Pyruvate Formate Lyase Is Required for Pneumococcal Fermentative Metabolism and Virulence

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Knowledge of the in vivo physiology and metabolism of Streptococcus pneumoniae is limited, even though pneumococci rely on efficient acquisition and metabolism of the host nutrients for growth and survival. Because the nutrient-limited, hypoxic host tissues favor mixed-acid fermentation, we studied the role of the pneumococcal pyruvate formate lyase (PFL), a key enzyme in mixed-acid fermentation, which is activated posttranslationally by PFL-activating enzyme (PFL-AE). Mutations were introduced to two putative pfl genes, SPD0235 and SPD0420, and two putative pflA genes, SPD0229 and SPD1774. End-product analysis showed that there was no formate, the main end product of the reaction catalyzed by PFL, produced by mutants defective in SPD0420 and SPD1774, indicating that SPD0420 codes for PFL and SPD1774 for putative PFL-AE. Expression of SPD0420 was elevated in galactose-containing medium in anaerobiosis compared to growth in glucose, and the mutation of SPD0420 resulted in the upregulation of pfa and pyk, encoding, respectively, fructose 1,6-bisphosphate aldolase and pyruvate kinase, under the same conditions. In addition, an altered fatty acid composition was detected in SPD0420 and SPD1774 mutants. Mice infected intranasally with the SPD0420 and SPD1774 mutants survived significantly longer than the wild type-infected cohort, and bacteremia developed later in the mutant cohort than in the wild type-infected group. Furthermore, the numbers of CFU of the SPD0420 mutant were lower in the nasopharynx and the lungs after intranasal infection, and fewer numbers of mutant CFU than of wild-type CFU were recovered from blood specimens after intravenous infection. The results demonstrate that there is a direct link between pneumococcal fermentative metabolism and virulence.

Streptococcus pneumoniae is the leading cause of pneumonia in children and adults, and it is a major cause of otitis media, meningitis, and septicemia (22). Despite considerable progress in pneumococcal vaccine development, the serotype specificity and the genomic plasticity of the pneumococcus will hamper its success (36). In addition, the increasing frequency of antibiotic resistance (14) makes it important to understand the mechanisms of pathogenesis of pneumococcal disease.

Probably one of the most understudied fields of pneumococcal biology is that of how the pathogen generates its metabolic energy. The pneumococcus is strictly fermentative, and sugars are the major sources of energy for biosynthesis and growth (19, 52). Therefore, in vivo fitness is determined to a large extent by the processes governed by sugar metabolism. Accumulating evidence suggests that there is a direct link between pneumococcal virulence and sugar metabolism (6, 16, 20, 21, 50). This is not limited to proteins involved in polysaccharide degradation, transport, and regulation (16, 31) but also includes those involved in redox metabolism, such as NADH oxidase (Nox) (6), and in pyruvate metabolism (42, 50). For example, mutation of nox caused diminished virulence in a systemic infection model, and this was attributed to a probable change in the NADH/NAD+ ratio or the increased sensitivity of S. pneumoniae to oxidative stress (6). Mutation of spxB (pyruvate oxidase) led to a reduction in virulence in both pneumonia and sepsis models with mice, which was linked to a decrease in acetyl phosphate levels and downregulation of adhesive proteins (45, 50).

Pneumococcus is known to maintain a fermentative metabolism regardless of oxygen, since it lacks a complete set of genes required for respiration (19, 52). Breakdown of carbohydrates by the classical Embden-Meyerhof pathway results in generation of pyruvate, NADH, and a net gain of two ATP per mole of substrate (40). In homolactic bacteria, NAD+ regeneration is accomplished mainly via the lactate dehydrogenase-catalyzed conversion of pyruvate to lactate (37) (Fig. 1). However, under certain conditions, such as aerobiciosis, sugar limitation, or the presence of sugars less preferred than glucose, such as galactose, there is a metabolic shift from homolactic (lactate production) to mixed-acid fermentation, with the formation of products other than lactate (e.g., ethanol, acetate, and formate) (34, 37, 40) (Fig. 1). Mixed-acid fermentation is mediated in part by the activities of the pyruvate dehydrogenase complex (PDHC) or pyruvate formate lyase (PFL) (34, 37, 40). While PDHC catalyzes the oxidative decarboxylation of pyruvate to form acetyl-coenzyme A (CoA) and CO2 in aerobiciosis, under microaerobic and anaerobic conditions, most of the pyruvate is converted to acetyl-CoA and formate by the...
The sequenced genome of the S. pneumoniae D39 strain has two copies of both pfl genes, annotated as SPD0235 and SPD0420, and pflA homologs, annotated as SPD0229 and SPD1774 (19, 28, 52). However, the role of the PFL/PFL-AE system in pneumococci is not known, despite the fact that SPD0420 and SPD1774 were predicted to be among the high-expressed genes, based on codon usage (24). In addition, it is not clear why the pneumococcus has multiple copies of these genes. Given that host tissues are limited environments for free, readily fermented sugars (44), such as glucose, but are rich in glycoproteins with O- and N-linked glycans that contain monosaccharides, including galactose (48), and because deep tissue sites are hypoxic, both conditions that favor mixed-acid fermentation (38), we hypothesized that the PFL/PFL-AE system must be important for the in vivo fitness of the pneumococcus. Hence, we identified the genes responsible for pneumococcal PFL activity and studied the impact of these genes on the expression of selected genes involved in glycolysis. Furthermore, we demonstrated that the lack of PFL results in altered lipid composition in the cell membrane and attenuates pneumococcal virulence.

