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Effect of hydrogen peroxide production and the Fenton reaction on membrane composition of *Streptococcus pneumoniae*☆

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Abstract

As part of its aerobic metabolism, *Streptococcus pneumoniae* generates high levels of H_2O_2 by pyruvate oxidase (SpxB), which can be further reduced to yield the damaging hydroxyl radicals via the Fenton reaction. A universal conserved adaptation response observed among bacteria is the adjustment of the membrane fatty acids to various growth conditions. The aim of the present study was to reveal the effect of endogenous reactive oxygen species (ROS) formation on membrane composition of *S. pneumoniae*. Blocking carbon aerobic metabolism, by growing the bacteria at anaerobic conditions or by the truncation of the *spxB* gene, resulted in a significant enhancement in fatty acid unsaturation, mainly *cis*-vaccenic acid. Moreover, reducing the level of $OH^{\cdot}$ by growing the bacteria at acidic pH, or in the presence of an $OH^{\cdot}$ scavenger (salicylate), resulted in increased fatty acid unsaturation, similar to that obtained under anaerobic conditions. RT-PCR results demonstrated that this change does not originate from a change in mRNA expression level of the fatty acid synthase II genes. We suggest that endogenous ROS play an important regulatory role in membrane adaptation, allowing the survival of this anaerobic organism at aerobic environments of the host.

Keywords: *Streptococcus pneumoniae*; Membrane lipid; Hydrogen peroxide; Fenton reaction; Fatty acid

1. Introduction

Streptococcus pneumoniae is a facultative anaerobic organism. It lacks the cytochromes and heme containing proteins involved in aerobic respiration, as well as many other proteins that have been shown to protect against oxidative burst (e.g. catalase). Without such mechanisms for handling oxidative stress, it seems that aerobic atmosphere should severely restrict pneumococcal growth, yet *S. pneumoniae* is aerotolerant and is able to grow under atmospheric oxygen pressure [1]. This suggests the existence of other bacterial factors that are important for aerobic pneumococcal growth.

The presence of oxygen has a distinct effect on carbon metabolism due to oxidase activities. Under aerobic conditions, pyruvate metabolism is shifted from homolactic towards mixed acid fermentation, thereby increasing ATP production via acetate generation [2]. The first step in the aerobic pathway i.e., conversion of pyruvate to acetyl phosphate is catalyzed by pyruvate oxidase (SpxB) (2). SpxB catalyzes a two-electron reduction of O_2, thereby forming the potentially damaging compound H_2O_2, which can be further reduced to the highly reactive hydroxyl radicals via the Fenton reaction, as follows:

$$H_2O_2 + Fe^{+2} \rightarrow Fe^{+3} + OH^{\bullet} + OH^{-}.$$

Production of H_2O_2 by pneumococci, previously shown to have cytotoxic effects on human epithelial cells, is an effective mechanism for limiting or eliminating competitive flora, including common pathogens such as *H. influenzae* and *N. meningitidis*, which share the same microenvironment [3]. In

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addition, *SpxB* expression is required for resistance to killing by its own toxic by-product H_2O_2, presumably by retaining a metabolism that maximizes ATP production under aerobic conditions, thereby increasing its viability during oxidative stress [4]. Mutations in SpxB resulted in reduced experimental virulence and bacterial persistence in tissues of infected mice [2]. Oxidative metabolism is thus a major determinant in the physiological specialization of pneumococci at various biological niches. Since *S. pneumoniae* exists primarily in aerobic environments, one would expect it to cope with the toxic effects of its own oxidative metabolism, mainly H_2O_2 and $OH^{\bullet}$.

A universal conserved adaptation response observed among bacteria is the adjustment of the membrane to various growth conditions [5,6]. The aim of the present study was to reveal the effect of endogenous reactive oxygen species (ROS) formation, due to oxidative metabolism, on membrane composition of *S. pneumoniae*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Three clinical isolates of *S. pneumoniae* carrying serotypes 2 (D39), 6A and 18C and one mutant of D39 containing an in-frame fusion of Tn*phoA* to the pyruvate oxidase gene (*spxB*::Tn*phoA*) [2] were used in this study. The strains were kindly provided by JN Weiser (University of Philadelphia). The capsular type was confirmed by the quellung reaction [7] using antisera provided by the Statens Serum Institute of Copenhagen (Denmark). The strains were cultured in brain heart infusion (BHI) broth (Difco Laboratories, Becton, Dickinson and Company, MD) at 37 °C, pH=7.4±0.2, unless otherwise indicated. Bacteria were grown to OD_{620}=0.2 with aeration, for aerobic conditions, or without shaking in closed bottles filled with BHI for anaerobic conditions. To verify aerobic/anaerobic conditions, oxygen levels in the medium were measured by a Dissolved Oxygen Meter (YSI, Cole-Parmer International, IL). Sodium salicylate (MP Biomedicals, CA) was prepared as previously described [8]. Bovine liver catalase was purchased from Worthington Biochemicals, NJ.

