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# Electron Paramagnetic Resonance (EPR) Spectroscopy to Detect Reactive Oxygen Species in *Staphylococcus aureus*

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## Abstract

Under aerobic conditions, *Staphylococcus aureus* (*S. aureus*) primarily metabolizes glucose to acetic acid. Although normally *S. aureus* is able to re-utilize acetate as a carbon source following glucose exhaustion, significantly high levels of acetate in the culture media may not only be growth inhibitory but also potentiates cell death in stationary phase cultures by a mechanism dependent on cytoplasmic acidification. One consequence of acetic acid toxicity is the production of reactive oxygen species (ROS). The present protocol describes the detection of ROS in *S. aureus* undergoing cell death by electron paramagnetic resonance (EPR) spectroscopy. Using 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) as a cell permeable spin probe, we demonstrate the detection of various oxygen radicals generated by bacteria. Although standardized for *S. aureus*, the methods described here should be easily adapted for other bacterial species. This protocol is adapted from Thomas *et al.* (2014) and Thomas *et al.* (2010).

## Materials and Reagents

1. Staphylococcus aureus
2. Bacto tryptic soy broth without dextrose (TSB) (BD Diagnostic Systems, catalog number: DF0862178)
3. Glucose (Sigma-Aldrich, catalog number: G8270).
4. 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (Noxygen Science Transfer & Diagnostics GmbH, catalog number: NOX-2.2–100mg)
5. Superoxide dismutase (SOD) (Sigma-Aldrich, catalog number: S7571)
6. Dimethyl thiourea (DMTU) (Sigma-Aldrich, catalog number: D188700)
7. Critoseal (Thermo Fisher Scientific, catalog number: 0267620)
8. 5 μM DETC (Noxygen, Catalog number: NOX-10.1)

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9. 25 μM deferoxamine (Noxygen, Catalog number: NOX-10.1)
10. Culture flask (250 ml)
11. Culture tubes
12. 1.5 ml Eppendorf tubes
13. Krebs-HEPES buffer (KDD buffer) (see Recipes)

## Equipment

1. 37 °C shaker-incubator (250 rpm per min)
2. Leica Biosystems Critoseal capillary tube sealant (Leica Microsystems, catalog number: MS215003A)
3. Bruker e-Scan EPR Spectrometer and Noxygen Temperature Controller Bio-I (Bruker, model: NOX-E.11-ESR)
4. Micropipettes (50 μl, EPR tubes) (Noxygen Science Transfer & Diagnostics GmbH, catalog number: MS215003A)
5. Spectrophotometer
6. Vortex-Genie 2

## Software

1. Bruker WinEPR Data Processing software

## Procedure

1. To obtain starter culture, inoculate a single colony of *S. aureus* into 3 ml sterile TSB supplemented with 14 mM glucose and incubate overnight in a 37 °C shaker-incubator adjusted to 250 rpm.
2. Inoculate 25 ml sterile TSB (suppl. with 35 mM glucose) in a 250 ml flask with starter culture to a final $OD_{600}$ of 0.06 and incubate in a 37 °C shaker-incubator adjusted to 250 rpm for a period of 72 h.
3. Following the 72 h incubation period, 10 $OD_{600}$ units (~$7\times10^9$ cfu/ml) is harvested in a 1.5 ml Eppendorf tube and suspended in 1 ml of ice cold KDD buffer. The bacterial suspension is placed on ice until further use.
4. Prior to EPR measurements, the ROS sensitive spin probe CMH (working stock ~4 mM prepared in KDD buffer) is added to a final concentration of 200 μM in 1 ml bacterial suspension (step 3), briefly vortexed (2 sec) and allowed to stand at room temperature for 15 min.
5. The bacterial suspension (50 μl) is then transferred into micropipettes by capillary action and sealed at the distal end using Critoseal, immediately prior to EPR analysis.

6. EPR acquisition parameters are as follows: Field sweep width, 60 gauss; microwave frequency, 9.75 kHz; microwave power, 21.9 mW; modulation amplitude, 2.37 gauss; conversion time, 10.24 ms; time constant, 40.96 ms.

7. Record 2-D spectra and average 10 scans for each sample to reduce background noise. Quantitation of EPR spectra and baseline correction can be accomplished using Bruker WinEPR Data Processing software.

