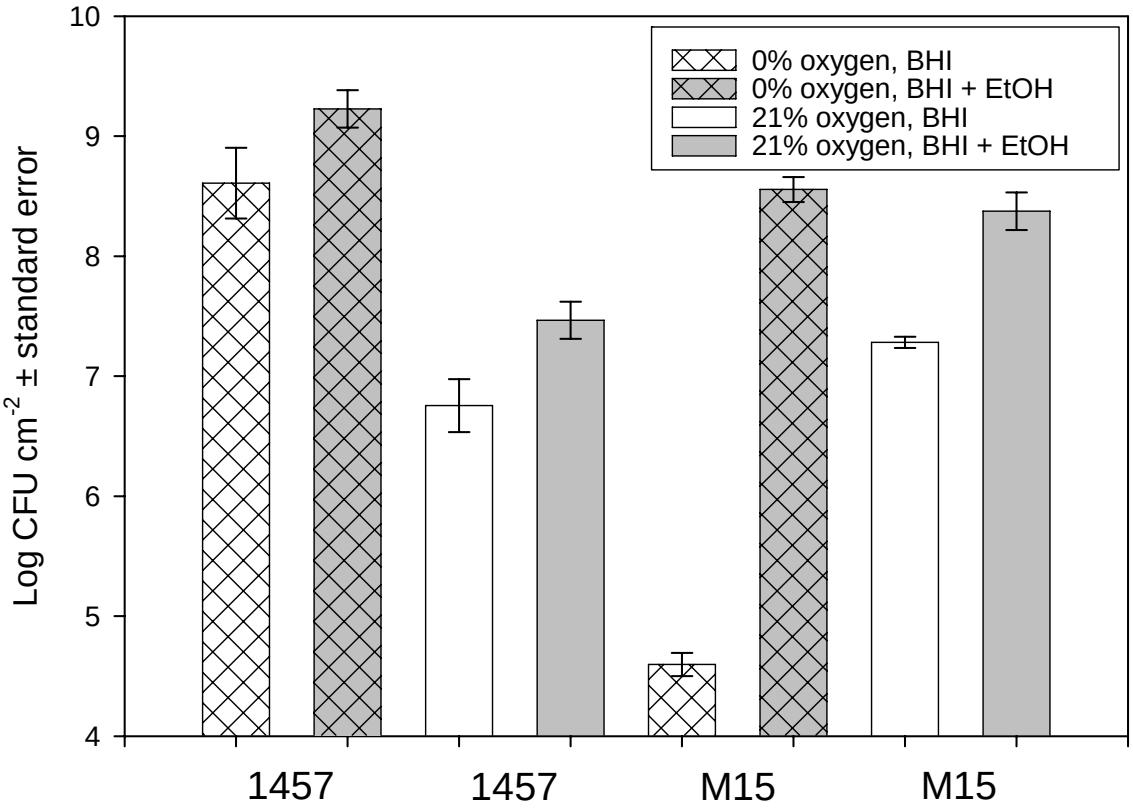
0% oxygen, BHI
0% oxygen, BHI + EtOH
21% oxygen, BHI
21% oxygen, BHI + EtOH
Log CFU cm-2 ± standard error
10
9
8
7
6
5
4
1457
1457
M15
M15

Fig. 5