MATERIALS AND METHODS

Bacterial growth conditions. S. pneumoniae type 2 strain D39 and its isogenic mutants were used in this work. Routinely, pneumococci were grown at 37°C in microaerophilic conditions either in brain heart infusion broth (BHI) (Oxoid, Basingstoke, United Kingdom) or on blood agar base (Oxoid) supplemented with 5% (vol/vol) horse blood. When appropriate, the growth medium was supplemented with 100 μg/ml spectinomycin or 500 μg/ml kanamycin.

Bacteria were also grown in chemically defined medium (CDM) (39) containing disodium β-glycerophosphate (21 g/liter), sodium pyruvate (0.01% [wt/vol]), and choline (0.001% [wt/vol]) at 37°C without pH control (initial pH 6.5). Glucose (1% [wt/vol]) or galactose (1% [wt/vol]) was used as the carbon source. S. pneumoniae was grown under anaerobic conditions in rubber-stoppered bottles (200 ml). Aerobic growth was established under low oxygen tension in Erlenmeyer flasks with an agitation of 50 rpm. Growth was monitored by measuring the optical density at 600 nm (OD600). Growth rates (μ) were calculated through linear regressions of the plots of ln(OD600) versus time during the exponential growth phase.

Mariner mutagenesis. In vitro mariner mutagenesis was used to construct the mutants (32). Approximately 2-kb genomic regions containing the target genes were amplified with the appropriate primers (Table 1). For transposition reactions, approximately 200 ng of PCR fragments was mixed with 200 to 400 ng of donor mariner plasmid pMR12, which contains a spectinomycin resistance cassette, and incubated in the presence of purified HinMar transposase, as described previously (32). Gaps in transposition products were repaired with T4 DNA polymerase (New England Biolabs, Ipswich, MA) and subsequently by Escherichia coli ligase (New England Biolabs). Repaired transposition products were transformed into S. pneumoniae D39 using synthetic competence-inducing peptide (1). Transformants isolated from selective medium were tested for the presence of mariner minitransposons through PCR and sequencing. The PCR used one of the transposon-specific primers, MP127 and MP128 (Table 1), together with an appropriate chromosomal primer. In addition, chromosomal primers were also used to confirm the absence of an intact copy of the gene that may result from duplication. Typical PCR conditions consisted of 1 cycle of 95°C denaturation, 35 cycles of amplification (30-s denaturation at 94°C, 60-s annealing at 53 to 55°C, and 3-min extension at 72°C) with a 3-min final extension at 72°C. The amplification products were analyzed by agarose (1% [wt/vol]) gel electrophoresis. In addition, the exact insertion sites and direction of the anti-bacterial cassette were also determined by sequencing. The amplicons for sequencing were prepared by three rounds of PCR using MP128 primer as described previously (25), and then the purified products were sequenced using MP127 primer. Sequencing indicated that the spectinomycin cassette had been inserted 608 bp, 833 bp, 650 bp, and 116 bp away from the 5′ end of SPD0229, SPD0235, SPD0420, and SPD1774, respectively. SPD0229M, SPD0235M, SPD0420M, and SPD1774M (mutated in SPD0229, SPD0235, SPD0420, and SPD1774, respectively) (Fig. 2) were selected for further study.

