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# Endogenous $H_2O_2$ produced by *Streptococcus pneumoniae* controls FabF activity

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ABSTRACT

FabF elongation condensing enzyme is a critical factor in determining the spectrum of products produced by the FASII pathway. Its active site contains a critical cysteine-thiol residue, which is a plausible target for oxidation by $H_2O_2$. *Streptococcus pneumoniae* produces exceptionally high levels of $H_2O_2$, mainly through the conversion of pyruvate to acetyl-P via pyruvate oxidase (SpxB). We present evidence showing that endogenous $H_2O_2$ inhibits FabF activity by specifically oxidizing its active site cysteine-thiol residue. Thiol trapping methods revealed that one of the three FabF cysteines in the wild-type strain was oxidized, whereas in an *spxB* mutant, defective in $H_2O_2$ production, none of the cysteines was oxidized, indicating that the difference in FabF redox state originated from endogenous $H_2O_2$. *In vitro* exposure of the *spxB* mutant to various $H_2O_2$ concentrations further confirmed that only one cysteine residue was susceptible to oxidation. By blocking FabF active site cysteine with cerulenin we show that the oxidized cysteine was the catalytic one. Inhibition of FabF activity by either $H_2O_2$ or cerulenin resulted in altered membrane fatty acid composition. We conclude that FabF activity is inhibited by $H_2O_2$ produced by *S. pneumoniae*.



## 1. Introduction

$H_2O_2$ is an unavoidable cytotoxic by-product of the aerobic life of most living organisms. Under aerobic conditions, $H_2O_2$ is relatively stable and less reactive compared to other reactive oxygen species, but is able to perform a number of rather specific chemical reactions [1]. $H_2O_2$ is a mild oxidant that can oxidize specific protein thiol groups, producing proteins that can easily be reduced back by various cellular reductants [2]. Consequently, intracellular levels of $H_2O_2$ should be strictly controlled by the cell.

*Streptococcus pneumoniae* produces exceptionally high levels of $H_2O_2$ (up to 1 mM), mainly through the conversion of pyruvate to acetyl phosphate via pyruvate oxidase (SpxB) [3]. We have previously shown [4] that reducing $H_2O_2$ production, by growing bacteria at anaerobic conditions or by the truncation of the *spxB* gene, resulted in a dramatic change in membrane fatty acid (FA) composition: a significant enhancement in FA unsaturation and chain length. *S. pneumoniae* lacks FA desaturases and the β-oxidation enzyme set [5]. Thus, the alterations in FA composition probably result from *de novo* synthesis through the anaerobic type II, FA synthase (FASII) pathway.

FabF is a critical factor in determining the spectrum of products produced by the FASII pathway [6]. It catalyzes the irreversible addition of two-carbon units into the growing acyl chain, during the elongation phase of FA synthesis [7]. FabF active site is a catalytic triad composed of a central cysteine$^{164}$-thiol residue [7], which is a plausible target for oxidation by $H_2O_2$. Thus, $H_2O_2$ may inhibit FabF activity, thereby controlling the products of the FASII system.

Over the past few years, an increasing number of thiol-containing proteins have been identified, that use $H_2O_2$ as a mediator, to quickly regulate their protein activity. Importantly, the activity of these enzymes usually depends on the preservation of the reduced state of the cysteine residues involved [8]. This is also the case with FabF, the activity of which requires the covalent binding of the acyl intermediates to the active site cysteine-SH group [7]. The aim of the present study was to demonstrate that endogenous $H_2O_2$ produced by *S. pneumoniae* specifically oxidize the active site cysteine-thiol residue of FabF, thus controlling membrane FA composition.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

A clinical isolate of *S. pneumoniae* carrying serotypes 2 (D39), and its isogenic mutant containing an in-frame fusion of Tn*phoA* to the pyruvate oxidase gene (*spxB*::Tn*phoA*) [9], kindly provided by J.N. Weiser (University of Philadelphia), were used in this study. Bacteria were grown to $OD_{620nm} = 0.2$ under aerobic or anaerobic conditions as previously described [4].

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### 2.2. FA extraction and analysis

Bacteria were centrifuged and kept in liquid nitrogen. Lipids were transmethylated with 2% $H_2SO_4$ in methanol at 80 °C for 1 h. The resulting FA methyl esters were analyzed by gas chromatography on a Supelcowax 10 capillary column using a temperature gradient from 180 to 225 °C as previously described [10]. A known amount of an internal standard (C17:0) was added to the samples for lipid quantitation. FA methyl esters were identified by co-chromatography with authentic standards and by comparison of their equivalent chain length. The proportions of the various FA were also measured in bacteria grown in the presence of 22.4 μM cerulenin.

### 2.3. Construction, expression, and purification of FabF

The *fabF* gene was amplified by PCR from a single colony of *S. pneumoniae* D39 and subcloned into pRSETc vector (Invitrogen) between BglII and KpnI sites. *E. coli* BL21 cells harboring the constructed plasmid were grown in LB medium supplemented with ampicillin (100 μg/ml) to $O.D._{600\ nm} = 0.5–0.7$ and induced with 0.5 mM IPTG for additional 3 h. Cells were harvested by centrifugation and stored at −70 °C. The pellet was suspended in lysis buffer (50 mM Tris pH 8, 100 mM PMSF), disintegrated by sonication, and centrifuged at 4000 × *g* for 1 h. Proteins in the supernatant were loaded onto a Ni-Nta column (Adar biotec, Israel), and incubated for 1 h at 4 °C. The column was then washed with buffer (10 mM imidazol), and the recombinant protein recovered from the column with elution buffer (100 mM imidazol).