2.2. Lipid separation and analysis

Bacteria were grown to OD_{620}=0.2, centrifuged and kept in liquid nitrogen. Lipids were extracted from lyophilized biomass with chloroform and methanol according to the procedure of Bligh and Dyer [9]. Total lipid extract was separated into individual lipids by two dimensional thin layer chromatography (TLC) (Silica Gel 60, 10×10 cm plates, 0.25 mm thickness, MERCK, Darmstadt, Germany) using a solvent system of chloroform:methanol:water (65:25:4, v/v/v) for the first direction, and chloroform:methanol:acetic acid:water (80:10:14: 3, v/v/v/v) for the second direction [10]. Neutral lipids were resolved using a solvent system of petroleum ether:diethyl ether:acetic acid (70:30:1, v/v/v). Lipids were visualized by brief exposure to iodine vapors. Lipids were identified by spraying the plates with specific reagents [11], and by comparison of Rf values to commercial lipid standards (Sigma, St. Louis, MO). For quantitation and FA analysis of individual lipids, lipid spots were scraped out from TLC plates, transmethylated and analyzed by gas chromatography as described below.

2.3. FA extraction and analysis

Lipids were transmethylated with 2% H_2SO_4 in methanol at 80 °C for 1 h [12]. 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 [12]. A known amount of an internal standard (C17:0) was added to each sample to allow for lipid quantitation. FA methyl esters were identified by co-chromatography with authentic standards (Sigma Co. St. Louis, MO) and by comparison of their equivalent chain length [13].

2.4. Hydrogen peroxide production assay

Endogenous peroxide production was analyzed by a spectrophotometric assay, adapted from Pericon et al. [3] with minor modifications. Briefly, bacteria were grown in BHI medium at 37 °C under aerobic and anaerobic conditions until they reached OD_{620}=0.2, centrifuged at 4 °C for 20 min at 4000×*g*, washed twice in ice cold phosphate buffered saline (PBS), pH=7.4, and resuspended in PBS containing 0.5 mM glucose to twice the original culture volume. Hydrogen peroxide production was measured in PBS to minimize the Fenton reaction. After 1 h of incubation at 37 °C under aerobic or anaerobic conditions, the cultures were harvested by centrifugation for 10 min at 10,000×*g*, and filtered through a 0.2 μm (pore size) membrane. Immediately prior to the assay, phenol red and horseradish peroxidase were added to peroxide assay buffer (PBS containing 0.5 mM glucose; pH=7.4) at final concentrations of 0.46 mM and 0.046 U/mL, respectively. Aliquots of filtered supernatant were added to the assay mixture at a ratio of 1:4 and incubated for 30 min at 37 °C. The reaction was stopped by the addition of NaOH (final concentration 0.004 N), and the absorbance recorded at 610 nm. Concentrations were calculated in comparison to a standard curve with known amounts of H_2O_2.

2.5. RNA quantitation

FA synthase II (FASII) expression was assessed by reverse transcribed polymerase chain reaction (RT-PCR). Bacterial RNA was prepared by Master Pure™ RNA Purification kit (EPICENTRE, Madison, WIS) according to the manufacturer's instructions, and quantified by absorbance measurements at 260 and 280 nm; cDNA was prepared by the Reverse Transcription System kit (Promega, Madison, WIS). Transcripts were used for PCR using specific primers designed by the "Primer 3" program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). 16S rRNA was selected as an internal control because rRNAs are known to be maintained at constant level [14]. The PCR reaction was carried out by the Master mix PCR kit (Promega, Madison, WIS). The PCR primers used are: *fabM*; 5′-GCC TTT GTT GGT GTT GGT TT-3′ and 5′-ACC AAC TTC TTA ATG GCA GCA-3′, *fabT*; 5′-GTC ATC GGT AAG GCT CCA GA-3′ and 5′-TTG TGG AAG CGT TTA TGC AG-3′, *fabH*; 5′-TGC TCA TTA TGT GCC AGA GC-3′ and 5′-GGC ATC ATC GAA TCT GGA GT-3′, *fabK*; 5′-GCC CAT CCA AAC TAC AAG GA-3′ and 5′-TAA CAG AGC CAC CAT CCA CA-3′, *fabF*; 5′-GGA ATG TAG CCA TGC GTT TT-3′ and 5′-ATC AAA TGG GAT CGA AGC AC-3′, 16S rRNA; 5′-CCA TGT GTA GCG GTG AAA TG-3′ and 5′-TAA GGT TCT TCG CGT TGC TT-3′. Separate PCR reactions were set up on ice from a master mix differing only in the constituent primers. Denaturation at 94 °C for 5 min was carried out prior to the PCR amplification: 20 PCR cycles were performed comprising denaturation at 94 °C for 30 s, primer annealing at 54.0 °C for *fabF*, 55 °C for *fabH*, 58 °C for *fabM* and *fabK*, and 60 °C for *fabT* for 45 s, and extension at 72 °C for 30 s. For 16S rRNA, 12 PCR cycles were performed comprising denaturation at 94 °C for 30 s, primer annealing at 57 °C for 45 s, and extension at 72 °C for 30 s. The reactions were terminated by extension at 72 °C for 5 min. The reaction products were separated by electrophoresis on 1.7% agarose gel, and quantified by FluoChem™ 8000 (Alpha Innotech Corp. CA).