8. To identify the contribution of superoxide ($O^{2-}$) and hydroxyl radicals ($OH^{\cdot}$) to the overall EPR spectra, SOD (400 units), an $O^{2-}$ scavenging antioxidant enzyme and/or DMTU (20 mM), a $OH^{\cdot}$ scavenger, may be added to the bacterial suspension (step 3) prior to the addition of CMH and incubated at room temperature for 15 min. Continue with step 4 of this protocol.

   *Note: It is crucial to have a negative control (step 4; KDD buffer containing 200 μM CMH) when acquiring EPR signals. This control will report the degree of auto-oxidation CMH has undergone over the period of the experiment.*

## Representative data

### Recipes

1. Krebs-HEPES buffer (KDD buffer) (pH 7.4)

   99 mM NaCl

   4.69 mM KCl

   2.5 mM $CaCl_2$

   1.2 mM $MgSO_4$

   25 mM $NaHCO_3$

   1.03 mM $KH_2PO_4$

   5.6 mM D-glucose

   20 mM HEPES

   5 μM DETC

   25 μM deferoxamine

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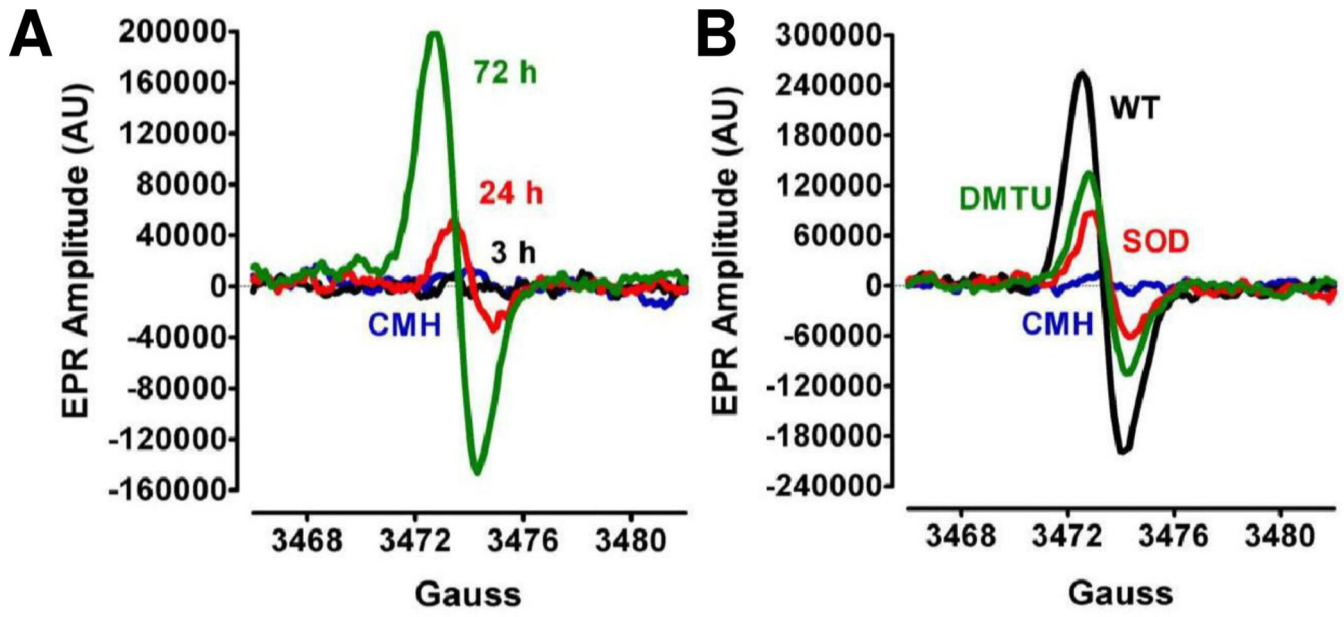


**Figure 1. EPR detection of *S. aureus* ROS production**

A. Following inoculation of *S. aureus* to an initial $OD_{600}$ of 0.06, cultures were incubated aerobically (flask: volume ratio = 10:1, 250 rpm). Culture samples for EPR analysis were withdrawn at 3 h, 24 h and 72 h of bacterial growth. B. *S. aureus* culture samples (72 h) were treated with either 400 units of SOD, 20 mM DMTU, or vehicle (wild-type, WT) before addition of the spin probe, CMH. The basal extent of CMH autoxidation in KDD buffer (blue line) was determined as control.