Complementation of SPD0420M. To eliminate the possibility of polar effect, SPD0420M was complemented by introduction of an intact copy of SPD0420 using pCEP, which is a nonreplicative plasmid and allows controlled gene expression following ectopic integration into the chromosome (18). The plasmid integrates immediately downstream of the well-studied ami operon (1). This site is believed to be transcriptionally silent and, as far as it is known, does not affect any cellular functions (18). The intact copy of SPD0420 was amplified with SPD0420CF and SPD0420CR primers, which incorporate the NcoI and BamHI sites to the 5′ and 3′ ends of the gene, respectively (Table 1). The amplicons were digested with NcoI and BamHI and the digested products were ligated with NcoI/BamHI-digested pCEP. An aliquot of ligation mixture was directly transformed into SPD0420M as described previously (1), and the transformants were selected in the presence of spectinomycin and kanamycin. The successful introduction of the intact copy of the gene was confirmed by PCR using the malF and

FIG. 1. Schematic representation of reactions downstream of pyruvate in lactic acid bacteria. After entering bacteria, galactose is converted to pyruvate, which is then further catabolized by homolactic or mixed-acid fermentation pathways. iPFL, inactive PFL; PFL, pyruvate dehydrogenase; POX, pyruvate oxidase; ADH, alcohol dehydrogenase; ACK, acetate kinase; PTA, phosphotransacetylase.
pCEPR primers (Table 1), whose recognition sites are localized immediately up- and downstream, respectively, of the cloning site. One of the positive transformants, SPD0420Comp, was selected for further analysis.

**Quantification of fermentation products during growth.** Strains were grown as described above. Culture samples (2 ml) were taken at the beginning of the stationary phase of growth and centrifuged (16,000 × g, 2 min, 4°C), and the supernatants were stored at −20°C until analysis by high-performance liquid chromatography or 1H nuclear magnetic resonance (NMR) (40). End products 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 each sample to enable fatty acid quantitation. Fatty acid methyl esters were identified by cochromatography with authentic standards (Sigma Co., St. Louis, MO) (43). A P value of <0.05 was considered significant, except as otherwise noted.

**Quantitative reverse transcriptase-PCR.** The extraction of RNA was done by the Trizol method using mid-log-phase cultures as described previously (51). Before use, the RNA was treated with amplification-grade DNase I (Qiagen, Crawley, United Kingdom) and subsequently purified with RNAeasy minikit (Qiagen). First-strand cDNA synthesis was performed on approximately 1 μg DNase-treated total RNA immediately after isolation using 200 U of SuperScript II reverse transcriptase (Invitrogen, Paisley, United Kingdom) at 42°C for 55 min and random hexamers (59). The transcription level of specific genes was normalized to grfA transcription and amplified in parallel with the SPD0709RTF and SPD0709RTR primers. To reduce the bias in the quantitative reverse transcriptase-PCR, we used primer pairs with similar PCR efficiencies. The results were analyzed by the comparative threshold cycle method (30).

**In vivo virulence studies.** Female MFI outbred mice (Harlan Olac) were used for virulence testing. A standardized inoculum was prepared as described previously (58, 60). Briefly, after overnight growth in BHI (Oxoid) in microaerophilic conditions, the OD500 of the pneumococcal cultures was adjusted to 1.6 with phosphate-buffered saline (PBS), and 100 μl of this was administered intraperitoneally to mice. Once the animals reached the lethargic state, blood specimens were collected by cardiac puncture under deep anesthesia with 5% (vol/vol) fluothane (Astra Zeneca, Macclesfield, United Kingdom) over oxygen (1.5 to 2 liter/min). An aliquot of blood specimens was used to inoculate BHI containing 20% bovine serum. The cultures were allowed to grow until they reached an OD500 of 1.6, at which point the growth ceased and bacteria were stored at −80°C until needed.

To determine the virulence of pneumococcal strains, mice were lightly anesthetized with 2.5% (vol/vol) fluothane over oxygen (1.5 to 2 liter/min). A 50-μl sample of PBS containing approximately 5 × 106 prepassaged S. pneumoniae CFU was given through the nostrils. The inoculum dose was confirmed by viable counting on blood agar plates. Mice were monitored for disease signs (hunched, piloerect, or lethargic) for 7 days (35), and those that reached the severely lethargic stage were considered to have reached the end point of the assay and were killed humanely. This point was set to the maximum time."
that were alive 7 days after infection were deemed to have survived the infection. To express the disease signs numerically, a mouse was given a score of 2 if it was moribund, a score of 4 if it had a starry coat (piloerection), and a score of 6 if it was moribund and had a starry coat. To determine the development of bacteremia in each mouse, approximately 20 μl of venous blood specimens was obtained from intranasally infected mice at predetermined time points after infection. Viable counts in blood specimens were determined by serial dilution in sterile PBS and plating onto blood agar plates supplemented with 5% (vol/vol) defibrinated horse blood with appropriate antibiotic. Median survival times were analyzed using the Mann-Whitney U test.