2.6. Statistical analysis

The significance of differences was determined by the unpaired *t*-test. $p<0.05$ was considered significant.

3. Results

3.1. Hydrogen peroxide production under aerobic versus anaerobic growth conditions

Hydrogen peroxide production by *S. pneumoniae* requires aerobic carbohydrate metabolism, and can be diminished by the use of a mutant lacking pyruvate oxidase activity [3]. H_2O_2 concentrations were measured in the supernatants of cultures

grown aerobically and anaerobically for 60 min. As expected, H_2O_2 production decreased by 2–3 folds when bacteria were grown under anaerobic condition: from 575±27 μM to 227±31 for serotype 18C ($p<0.05$), from 428±25 to 176±1 for serotype 6A ($p<0.05$), and from 750±54 to 205±21 for serotype 2 (D39) ($p<0.05$). The *spxB*-mutant showed even lower levels of H_2O_2 under both aerobic and anaerobic conditions: 127±6 μM and 55±5, respectively ($p<0.05$), indicating that most of the H_2O_2 produced by the pneumococcus originates from pyruvate oxidation by *SpxB*.

3.2. FA composition under aerobic versus anaerobic growth conditions

Gas chromatographic analysis of the FA methyl esters was performed on three wild type strains belonging to serotypes 18C, 6A and 2 (D39), and on the *spxB*-mutant of D39 under aerobic and anaerobic conditions, at various culture densities. For each data point we have calculated the unsaturation index (weighted average of the number of double bonds per FA), and the C18:C16 ratio.

As shown in Fig. 1 the unsaturation index increased gradually with the increase in culture density under both aerobic and anaerobic conditions (Fig. 1). To ensure the reproducibility of results and their proper interpretation, experiments were performed under steady state exponential growth conditions ($OD_{620}=0.2$) [15], where the variability in fatty acid composition is minor (Fig. 1).

The transition from aerobic to anaerobic conditions was accompanied by a remarkable change in the proportion of the fatty acyl residues, mainly the level of *cis*-vaccenic acid (C18:1ω7): from 8.7±0.53% (of total FA) at aerobic conditions to 22.1± 0.23% at anaerobic conditions for serotype 18C, from 12.4±3.5% to 20.2±2.8% for serotype 6A, and from 11.8±1.4% to 18.6±0.4% for serotype 2 (D39). Concomitantly, a decrease in the proportions of C12:0, C14:0 and C16:0 was observed. As a result, membrane composition was changed from a short-chained saturated FA (SFA) profile at aerobic conditions to a long-chained, monounsaturated profile at anaerobic conditions. Indeed, the unsaturation index and the length of the fatty acyl chains, represented by the ratio of C18 to C16, increased under anaerobic compared to aerobic growth conditions (Table 1).

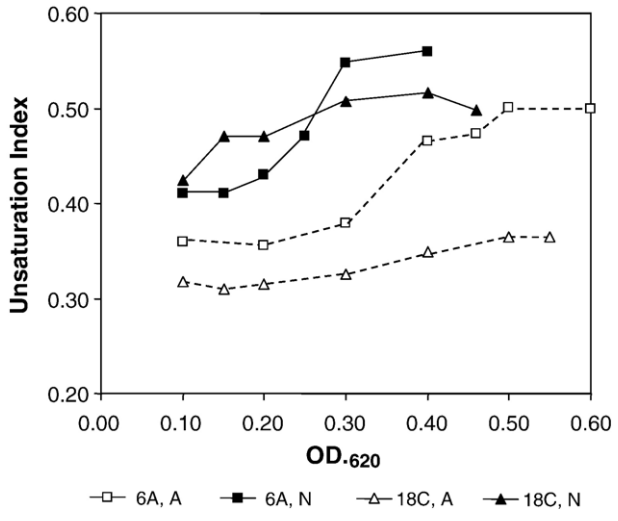

Fig. 1. Effect of culture density on fatty acid unsaturation index. Cultures of *S. pneumoniae* were grown under aerobic and anaerobic conditions up to their stationary phase. The extracted lipids were transmethylated and fatty acids analyzed by gas chromatography. Unsaturation index is defined as the weighted average of the number of double bonds per FA. Values are the means of triplicate determinations of 2 independent experiments.

