Characterization of the dihydrolipoamide dehydrogenase from *Streptococcus pneumoniae* and its role in pneumococcal infection

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Summary

In the present study, we have characterized the dihydrolipoamide dehydrogenase (DLDH) of *Streptococcus pneumoniae* and its role during pneumococcal infection. We have also demonstrated that a lack of DLDH results in a deficiency in α-galactoside metabolism and galactose transport. DLDH is an enzyme that is classically involved in the three-step conversion of 2-oxo acids to their respective acyl-CoA derivatives, but DLDH has also been shown to have other functions. The *dldh* gene was virtually identical in three pneumococcal strains examined. Besides the functional domains and motifs associated with this enzyme, analysis of the pneumococcal *dldh* gene sequence revealed the presence of an N-terminal lipoyl domain. DLDH-negative bacteria totally lacked DLDH activity, indicating that this gene encodes the only DLDH in *S. pneumoniae*. These DLDH-negative bacteria grew normally in vitro but were avirulent in sepsis and lung infection models in mice, indicating that DLDH activity is necessary for the survival of pneumococci within the host. The lack of virulence was not associated with a loss of 2-oxo acid dehydrogenase activity, as the wild-type pneumococcal strains did not contain activity of any of the known 2-oxo acid enzyme complexes. Instead, studies of carbohydrate utilization demonstrated that the DLDH-negative bacteria were impaired for α-galactoside and galactose metabolism. The DLDH mutants lost their ability to oxidize or grow with galactose or melibiose as sole carbon source and showed reduced oxidation and growth on raffinose or stachyose. The bacteria had an 85% reduction in α-galactosidase activity and showed virtually no transport of galactose into the cells, which can explain these phenotypic changes. The DLDH-negative bacteria produced only 50% of normal capsular polysaccharide, a phenotype that may be associated with impaired carbohydrate metabolism.

Introduction

Dihydrolipoamide dehydrogenases (DLDH; EC 1.8.1.4) are homodimeric flavoproteins that catalyse the NAD⁺-dependent reoxidation of dihydrolipoamide (DLA) in a number of multienzyme complexes (Perham et al., 1987; Carothers et al., 1989; Williams, 1992; de Kok et al., 1998). These complexes are primarily involved in the conversion of 2-oxo acids to their corresponding acyl-CoA derivative and are involved in important steps in aerobic and anaerobic metabolism (Carothers et al., 1989; de Kok et al., 1998). DLDH makes up the E3 component of the pyruvate dehydrogenase, 2-oxo glutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes. Additionally, DLDH functions in the glycine cleavage multienzyme complex (where it is referred to as the L protein), as well as in the acetoin dehydrogenase complex in bacteria such as *Bacillus subtilis*, *Clostridium magnum* and *Pelobacter carbinolicus* (Wieland, 1983; Dietrichs and Andreesen, 1990; Kruger et al., 1994; Oppermann and Steinbuchel, 1994; Berg and de Kok, 1997; de Kok et al., 1998; Aervarsson et al., 1999; Huang et al., 1999).

Even though the main function of DLDH is associated with its role in 2-oxo acid dehydrogenase complexes, the fact that DLDH is present in organisms that lack these complexes suggests that the enzyme may have additional functions (Danson et al., 1987; Danson, 1988a). *Trypanosoma brucei* as well as various archaebacteria exhibit simpler ways of converting 2-oxo acids, while still containing a DLDH enzyme (Danson et al., 1987; Danson, 1988b). This suggests that the DLDH must have a separate function in these organisms, and that this function is possibly retained in species in which the DLDH is also part of a 2-oxo acid dehydrogenase complex. Richarme and Heine (1986) and Richarme (1989) proposed that DLDH may be involved in regulation of the transport of galactose, maltose and ribose across the membrane of...
Fig. 1. Sequence comparisons between *S. pneumoniae* C08 ORF and known DLDH sequences.