Growth of bacteria in the nasopharynx and lungs was also determined. To do this, at predetermined time intervals following intranasal infection, groups of mice were deeply anesthetized as described previously (58, 60), and subsequently the lungs and nasopharynx were transferred separately into 10 ml of sterile PBS, weighed, and then homogenized in a stomacher lab blender (Seward Medical, London, United Kingdom) (58, 60). Viable counts in homogenates were determined as described above. For intra-venous infections, 1 l of venous blood specimens was obtained from intranasally infected mice, and then homogenized in a stomacher lab blender (Seward Medical, London, United Kingdom) (59, 60).

### RESULTS

#### Bioinformatic Analysis

The putative pfl genes, SPD0235 and SPD0420, are found in all sequenced pneumococcal genomes (19, 28, 52). The predicted amino acid sequences of SPD0235 and SPD0420 exhibit 20% identity over 770 amino acids. While the SPD0420 protein has conserved adjacent cysteinyl residues characteristic of a PFL active site (EMSCISCVCVSLD) (47), as well as the highly conserved peptide sequence around the C-terminal glycyl residue (RISGY) (47), the SPD0235 protein does not have the conserved cysteine residues. This implies that SPD0235 is unlikely to encode a PFL, in spite of the adjacent glycyl residue (RISGY) (47), the SPD0235 protein has conserved adjacent cysteinyl residues (19, 28, 52). The predicted amino acid sequences of the putative PFL-AE genes SPD0235 and SPD0420 are transcribed in opposite directions (Fig. 2), implying that the mutation of SPD0420 is unlikely to create a polar effect, and furthermore, a strong rho-independent transcription terminator with an estimated ΔGf of −12.3 kcal/mol is located 17 bp downstream of the SPD0420 stop codon. While dinP is conserved universally in all sequenced pneumococcal genomes, the hypothetical gene (SPD0421) either exhibits sequence variation or is absent in certain pneumococcal genomes, such as in TIGR4 or Hungary19A_6, indicating that recent evolutionary genetic events have taken place in the region (www.ncbi.nlm.nih.gov). In contrast to the genes in E. coli, Haemophilus influenzae, and Clostridium pasteurianum (15, 46, 54), in S. pneumoniae, the last gene of the pflA operon (13) and the carbon source (Table 2 and Fig. 3).

#### In vitro growth characteristics of pfl and pflA mutants

The predicted amino acid sequences of the putative FFL-AE proteins SPD0229 and SPD1774 share 32% identity over 236 amino acids, and they are found in all sequenced pneumococcal genomes (www.ncbi.nlm.nih.gov). Both of these proteins have a CXXXXXXCC consensus sequence motif close to the N-terminus of the protein, which is reported to be the catalytic site in the E. coli enzyme (7). SPD1774 is the last gene of a predicted operon (13) and surrounded by genes responsible for protein export (SPD1773) and pH homeostasis (SPD1775), while the locus encompassing SPD0229 contains genes coding for transcriptional regulators.

#### In vitro growth rates of pneumococcal strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose aerobic</th>
<th>Glucose anaerobic</th>
<th>Galactose aerobic</th>
<th>Galactose anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>D39</td>
<td>0.63 (0.02)</td>
<td>0.45 (0.02)</td>
<td>0.41 (0.01)</td>
<td>0.33 (0.02)</td>
</tr>
<tr>
<td>SPD0420 M</td>
<td>0.55 (0.03)</td>
<td>0.47 (0)</td>
<td>0.35 (0.02)</td>
<td>0.2 (0.03)</td>
</tr>
<tr>
<td>SPD0420Comp</td>
<td>0.6 (0)</td>
<td>0.41 (0.008)</td>
<td>0.22 (0.01)</td>
<td>0.3 (0)</td>
</tr>
<tr>
<td>SPD0229 M</td>
<td>0.6 (0)</td>
<td>0.45 (0)</td>
<td>0.41 (0.03)</td>
<td>0.3 (0.03)</td>
</tr>
<tr>
<td>SPD0235 M</td>
<td>0.62 (0.06)</td>
<td>0.44 (0.07)</td>
<td>0.43 (0.04)</td>
<td>0.35 (0.03)</td>
</tr>
</tbody>
</table>

*Pneumococcal strains were grown in CDM containing the indicated sugars as the sole carbon source in aerobiosis or anaerobiosis. The mean rate, where rate is defined as the increase in optical density per hour, was calculated from three independent experiments. Values are the mean rate, with the standard deviation given in parentheses.
The growth yields of the strains were higher aerobically than anaerobically, with the exception of SPD1774M, whose yield was higher anaerobically than aerobically. In addition, SPD0420M exhibited an extended lag phase during anaerobic growth. With galactose, the growth rate that was observed for all strains in aerobiosis and anaerobiosis \( P < 0.01 \) was lower than that for glucose-containing medium.