Table 1
Unsaturation index and C18:C16 ratio of the major fatty acyl chains under aerobic versus anaerobic conditions

Strain	Unsaturation index [a]		C18:C16 ratio [b]	
	Aerobic	Anaerobic	Aerobic	Anaerobic
18C	0.31±0.01	0.47±0.01	0.25±0.04	0.52±0.01
6A	0.35±0.06	0.43±0.03	0.41±0.06	0.58±0.08
D39	0.37±0.02	0.48±0.01	0.38±0.08	0.43±0.00
D39-*spxB*-mutant	0.72±0.00	0.79±0.01	0.83±0.02	1.07±0.04

[a] Unsaturation index is defined as the weighted average of the number of double bonds per FA.
[b] Ratio between the proportions of FA with 18 carbons and FA with 16 carbons.

Loss of pyruvate oxidase activity in the *spxB*-mutant resulted in exceptionally elevated degree of FA unsaturation under both aerobic and anaerobic growth conditions, with unsaturation index of 0.72±0.01 under aerobic conditions, and with almost no change, 0.79±0.02 under anaerobic conditions. The isogenic wild type strain, on the other hand, showed significantly reduced levels under both aerobic and anaerobic conditions ($p<0.005$): 0.37±0.02 and 0.48±0.01, respectively.

3.3. Lipid composition under aerobic versus anaerobic growth conditions

As previously shown by Trombe et al. [16] the membrane of *S. pneumoniae* contains mainly two glycolipids: monoglucosyldiacylglycerol (MGDG) and galactosylglucosyldiacylglycerol (DGDG), and two acidic phospholipids: phosphatidylglycerol (PG) and cardiolipin (CL). The cell contains also one neutral lipid, diacylglycerol (DAG) (Fig. 2). For each of the wild type strains, the proportions of the various lipids were found to be significantly different under aerobic versus anaerobic conditions: generally, the levels of the two glycolipids and, to a lesser extent, PG, increased under anaerobic conditions while the levels of CL and DAG decreased. On average (for 18C, 6A and D39), the level of MGDG increased from 12.9±2.0% at aerobic conditions to 19.6±2.9% at anaerobic conditions ($p<0.05$), the level of DGDG increased from 21.2±2.0 to 27.8±1.8 ($p<0.05$), and the level of PG increased from 12.7±1.8 to 16.3±1.2 ($p<0.05$). On the other hand, the level of DAG decreased from 37.8±0.7 to 28.0±1.8 ($p<0.005$) and the level of CL decreased from 15.3±0.9 to 8.2±1.6 ($p<0.005$). The proportions of the various FA of each of the lipids were also examined. We found that the change in the fatty acyl chains was very similar for all the lipid components when shifting from aerobic to anaerobic conditions, indicating an across the board increase in the level of UFA regardless of their head group (data not shown).