A. **CLUSTALW** alignment of the *S. pneumoniae* C08 ORF, the C37 ORF and selected DLDH proteins from different species. Sequences were aligned by the CLUSTALW algorithm and the BLOSUM30 amino acid scoring matrix using the MACVECTOR software. The figure shows amino acids with over 51% similarity or identity between sequences in shaded boxes. Common motifs in DLDHs were obtained by searching the PROSITE database (http://www.expasy.ch/prosite/) with the C08 ORF as a query sequence, and information on conserved residues was provided through the information from the crystal structure of DLDH from *Azotobacter vinelandii* (Mattevi et al., 1991). These conserved residues and domains are marked with white letters on a black background. Letter D above the sequence indicates residues involved in dimerization, and F indicates residues involved in FAD binding. DLDH sequences used for the alignment were obtained from GenBank and were as follows: *Bacillus subtilis* (accession no P21880), *Clostridium magnum* (accession no I40794), *Streplococcus pyogenes* (accession no P21795), *Azotobacter vinelandii* (accession no P18925), *Escherichia coli* (accession no P00395) and *Neisseria meningitidis* (accession no CAB84783).

B. **Dendrogram** showing the relatedness of DLDH sequences based on the nearest neighbour joining method showing bootstrap values calculating real differences between the sequences after 1000 iterations. The tree is based on the homologous domains of all sequences and thus excludes the lipoyl domain. Sequences were the same as in (A) with the addition of *Bacillus halodenans* (accession no P18925), *Escherichia coli* (accession no P00395) and *Neisseria meningitidis* (accession no CAB84783). The C08 *S. pneumoniae* sequence had a FAD-binding domain (amino acids 113–248) that contains a nucleotide-binding motif (GXGXXG, amino acids 117–123) and a class I pyridine nucleotide disulphide oxidoreductase active site (amino acids 146–156, PROSITE accession no PDOC0071). Within the oxidoreductase active site are two catalytic cysteine residues (C149 and C154) involved in electron transfer, as well as a conserved glycine residue at position 150 and an isoleucine residue at position 155 (Carothers et al., 1989). Furthermore, the C08 sequence also contains an NAD-binding domain (amino acids 249–375) with a conserved nucleotide-binding motif (GXGXXG, amino acids 285–390), followed by a central domain (amino acids 390–442) and an interface domain (amino acids 443–568). Within this domain resides the conserved enzymatic active site base (H542) as well as residues important for DLDH dimerization (E521 and E527).

C. **Dendrogram** showing the relatedness of DLDH sequences that encode a lipoyl domain. The relatedness was calculated as described above.
Escherichia coli cells. One of two DLDH enzymes in E. coli was shown to be upregulated by the transport of these sugars and was also co-purified with the MgIC component of the galactose transport system. DLDH has also been suggested to be involved in cell cycle progression in fission yeast (Jang et al., 1997) and is a highly immunogenic surface antigen in Neisseria meningitidis (Li de la Sierra et al., 1997; Exposito Raya et al., 1999).

In this study, we describe the identification and characterization of the dldh gene in Streptococcus pneumoniae. The gene was sequenced in three strains of pneumococci and inactivated by insertion–duplication mutagenesis. DLDH-negative bacteria lacked DLDH activity and were avirulent in animal infection models. It was discovered that pneumococci lack detectable 2-oxo acid dehydrogenase complex activities, suggesting that the pneumococcal DLDH is used for a different function. Instead, we provide evidence that the pneumococcal DLDH is critical for α-galactoside metabolism and galactose transport, and that DLDH mutations result in decreased capsule production.

Results

Identification of the dldh gene

While studying sequences encoding oxidoreductase-type enzymes, we identified three open reading frames (ORFs C08, C37 and C42) from the recently published pneumococcal genome sequence (Tettelin et al., 2001), each of which had homology to DLDHs of other species. The C42 ORF was identical to the previously characterized enzyme NADH oxidase (Auzat et al., 1999). Of the other two sequences, only the C08 ORF fully met the required sequence criteria for a DLDH (see Fig. 1A). The sequence
criteria used included an FAD-binding domain, a conserved pyridine redox active site, an NAD-binding domain, conserved dimerization residues and a highly conserved active site histidine (Mattevi et al., 1991; Li de la Sierra et al., 1997). The disqualified C37 ORF lacked the initial nucleotide binding site of the FAD-binding domain, had an atypical isoleucine at amino acid position 7 and lacked several of the conserved residues involved in dimerization (Fig. 1A). Thus, the C08 ORF was the likely candidate for the only DLDH in the sequenced type 4 S. pneumoniae genome (Fig. 1A). This prediction was later verified by mutational analysis of the ORFs C08 and C37 (see below).