**End-product analysis.** The PFL enzyme can be measured directly (57) or indirectly (2). The indirect assay relies on formate detection in spent bacterial culture supernatants to indicate the presence of active enzyme. In this study we used the indirect method to assay PFL activity. The fermentation end products of pneumococcal strains in aerobiosis and anaerobiosis, in CDM with glucose or galactose as the sole carbon source, were analyzed using late-exponential-culture supernatants. Regardless of aeration, when glucose was used, the main fermentation product of D39 was lactate, with a small amount of acetate (Fig. 4A). However, replacement of glucose with galactose led to the generation of mixed fermentation products, under both aerobic and anaerobic conditions, with for-
Gene expression analysis. The glycolytic pathway is composed of an integrated network of enzymes whose activity is dependent on environmental, allosteric, and transcriptional regulation. Disruption of a route leading to end products in a strictly fermentative organism can be expected to affect expression of the glycolytic enzymes, as the flux through glycolysis is intimately associated with the conversion of pyruvate to end products, which ultimately allows glycolysis to proceed by replenishing NAD^+ pools. To further understand the role of PFL in the regulation of pneumococcal glycolysis, the expression of certain genes coding for glycolytic enzymes was determined for bacteria cultured under conditions of aerobicosis and anaerobiosis, using glucose or galactose as a carbon source (Table 3). Specifically, the expression of genes coding for FBA \( (fba) \), pyruvate kinase \( (pyk) \), pyruvate oxidase \( (spoB) \), GAP dehydrogenase \( (gap) \), lactate dehydrogenase \( (ldh) \), and \( \alpha \)-acetolactate dehydrogenase \( (aldB) \) was studied, along with SPD0420 and SPD1774. Differences in expression twofold or greater were considered significant, although some differences less than twofold are known to be biologically important (9).

\( pfl \) expression in other bacteria is known to be influenced by galactose and anaerobiosis (33, 34). Indeed, in strain D39 in anaerobiosis, SPD0420 expression increased nearly ninefold in galactose relative to that for glucose-grown bacteria. In addition, in aerobicosis, SPD0420 and SPD1774 were upregulated by 2.4- and 3.5-fold, respectively, in D39 grown in galactose relative to growth in glucose. The overexpression of these genes in aerobicosis reflects the readiness of the pneumococcus to adjust its metabolism when a shift from aerobicosis to anaerobiosis occurs. The expression of other genes did not show significant change under the experimental conditions employed.

In SPD0420M grown anaerobically in galactose, the expression of \( fba \) and \( pyk \) went up 5.7-fold \( (±1.4) \) and 4.8-fold \( (±1.7) \), respectively, compared to anaerobic growth in glucose. The expression of other genes in SPD0420M was similar to that for D39 in anaerobiosis, regardless of the carbon source \( (P > 0.05) \). When the expression of \( fba \) in SPD0420M grown anaerobically with galactose was compared to that for D39 cultured under the same conditions, the difference in expression levels was further pronounced, to >18-fold (Table 3).

**Fatty acid composition of bacterial cell membranes.** Because PFL activity leads to formation of acetyl-CoA in addition to formate and because acetyl-CoA is an important precursor of fatty acid biosynthesis (8), we hypothesized that in SPD0420M and SPD1774M, the fatty acid composition would exhibit altered patterns. Analysis of the fatty acid composition of D39 and the two mutant strains SPR0420M and SPD1774M. For each strain the weighted average of the number of double bonds per fatty acid, i.e., the unsaturation index, was calculated. Since the mean value of the unsaturation index is one or two orders of magnitude smaller than the proportions of fatty acids, we adjusted the critical \( P \) value by using a Bonferroni correction that considers the number of constituent fatty acids on which the unsaturation index is based; i.e., the initial critical \( P \) value, 0.05, was divided by 5, giving a new critical \( P \) value of 0.01.