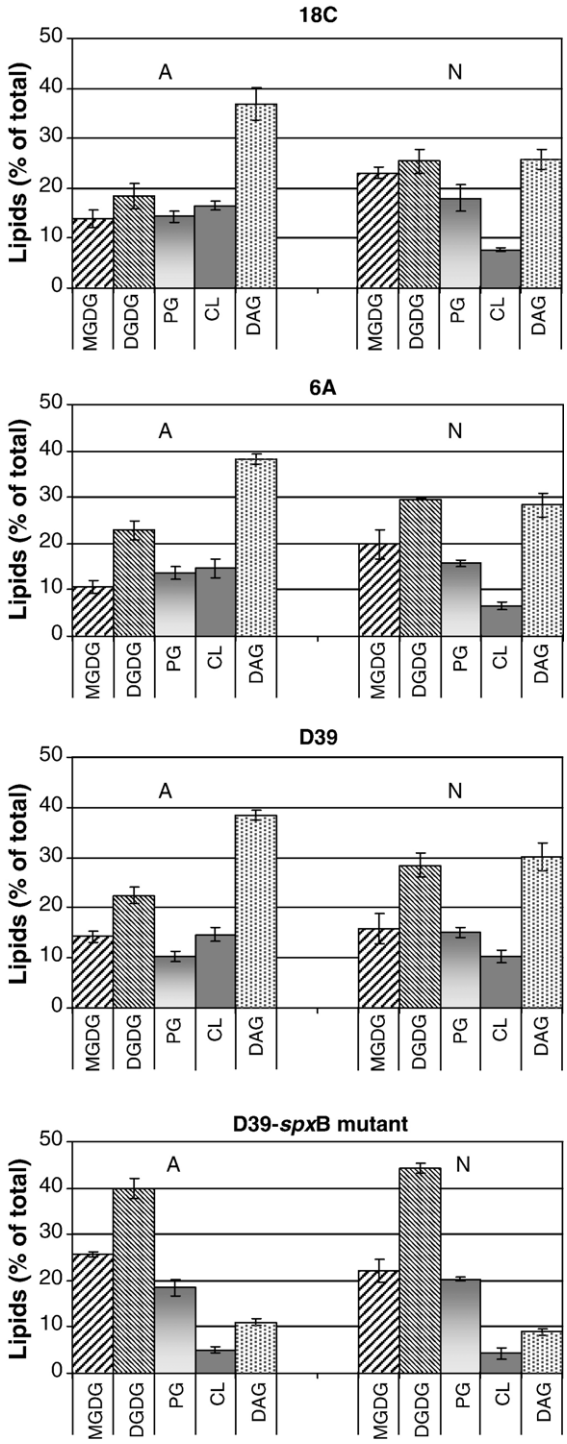

Fig. 2. Effect of oxygen availability on lipid distribution. Cultures of *S. pneumoniae* carrying serotype 2 (D39), 6A and 18C and an *spxB* mutant of D39, were grown under aerobic and anaerobic conditions to an OD_{620} of 0.2. Total lipid extract of each culture was separated into individual lipids by two dimensional-TLC. For quantitation, lipid spots were scraped out from the TLC plates, transmethylated and analyzed by gas chromatography. Values are the means of at least three independent experiments. Calculated standard deviation values for each lipid are specified as bars. MGDG, monoglucosyldiacylglycerol; DGDG, galactosylglucosyldiacylglycerol; PG, phosphatidylglycerol; CL, cardiolipin; DAG, diacylglycerol.

The *spxB*-mutant demonstrated a different lipid profile from that of the wild type strains (Fig. 2). Specifically, the proportion of DGDG in the mutant strain increased by almost two fold compared to its isogenic wild type (D39) strain under both anaerobic and aerobic conditions, while the proportions of CL and DAG decreased. Generally, the lipid profile of the *spxB*-mutant resembled that of the wild type strain under anaerobic conditions a lot more than its profile under aerobic conditions, namely: increased proportion of DGDG at the expense of CL and DAG. Here again, the proportions of the various FA in each of the lipid groups were very similar (data not shown).

3.4. FA composition under physiological versus acidic growth conditions

The rate constant of the Fenton reaction, and therefore $OH^{\cdot}$ formation, is much lower at acidic pH than under physiological pH [17]. Growth of 18C, 6A and D39 at acidic pH (6.5 and 6.0) was accompanied by a remarkable change in the proportion of the fatty acyl residues, mainly increased proportion of *cis*-vaccenic acid (C18:1ω7), similar to recent findings in other aciduric oral bacteria (18). Accordingly, an increase in the unsaturation index of the fatty acyl chains was noticed: from 0.36 ± 0.01 at pH=7.0 to 0.56 ± 0.01 at pH=6.0 ($p<0.005$) for 18C, from 0.34 ± 0.01 to 0.48 ± 0.04 ($p<0.05$) for 6A and from 0.42 ± 0.01 to 0.54 ± 0.02 ($p<0.05$) for D39 (Table 2). On the other hand, in the *spxB*-mutant, where the rate of the Fenton reaction is exceedingly low (independent of the pH conditions), due to the lack of peroxide production, the unsaturation index was not affected by the acidic conditions: 0.81 ± 0.01 at pH=7.0 and 0.79 ± 0.01 at pH=6.0 ($p>0.1$) (Table 2).

3.5. Effect of ROS scavenger

Salicylate is known for its ability to impair the hydroxyl radicals [19,20] formed by the Fenton reaction. Addition of sodium salicylate (5 mM) to the culture medium of D39 under aerobic conditions resulted in a significant increase in the unsaturation index of the fatty acyl chains, from 0.37 ± 0.02 to 0.50 ± 0.01 ($p<0.001$), (Fig. 3 and Table 3). On the other hand, under anaerobic conditions, where the levels of H_2O_2 and $OH^{\cdot}$ produced by the organism are expected to be much lower because of oxygen deficiency, salicylate had negligible effect on the unsaturation index: 0.49 ± 0.01 and 0.48 ± 0.01 with and without salicylate ($p>0.1$), respectively.