Sequence characteristics of the dldh gene

The C08 ORF (dldh gene) from S. pneumoniae strains D39, TIGR4 and EF3030 were sequenced using polymerase chain reaction (PCR) amplification and dye terminator chemistry. The strains express capsular types 2, 4 and 19F respectively. Worth noting is that, even though we used the same isolate (TIGR4) that was sequenced by The Institute for Genomic Research, our sequence data differed at one position (A1184C) from the sequence reported (Tettelin et al., 2001). This would lead to one amino acid change (N395T). The dldh gene sequence was highly conserved between the strains. Only three nucleotides differed between the TIGR4 and D39 sequences and only six between the TIGR4 and EF3030 sequences. These nucleotide differences resulted in two amino acid changes between the TIGR4 and D39 protein sequences (N253S and T395N) and four changes between the TIGR4 and EF3030 protein sequences (P95S, G106D, N253S and T395N). None of these amino acid changes occurred within the known active domains of the enzyme (Mattevi et al., 1991; Williams, 1992).

Interestingly, the pneumococcal DLDH, unlike most other DLDH proteins, contains an extended N-terminal region that encodes a lipoyl-binding motif (PROSITE, accession no PDOC00168; http://www.expasy.ch/prosite). This lipoyl domain (amino acids 1–112) contains a conserved lysine (K43), to which lipoamide (LA) can be covalently attached through an amide linkage, and has the appropriate conserved surrounding residues that are involved in the overall lipoyl domain structure (Russell and Guest, 1991). The pneumococcal protein shares this feature with the DLDHs from Streptococcus pyogenes, C. magnum, N. meningitidis and Mycoplasma capricolum. The role of this extra domain in DLDH enzymes remains unknown.

To determine the relatedness of the pneumococcal DLDH to other DLDH proteins, two dendrograms were generated using the nearest neighbour joining method set to calculate bootstrap values based upon 1000 repeated resamplings of the sequences. The first dendrogram (Fig. 1B) was produced from the pairwise alignment of the pneumococcal DLDH with DLDH sequences from the GenBank database, comparing only the domains common between the sequences. As this excluded comparisons of the lipoyl domain, a second dendrogram was generated comparing only those DLDH sequences that contain an N-terminal lipoyl domain (Fig. 1C). Both trees showed that the pneumococcal DLDH was most closely related to the DLDH of S. pyogenes with a bootstrap value of 100, but also suggested that the pneumococcal DLDH is more closely related to DLDH sequences from bacterial intracellular parasites than to some of the other Gram-positive sequences (Fig. 1B).

Measurement of DLDH activity in crude extracts from S. pneumoniae

DLDH catalyses the oxidation of DLA to LA using NAD+ as an electron acceptor. The activity of the enzyme can be monitored by a decrease in absorbance at 365 nm through the LA-dependant conversion of NADH to NAD+ or as the increase in absorbance at 340 nm as NAD+ is converted to NADH in the presence of DLA.

Crude extracts from S. pneumoniae were made by sonication of bacteria followed by collection of the soluble fraction. Each sample was then measured for the LA-dependant conversion of NADH to NAD+. The D39, TIGR4 and EF3030 extracts all contained a high capacity to convert NADH to NAD+ that was independent of LA, making the LA-dependant activity to be ascribed to the recently characterized NADH oxidase (NOX) (Auzat et al., 1999) and, using a NOX-negative strain (CP8056), the LA-dependant NADH oxidation (DLDH activity) could easily be measured in the bacterial extracts. Extracts from CP8056 bacteria contained an LA-dependant DLDH activity of 7–17 mU mg–1 protein (Table 1). When the activity was measured in the opposite reaction direction (conversion of NAD+ to NADH), an activity of 45–71 mU mg–1 protein

Table 1. DLDH activity in crude extracts of S. pneumoniae CP8056.

