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<td><strong>Publication date</strong></td>
<td>2009-01</td>
</tr>
<tr>
<td><strong>Publication information</strong></td>
<td>Applied and Environmental Microbiology, 75 (1): 261-264</td>
</tr>
<tr>
<td><strong>Publisher</strong></td>
<td>ASM</td>
</tr>
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<td><strong>Link to online version</strong></td>
<td><a href="http://dx.doi.org/10.1128/AEM.00261-08">http://dx.doi.org/10.1128/AEM.00261-08</a></td>
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<td><strong>Item record/more information</strong></td>
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<tr>
<td><strong>Publisher's statement</strong></td>
<td>All Rights Reserved.</td>
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<td><strong>Publisher's version (DOI)</strong></td>
<td>10.1128/AEM.00261-08</td>
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Oxygen-mediated regulation of biofilm development is controlled by the alternative sigma factor $\sigma^B$ 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 $\sigma^B$. Under anaerobic conditions increased $\sigma^B$ 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, $\sigma^{B}$ (13). Thus environmental conditions that activate $\sigma^{B}$ 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 RNALater (Ambion, TX) to maintain the biofilm mRNA expression profile. Prior to RNA purification, RNALater 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 icaADB 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 σB by measuring transcription of asp23.
Significantly asp23 expression was dramatically activated under anaerobic conditions,
indicating that σB 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 σB 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 σB-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 σB regulatory pathway. Under high oxygen conditions, it appears that
that σB is less important for biofilm, as the wild type (1457) and the mutant exhibit
similar biofilm phenotypes. In \textit{S. aureus}, the staphylococcal respiratory response regulator SrrA directly activates ica transcription under anaerobic conditions and does not modulate ica\textsubscript{R} expression (23). A potential role for SrrA in \textit{S. epidermidis} has yet to be investigated, but amino acid sequence alignments suggest that no SrrA homologue exists in \textit{S. epidermidis}. These findings may suggest that $\sigma^B$ is less important for oxygen-dependent biofilm regulation in \textit{S. aureus} and are consistent with previous studies indicating that $\sigma^B$ plays a more important role in \textit{S. epidermidis} biofilm regulation than in \textit{S. aureus} (5, 12, 13, 24).

This research was funded by Science Foundation Ireland (SFI) grant 04/BRG/E0072. We thank Liam Morris, Tom Burke, Frank Mac Loughlin, Eoin Syron and Barry Heffernan for engineering expertise; Linda Holland, Sinéad O’Donnell and Kate Malone for RNA advice and assistance.
References


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 insert 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 cm². 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 cm². Error bars represent standard error from three independent experiments.
Fig. 1
Fig. 2
<table>
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Fig. 3
Fig. 4

![Banding patterns for icaA, icaR, asp23, gyrB, and 16s rRNA under 0% and 21% conditions.](image)

- **icaA**: Shows a band in the 21% condition but not in the 0% condition.
- **icaR**: Shows a band in both conditions.
- **asp23**: Shows a band in both conditions with a slightly stronger intensity in the 21% condition.
- **gyrB**: Shows a band in both conditions with a notable difference in intensity.
- **16s rRNA**: Shows a band in both conditions with a darker intensity in the 21% condition.

16s rRNA is a standard control marker in such experiments.
Fig. 5