We have also looked at the effect of salicylate on FA composition in the *spxB*-mutant, and found almost no change under both aerobic and anaerobic conditions, which is due, most probably, to the low H_2O_2 production by the mutant strain (Table 3). Replacing salicylate with catalase (500 U/ml) in the growth medium at aerobic conditions had no effect on membrane FA content in both D39 and its *spxB* mutant.

3.6. FASII expression

The increased unsaturation level under anaerobic conditions may originate from a change in the regulation of enzymes in the

Table 2
Unsaturation index and C18:C16 ratio of the major fatty acyl chains at various pH levels

Strain	pH	Unsaturation index [a]	C18:C16 ratio [b]
Serotype 18C	7.0	0.36±0.01	0.38±0.02
	6.5	0.49±0.01	0.85±0.02
	6.0	0.56±0.01	1.42±0.12
Serotype 6A	7.0	0.34±0.01	0.41±0.01
	6.5	0.43±0.01	0.61±0.01
	6.0	0.48±0.04	0.88±0.14
D39 (serotype 2)	7.0	0.42±0.01	0.44±0.03
	6.5	0.44±0.02	0.53±0.09
	6.0	0.54±0.02	1.08±0.02
D39-*spxB*-mutant	7.0	0.81±0.01	1.21±0.03
	6.5	0.80±0.01	1.10±0.07
	6.0	0.79±0.01	1.47±0.01

[a] Unsaturation index is defined as the weighted average of the number of double bonds per FA.
[b] Ratio between FA with 18 carbons to FA with 16 carbons.

FASII system. To test this assumption, mRNA expression levels of *fabM, fabT, fabH, fabK* and *fabF* were examined in D39 under aerobic and anaerobic condition. The semi-quantitative RT-PCR data showed no significant change in mRNA expression levels of *fabT, fabH* and *fabK* ($p>0.1$), while *fabM* and *fabF* levels decreased under anaerobic conditions by 34±3 and 24±12%, respectively. Three enzymes act in the branch point between SFA:UFA biosynthesis [21,22]: FabK, which directs to the production of SFA, and FabM and FabF that are required for the synthesis of UFA. The reduction in *fabM* and *fabF* mRNA expression suggests that the increased unsaturation level under anaerobic conditions apparently does not originate from increased transcription of the FASII cluster.

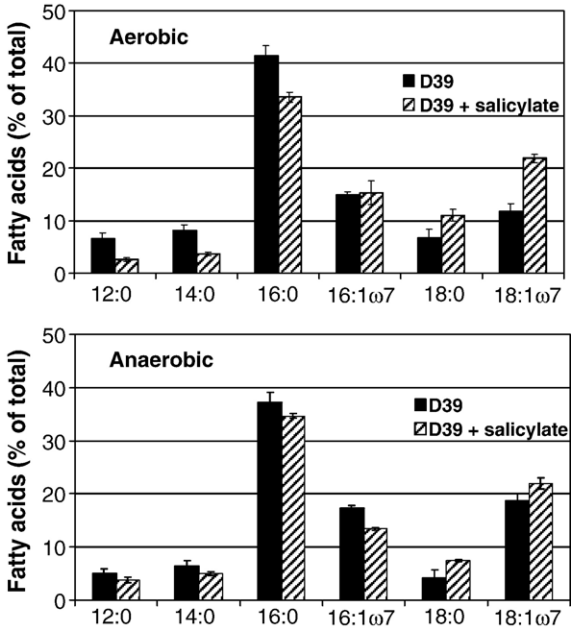

Fig. 3. FA distribution in D39 under aerobic and anaerobic conditions: effect of salicylate. Cultures were grown in the presence of salicylate (5 mM) to an OD_{620} of 0.2. FA-methyl ester derivatives of the bacterial isolates were analyzed by gas chromatography. The figure shows the 6 major fatty acyl residues. Values are the means of triplicate determinations of at least 2 independent experiments.

Table 3
Unsaturation index and C18:C16 ratio of the major fatty acyl chains in D39 and D39-*spxB*-mutant under aerobic versus anaerobic conditions: effect of salicylate

Strain		Unsaturation index [a]		C18:C16 ratio [b]	
		(−) sal [c]	(+) sal	(−) sal	(+) sal
D39	Aerobic	0.37±0.02	0.50±0.01	0.38±0.08	0.73±0.08
	Anaerobic	0.48±0.01	0.49±0.01	0.43±0.00	0.68±0.03
D39-*spxB*-mutant	Aerobic	0.72±0.01	0.72±0.01	0.83±0.02	1.07±0.04
	Anaerobic	0.79±0.01	0.76±0.02	1.07±0.04	1.16±0.10

[a] Unsaturation index is defined as the weighted average of the number of double bonds per FA.
[b] Ratio between FA with 18 carbons to FA with 16 carbons.
[c] sal; salicylate.