As shown in Table 4, the absence of PFL activity resulted in a decreased unsaturation index, from 0.31 \( (±0.01) \) to 0.25 \( (±0.02) \) \( (P = 0.029) \) in the wild-type strain (D39) to 0.25 \( (±0.02) \) \( (P = 0.0056) \) in SPR0420M and SPD1774M, respectively, when bacteria were grown in BHI. Fatty acid analysis of bacteria grown in CDM supplemented with galactose resulted in only a

### Table 3. Fold change in gene expression under different conditions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Galactose vs glucose</th>
<th>SPD0420M vs D39 in galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolytic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( fba )</td>
<td>0.7 (0.12)</td>
<td>0.86 (0.35)</td>
</tr>
<tr>
<td>( pyk )</td>
<td>1.3 (0.05)</td>
<td>1.5 (0.21)</td>
</tr>
<tr>
<td>( gap )</td>
<td>1.3 (0.01)</td>
<td>8.6 (0.35)</td>
</tr>
<tr>
<td>Pyruvate breakdown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( spdh )</td>
<td>1.2 (0.04)</td>
<td>1.5 (0.21)</td>
</tr>
<tr>
<td>( ldh )</td>
<td>1.8 (0.17)</td>
<td>2.0 (0.47)</td>
</tr>
<tr>
<td>( aldB )</td>
<td>1.6 (0.09)</td>
<td>1.5 (0.21)</td>
</tr>
<tr>
<td>SPD1774</td>
<td>1.5 (0.21)</td>
<td>1.5 (0.21)</td>
</tr>
<tr>
<td>SPD0420</td>
<td>8.6 (0.35)</td>
<td>3.5 (0.32)</td>
</tr>
</tbody>
</table>

*Pneumococcal strains grown in CDM containing the indicated sugars as the sole carbon source in aerobiosis or anaerobiosis. See the text for gene names. The relative expression was calculated from three independent experiments, and the standard deviation is indicated in parentheses.*
slight change in SPD1774M (data not shown). Since CDM contains a high acetate concentration (7.3 mM), the organism can bypass the need for PFL/PFL-AE by converting acetate to acetyl-CoA via acetate kinase and phosphotransacetylase (50), supplying the PFL mutants with sufficient acetyl-CoA for fatty acid biosynthesis.

**In vivo studies.** The SPD0420M and SPD1774M strains were tested for their ability to cause disease in mice after intranasal infection. It was found that the SPD0420M- and SPD1774M-infected groups (108 ± 14 h, n = 10, and 90 ± 13 h, n = 10, respectively) survived significantly longer than the wild-type-infected group (51 ± 2 h, n = 28) (P < 0.01 and P < 0.05, respectively). There was no difference in the survival times of the SPD1774M- and SPD0420M-infected groups (P > 0.05). SPD0420Comp-infected mice did not survive (61 ± 8 h, n = 10) significantly longer than the wild-type-infected group (P > 0.05). Furthermore, the disease sign scores for the groups infected with mutants at 24, 36, and 48 h postinfection (for SPD0420M, 0 ± 0 at 24 h, 1.6 ± 0.3 at 36 h, and 3.4 ± 0.2 at 48 h; for SPD1774M, 0 ± 0, 1.7 ± 0.2, and 3.6 ± 0.2, respectively; n = 10 for both) were lower than those for the wild-type group (1.1 ± 0.3, 3.3 ± 0.4, and 4.8 ± 0.3, respectively; n = 20) (P < 0.01 for 24 and 36 h, and P < 0.05 for 48 h postinfection). For example, by 24 h postinfection, signs of disease could be detected only in the wild-type-infected cohort, and the signs increased swiftly thereafter. However, the signs of disease emerged after 24 h postinfection in the groups infected with SPD0420M and SPD1774M. Moreover, by sampling blood at predetermined time intervals following intranasal infection, it was demonstrated that bacteremia in the groups infected with SPD0420M or SPD1774M (between 12 and 24 h postinfection) occurred later than with D39 or SPD0420Comp (0 to 12 h postinfection) (P < 0.05) (Fig. 5A). However, once in the blood the mutants grew as well as the wild type.