### 2.4. Immunization of rabbits

Three-month-old albino rabbits (Harlan Laboratories, Israel) were immunized subcutaneously with 100–200 μg purified FabF in complete Freund's adjuvant. Booster immunizations were performed with incomplete Freund's adjuvant.

### 2.5. Immunoblotting

FabF was separated by SDS-PAGE, transferred to a 0.45 μm nitrocellulose membrane (Bio-Rad), and probed with Rabbit anti FabF serum. Antigen complexes were detected using peroxidase affinity pure goat anti-rabbit IgG (Jackson ImmunoResearch) and visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce). Bands were quantified using FluoChem™ 8000 system (Alpha Innotech Corp.), or by the TINA software (Raytest Isotopenmessgeräte, Straubenhardt, Germany). The linearity of the Western blot experiments was verified by using increasing volumes of D39 lysates and checking the intensity of the FabF band by densitometry.

### 2.6. Immunoprecipitation

FabF antibodies were incubated with protein A–agarose beads (Santa Cruz Biotechnology) for 1 h. Lysates were added and incubated overnight. Beads were boiled and sample resolved by SDS-PAGE. The Gel was stained with GelCode Blue Stain (Thermo Scientific).

### 2.7. Mass spectrometry analysis

Proteins in gel were reduced with 2.8 mM DTT (60 °C for 30 min), modified with 8.8 mM IAM in 100 mM ammonium bicarbonate (in the dark, room temperature for 30 min), and digested in 10% ACN and 10 mM ammonium bicarbonate with modified trypsin (Promega) overnight at 37 °C. The resulting tryptic peptides were resolved by reverse-phase chromatography on 0.075 × 200-mm fused silica capillaries (J&W) packed with Reprosil reversed phase material (Dr Maisch GmbH, Germany). Mass spectrometry was performed by an ion-trap mass spectrometer (Orbitrap, Thermo Scientific) in a positive mode using repetitively full MS scan followed by collision induced dissociation of the 7 most dominant ions selected from the first MS scan. The mass spectrometry data were clustered and analyzed using the Sequest software (J. Eng and J. Yates, University of Washington and Finnigan, San Jose) and Pep-Miner [11] searching against the *S. pneumoniae* database.

FabF antibodies yielded 2 bands at about 37 and 43 kDa in SDS-PAGE followed by Western blotting. By using immunoprecipitation and mass spectrometry we found that only the band at 43 kDa corresponds to the pneumococcal FabF (data not shown).

### 2.8. Determination of FabF cysteine-thiol redox state by double trapping with IAA and IAM

Alkylation was performed as previously described [12]. Briefly, reduced protein-thiols were blocked by resuspending PBS washed bacteria in urea buffer (0.1 M Tris pH 8.2, 1 mM EDTA, 8 M urea) containing 44 mM iodoacetic acid (IAA). Following sonication and 30 min incubation at 37 °C, excess IAA was removed with a micro bio-spin 6 chromatography column (Bio-Rad), and the oxidized thiols reduced with 5 mM dithiothreitol (DTT). Unblocked FabF thiols were then reacted with 10 mM iodoacetamide (IAM) in urea buffer for 30 min at 37 °C. Samples were resolved on a 17% polyacrylamide-urea gel and immunoblotted, using polyclonal antibodies that were raised against the cloned protein. Bands were quantified using the TINA software. Redox state standards were prepared from lysed bacteria that were incubated with 5 mM DTT for 30 min at 37 °C, to reduce all thiols. The standard was divided into 3 aliquots, which were incubated for 30 min at 37 °C with the following reagents: 1st aliquot: 44 mM IAA; 2nd aliquot: 11 mM IAM; the 3 rd aliquot was treated sequentially with IAA and IAM. To demonstrate the effect of cerulenin, bacteria were challenged with 67 μM cerulenin and then treated with IAA, DTT and IAM as described above.

### 2.9. Determination of FabF cysteine-thiol redox state by single trapping with AMS

Alkylation was performed as previously described [13]. Briefly, reduced protein-thiols were blocked by resuspending PBS washed bacteria in 100 μl ice cold AMS-buffer (0.1 M Tris pH 8, 1 mM EDTA, 1% SDS) supplemented with 20 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; Invitrogen), and sonicated for 10 s. Alkylation was performed at room temperature for 2 h and stopped by adding 1/4 volume of non reducing sample buffer (0.1 M Tris pH 6.8, 25 mM EDTA, 5% SDS, 20% glycerol). Alkylated samples were loaded on 18% polyacrylamide-SDS gels and immunoblotted using FabF polyclonal antibodies. Fully reduced standard was prepared by incubation of harvested bacteria with 10 mM DTT for 30 min, and then treatment as described above.

### 2.10. Effect of $H_2O_2$ on FabF redox state in vitro

D39-*spxB*-M cells were centrifuged and treated with various $H_2O_2$ concentrations, up to 1 mM, for the indicated times. $H_2O_2$ was removed from the culture by adding 2 unit/μmole/min of catalase. Cells were then sonicated and alkylated with AMS as described above.

### 2.11. RNA quantitation by real-time RT-PCR

RNA quantitation was done in bacteria grown at aerobic compared to anaerobic condition, and in the presence of 22.4 μM cerulenin. Bacterial RNA was prepared as previously described [4]. cDNA was synthesized with Reverse iT MAX 1st strand kit (ABgene, UK). PCR reactions contained Absolute™ blue QPCR SYBR mix ROX (ABgene, UK) and the following primers (designed with Primer Express® Software Version