The expression levels of the above FASII genes were also examined in D39 grown at physiological (pH=7.0) compared to acidic conditions (pH=6.0). Here again mRNA expression levels of *fabM* and *fabF* were significantly reduced at pH=6.0: 23±1 and 28±3%, respectively, while *fabH* and *fabK* were unchanged ($p>0.1$), suggesting that the increased unsaturation index at acidic pH is attributed, most probably, to a lower rate of hydroxyl radicals formation at acidic conditions, and not to a change in the mRNA expression levels.

The second gene in the FASII cluster, *fabT*, was predicted to encode a DNA binding protein of the MarR superfamily [22]. Salicylate is known for its activity as a MarR repressor [23]. Therefore we looked at the effect of salicylate on mRNA levels of *fabM, fabT, fabH, fabK* and *fabF* genes. No change in mRNA levels was noticed under both aerobic and anaerobic conditions (data not shown), suggesting that the effect of salicylate is apparently not due to its involvement in *fabT* regulation.

4. Discussion

Oxygen is a variable feature in the environment of *S. pneumoniae*, and the survival of the organism in different host environments is likely to require adaptive responses [24]. Differences in ambient oxygen concentration affect diverse factors like the regulation of capsular polysaccharide synthesis [25] and early competence genes (24). As part of its aerobic metabolism, the pneumococcus generates high levels of H_2O_2 (about 0.5–2 mM), similar to those produced by activated neutrophils [3]. One of the main defenses against environmental challenges is the bacterial membrane [26]. However, the casual relationship between membrane lipid composition and oxygen availability has not yet been established in *S. pneumoniae*. Findings in the current study show that membrane composition is altered in response to hazardous compounds produced by the pneumococcus, i.e., H_2O_2 and $OH^{\bullet}$, at aerobic atmosphere.

We found that anaerobiosis, which almost eliminates H_2O_2 production and therefore $OH^{\bullet}$ formation, is accompanied by a remarkable change in FA composition, mainly increased level of *cis*-vaccenic acid (C18:1ω7) at the expense of palmitic acid (C16:0). These compensatory shifts are geared towards maintaining homeostasis, so that membrane physical state is kept within limits consistent with normal (optimal) metabolic

function. Similar modifications in membrane composition were demonstrated in *E. coli* [27], where changes in the proportions of C18:1ω7 were coupled with C16:0. The link between H_2O_2 production and membrane alterations was further established by looking at membrane composition in an *spxB*-mutant. Our data demonstrate that blocking the pyruvate aerobic metabolism, by the truncation of the *spxB* gene, resulted in a dramatic change in membrane FA profile: a significant enhancement in FA unsaturation together with an increase in C18:C16 ratio compared to the wild type strain. In addition, FA composition of the *spxB*-mutant was very similar under aerobic and anaerobic conditions, suggesting that the trigger for membrane FA alterations could result from the formation of H_2O_2 and/or $OH^{\bullet}$.

To further establish this hypothesis, strains were grown aerobically at acidic pH, where the oxidation kinetics of iron through the Fenton reaction is much lower compared to physiological pH (17). FA composition at pH=6.0 was characterized by a tremendous increase in the unsaturation index compared to physiological pH, similar to data published by others on aciduric bacteria [18]. Likewise, addition of salicylate, a known $OH^{\bullet}$ scavenger [19,20], to the growth medium resulted in increased unsaturation index, similar to that observed under anaerobic conditions. On the other hand, salicylate and acidic pH had almost no impact on the unsaturation index of the *spxB*-mutant, due to the low levels of $OH^{\bullet}$ formation. These data suggest that $H_2O_2/OH^{\bullet}$ production is associated with lower level of double-bond formation in the membrane. It is tempting to speculate that UFA are less stable in oxidizing environments. However, it is not clear that lipid peroxidation can occur in bacteria lacking polyunsaturated fatty acyl chains [28,29].