To further investigate the nature of impaired virulence in strain SPD0420M, we went on to determine the growth of bacteria in the nasopharynx (Fig. 5B) and lungs (Fig. 5C) after intranasal infection. The D39 numbers in the nasopharynx were significantly greater than those for the mutant at 24 h (P < 0.01) and 48 h (P < 0.05) postinfection. D39 numbers progressively increased in the nasopharynx 12 h postinfection, whereas numbers for SPD0420M did not significantly change over 48 h. In the lungs, the wild-type numbers progressively increased after infection, from log$_{10}$ 2.99 ± 0.08 at the beginning of infection to log$_{10}$ 4.49 ± 0.65 at 48 h postinfection (P < 0.05) (Fig. 5C). However, SPD0420M was not as successful as the wild type in its ability to survive in the lungs, and by 24 h after infection an approximately 2-log decrease was observed with the numbers of SPD0420M (P < 0.05).

When the intravenous route was used for infection, the wild-type numbers increased from log$_{10}$ 5.07 ± 0.11 CFU/ml at the beginning of the infection to log$_{10}$ 7.12 ± 0.86 CFU/ml at 48 h postinfection, without apparent lag (Fig. 6). However, the mutant numbers started to increase only after 8 h postinfection, following an initial decline in viable counts. From this point onward the mutant grew as well as the wild type, suggesting the presence of alternative mechanisms to compensate the absence of PFL.

**DISCUSSION**

The transition from aerobiosis to an oxygen-depleted environment signifies passage from mucosal surfaces to deep-tissue sites for the pneumococcus; hence, it is integral to pneumo-

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**TABLE 4.** The fatty acid composition of pneumococcal strains grown in BHI

<table>
<thead>
<tr>
<th>Strain</th>
<th>14:0</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>P value for 18:1</th>
<th>UI</th>
<th>P value for UI</th>
</tr>
</thead>
<tbody>
<tr>
<td>D39</td>
<td>20.0 (0.6)</td>
<td>36.8 (1.5)</td>
<td>13.0 (0.2)</td>
<td>7.1 (0.9)</td>
<td>14.1 (0.6)</td>
<td>0.014</td>
<td>0.31 (0.01)</td>
<td></td>
</tr>
<tr>
<td>SPD0420M</td>
<td>19.1 (0.3)</td>
<td>38.7 (1.4)</td>
<td>13.0 (0.5)</td>
<td>6.8 (1.4)</td>
<td>9.4 (0.5)</td>
<td>0.014</td>
<td>0.25 (0.02)</td>
<td></td>
</tr>
<tr>
<td>SPD1774M</td>
<td>16.2 (0.5)</td>
<td>42.0 (0.9)</td>
<td>13.1 (0.1)</td>
<td>6.9 (0.8)</td>
<td>8.2 (0.8)</td>
<td>0.016</td>
<td>0.24 (0.01)</td>
<td></td>
</tr>
</tbody>
</table>

a The first figure shows the numbers of carbons, and the second the number of double bonds.

b The significance of difference was calculated in comparison to D39 by Student's t test; P < 0.05.

c UI, unsaturation index, defined as the weighted average of the number of double bonds per fatty acid.

d P < 0.05 based on the Bonferroni correction.

e Values are percentages of the five major fatty acyl residues; standard deviations are given in parentheses.

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**FIG. 5.** Growth of D39 (●), SPD0420M (■), SPD1774M (▲), and SPD0420Comp (▼) in blood specimens (A), nasopharynx (B), and lung (C) after intranasal infection. Each point is the mean of data from five mice. Error bars show the standard error of the mean.
coli enzyme (2, 56). This could be due to the presence of a
tively resistant to inactivation by oxygen compared to the
deactivation was suggested to occur through removal of the
deactivation system that may protect the PFL (34, 41). PFL
ously that PFL in
the pneumococcal PFL may not be as susceptible to oxygen as
inactivated in these oxygenated sites. Our results indicate that
of the nasopharynx and lungs, because PFL was expected to be
would be seen in the hypoxic environment of blood specimens
that PFL contributes to pneumococcal virulence in several
attenuated in its growth in the nasopharynx and lungs, showing
in anaerobiosis, consistent with PFL’s role in mixed-acid fer-
metabolism. In addition, we observed upregulation of
pyk. The increased
expression when grown in lactose-containing
medium (5). This may indicate concerted regulation of
fba and pyk during anaerobic growth in galactose-containing medium, compared
to growth in glucose. In Streptococcus bovis, overexpression of
fba increases pfl expression when grown in lactose-containing
medium (5). This may indicate concerted regulation of pfl, since the products of FBA, in addition to GAP and dihydroxy-
cell machinery is “optimized” to operate the PFL pathway. In
addition, we observed upregulation of pyk. The increased
expression in an E. coli pfl mutant was suggested to be due to
an increase in glycolytic flux. Higher glycolytic flux may be used to
achieve the energy requirement (62).
PFL is posttranslationally regulated by PFL-AE, which activ-
ates PFL by generation of a stable and catalytically essential
glycol free radical, which is required for the active PFL, by
alcohol dehydrogenase during the transition from anaerobic to
acid sequence). In E. coli, induction of pfl in anaerobiosis has been
shown to involve both FNR and ArcA/ArcB regulators
(33, 34, 47). The global transcription factor FNR was reported
to have a role in the expression of pfl via two recognition
sequences located in the promoter region of this gene (23). However, there is no
fnr homolog in S. pneumoniae, but the
coccal virulence (22). This move is expected to trigger a fund-
damental change in energy metabolism due to differences in
nutrient availability and oxygen concentration (44). We investi-
tigated biochemical aspects of these changes and the implica-
tion of this metabolic shift on pneumococcal virulence.
In many microorganisms, PFL is one of the key enzymes for
anaerobic energy metabolism, especially in the metabolic shift
to mixed-acid fermentation (2, 4, 11, 34). We demonstrated
through mutational studies and subsequent metabolite analysis
and by an in silico approach that the pneumococcal SPD0420
and SPD1774 genes are responsible for active PFL protein.
The mutant strains did not produce formate, which is gener-
ated only in the presence of active PFL, and a homology search
indicated conserved residues that are found in other PFLs (47).
SPD0420 and SPD1774 mutations led to reduced rates of
growth in galactose-containing medium compared to glucose in
anaerobiosis, consistent with PFL’s role in mixed-acid fer-
mentation. In addition, the mutation of SPD0420 and
SPD1774 led to a reduction in virulence, as manifested by the
increased survival times of animals infected with SPD0420M
and SPD1774M compared to the parental strain, and the bac-
teria in cohorts infected with SPD0420M and SPD1774M
was developed later than that in D39. Moreover, SPD0420M
attenuated in its growth in the nasopharynx and lungs, showing
that PFL contributes to pneumococcal virulence in several
tissue sites. Initially, we predicted that the main impact of PFL
would be seen in the hypoxic environment of blood specimens
(29), and no role was envisaged to be present during infection
of the nasopharynx and lungs, because PFL was expected to be
inactivated in these oxygenated sites. Our results indicate that
the pneumococcal PFL may not be as susceptible to oxygen as
other bacterial PFLs (26). Indeed, it has been reported previ-
ously that PFL in Streptococcus mutans and L. lactis is rela-
tively resistant to inactivation by oxygen compared to the E.
coli enzyme (2, 56). This could be due to the presence of a
deactivation system that may protect the PFL (34, 41). PFL
deactivation was suggested to occur through removal of the

FIG. 6. Time course of bacterial growth in blood specimens from
mice infected intravenously with D39 (●) or SPD0420M (■). Each
point is the mean of data from 6 to 10 mice, except 72 h for
SPD0420M, which is from 3 mice. Error bars show the standard error
of the mean.
pneumococcal genomes contain homologs of ArcA/ArcB transcriptional regulators (data not shown).

In addition to SPD0420 and SPD1774, SPD0235 and SPD0229 closely resemble the pfl and pflA genes, respectively. However, under the conditions tested, these genes are not involved in the synthesis of an active PFL. Genomic analysis of E. coli indicated the presence of several pfl- and pflA-like genes (46, 47). Many of these PFL-like enzymes are unlikely to have a role in the dissimilation of 2-keto acids, and therefore, they may represent new classes of glycolytic enzymes with novel enzymatic activities (46, 47).

In this study we identified the pneumococcal pfl and pflA genes and demonstrated their involvement in mixed-acid fermentation. It was found that the mutation of pfl results in reduced pneumococcal virulence, indicating a strong link between the ability to have flexible fermentative metabolism and virulence. The reduced virulence was probably due to a defect in ATP and acetyl-CoA biosynthesis, which affect fitness and fatty acid composition, respectively. At mucosal surfaces the concentration of glucose is low (44), but the pneumococcus is exposed to mucin at these sites, and mucin is rich in galactose-containing glycosides (48, 59). We recently demonstrated the mucin utilization ability of the pneumococcus (59). In light of available data, we conclude that the pneumococcus maintains mixed-acid fermentation in the nutrient-limited niches of the host, and active PFL plays a vital role in this metabolic event. Thus, future studies investigating pneumococcal virulence should not define virulence solely from the host perspective, but the intricacies of the environmental settings surrounding the microorganism should also be considered.

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REFERENCES


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