Growth of the *spxB*-mutant in the presence of exogenous H_2O_2 (0.05–1.0 mM) had a minor effect on FA composition: a 5% decrease in the unsaturation index at 1 mM (data not shown). However, higher concentrations were lethal. H_2O_2 is a highly reactive compound with a short half life in the medium [30]; its decomposition results in the formation of $OH^{\bullet}$, which tends to react with any molecule in its vicinity. Hence, bacteria grown in the presence of 1 mM H_2O_2 are expected to experience much lower concentrations inside the cell, resulting in a minimal effect on membrane FA content. An attempt to eliminate H_2O_2 in the growth medium by catalase had no effect. All together these data indicate that the change in FA composition originates from endogenous rather than exogenous H_2O_2. In the case of *S. pneumoniae*, which lacks FA desaturases and the β-oxidation enzyme set [31], alterations in FA unsaturation level and chain length could result from *de novo* synthesis through the anaerobic FASII pathway. It was shown [32] that the regulation of UFA:SFA ratio in *S. pneumoniae* is determined by the competition between FabF and FabK on the substrates of the FabM reaction: FabK (enoyl-ACP reductase) pulls the pathway towards SFA synthesis by utilizing the *trans*-2-C10:1 intermediate, whereas FabF pulls the pathway towards UFA synthesis by utilizing the *cis*-2-C10:1 intermediate. Our semi-quantitative RT-PCR data showed that, despite the increased unsaturation index when bacteria were grown under anaerobic compared to aerobic conditions or challenged with acidic pH, mRNA expression levels of *fabM* and *fabF* were significantly reduced, indicating that this change was not due to increased mRNA expression.

FabT was predicted to encode a DNA binding protein of the MarR family [22]. Since salicylate is known for its activity as a MarR repressor [23], changes observed in FA composition in the presence of salicylate could be due to FabT inactivation, thereby abolishing its function as a transcriptional regulator of the FASII genes [32]. Semi-quantitative RT-PCR data showed no change in the mRNA expression level of *fabT* in the presence of salicylate. Likewise, no change in *fabT* mRNA level was observed under anaerobic compared with aerobic atmospheres. Hence, these data suggest that the effect of salicylate on FA composition is due to its scavenging activity, rather than its possible regulation on *fabT* expression.

A unique property of biological membranes is that they are a complex mixture of several lipids with respect to head groups and FA composition [33]. The head groups determine many of the structural properties of the membrane surface, while the combination of head groups with different FA determines the collective physical properties and biological functionality of the membrane [34]. In order to adapt to conditions of high endogenous ROS production, the organism must be able to rapidly replace damaged acyl groups. However, *de novo* synthesis of these acyl groups is significantly slower than their oxidation by ROS. In *S. pneumoniae* the FA composition is rather regardless of the lipid head groups. Under equilibrium conditions cell membranes contain no significant amounts of diacylglycerols (DAG) [35]. However, they are important intermediates in the biosynthesis and degradation of glycerophospholipids and glyceroglycolipids. Therefore we hypothesize that under aerobic conditions, the organism would store enhanced amounts of DAG, the common building block of all polar lipids [36], which can be rapidly deployed to modify membrane composition. Indeed, the level of DAG increased dramatically when bacteria were grown at atmospheric compared to anaerobic conditions, at the expense of the share of the two major glycolipids, DGDG and MGDG (Fig. 2). However, the ratio between membrane polar lipids was not significantly altered. Indeed, pyrene lateral diffusion was similar in both aerobic and anaerobic conditions (data not shown), indicating there was no significant change in the physical properties of the membrane. In the *spxB*-mutant, an even more remarkable decrease in the proportion of DAG was observed under both aerobic and anaerobic conditions, in line with its inability to synthesize H_2O_2. Similarly, Khozin-Goldberg et al. [37] have shown that the alga *Parietochloris incisa* can accumulate arachidonyl-rich triacylglycerols that can be mobilized for the construction of membrane polar lipids, when a rapid decrease in ambient temperature takes place.

Oxygen-rich environments present a challenge to the survival of *S. pneumoniae*. This anaerobic organism is particularly susceptible to endogenous oxidants, since it lacks the known antioxidant defense mechanisms observed in aerobic organisms. The data presented in this study suggest that endogenous ROS play an important regulatory role in membrane adaptation to various biological niches. Changes in fatty acid composition can

only be seen as a long term adaptive response but not as a direct protective mechanism against ROS. Other mechanisms such as the activity and overexpression of repair enzymes, e.g. methionine sulfoxide reductase [38,39], may function as the urgent protection against the toxicity of oxygen radicals.

It was recently discovered that H_2O_2 is not only a deleterious oxidant for cells but also plays an important role as a beneficial signaling molecule, similar to other ubiquitous molecules such as nitric oxide and calcium [40]. There has long been skepticism about the signaling role of ROS, partly because it seemed illogical for nature to use a dangerous molecule such as H_2O_2 for such a crucial function [41]. However, if one considers that nitric oxide (NO), itself a reactive radical, is easily converted to the more reactive $OONO^-$ while also functioning as an established intracellular messenger, then the proposed H_2O_2/$OH^\bullet$ function is not implausible. 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 [42]. The FASII pathway involves the covalent attachment of fatty acyl intermediates to the SH groups of FabF and ACP (acyl carrier protein) [21]. Thus, oxidation by ROS may function as a specific modification used for signaling rather than being simply destructive. However, the identity of the pathway that controls this change is still under investigation.

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