<table>
<thead>
<tr>
<th>Strain</th>
<th>NADH conversion (± SEM)</th>
<th>NAD+ conversion (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP8056</td>
<td>11.1 (±1.15)</td>
<td>59.2 (±9.04)</td>
</tr>
<tr>
<td>CP8056 C0805</td>
<td>0.2 (±0.12)</td>
<td>0.0</td>
</tr>
<tr>
<td>CP8056 C0832</td>
<td>0.3 (±0.29)</td>
<td>0.0</td>
</tr>
<tr>
<td>CP8056 C3701</td>
<td>9.5 (±2.00)</td>
<td>ND</td>
</tr>
<tr>
<td>CP8056 C3702</td>
<td>10.5 (±1.50)</td>
<td>ND</td>
</tr>
</tbody>
</table>

a. Activity was measured as the change in the concentration of NADH per minute by measuring the absorbance at 340 nm or 365 nm and using the extinction coefficients for NADH or NAD+ of 6.22 mM–1 cm–1 and 3.02 mM–1 cm–1 respectively.

ND, not determined.
Dihydrolipoamide dehydrogenase in S. pneumoniae was recorded. As both reactions were run at similar substrate concentrations, the higher DLDH activity observed for the NAD$^+$ to NADH conversion suggested that the DLDH enzyme favours the reaction direction that it uses within a 2-oxo acid dehydrogenase complex.

DLDH activity for the CP8056 strain was followed throughout bacterial growth. Maximum activity of DLDH was observed during mid-logarithmic growth, and activity decreased as the bacteria approached and entered into stationary phase (data not shown). All additional experiments therefore used bacteria grown to mid-logarithmic phase.

To visualize DLDH activity from the wild-type bacteria, the S. pneumoniae extracts were analysed in native polyacrylamide gels (Fig. 2A). Full-length 6His-tagged recombinant pneumococcal DLDH protein expressed in E. coli (A. W. Smith, et al., manuscript in preparation) was used as a marker for enzyme activity. All wild-type extracts contained several protein bands with NADH oxidation activity. One of these could be identified as the pneumococcal DLDH based upon the absence of this band in DLDH mutant pneumococcal extracts (see below). It is interesting to note that the DLDH protein in the wild-type pneumococcal extracts seemed to migrate more slowly in the native gel than the recombinant DLDH. As DLDH is known to function as a dimer, these bands might correspond to aggregates of the DLDH protein with itself or other proteins.

Mutational inactivation of the dldh gene

The dldh sequence of S. pneumoniae was inactivated by insertion–duplication mutagenesis in strains D39 and CP8056. To control for polar effects, an insertion–duplication was also placed downstream of the dldh gene, resulting in strains D39-C08P1 and D39-C08P2. The reason for including such a control mutation is to verify that the phenotype investigated is not the result of a changed or disrupted expression of downstream genes. The gene sequence of the C37 ORF was also inactivated in the CP8056 strain to verify that this ORF does not encode for a second DLDH.

Two independent mutants of each insertion were selected for further studies. All mutant strains showed in vitro growth rates indistinguishable from the wild-type bacteria (data not shown). DLDH activity assessment in native polyacrylamide gels revealed that both D39 and CP8056 wild-type strains contained a protein band that could oxidize NADH, which was absent in the dldh mutant strains. This band was present in both control mutants, excluding the role of polar effects of the mutation, and verifying that the C08 ORF encoded for the pneumococcal DLDH (Fig. 2B). Additionally, a Western blot of the D39 and CP8056 strains and their DLDH mutants (CP8056-C0805, CP8056-C0832, D39-C0832:1 and D39-C0832:2), developed with antibodies from the serum of a patient with primary biliary cirrhosis (PBC),

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Fig. 2. The presence of DLDH in S. pneumoniae extracts.
A. Activity gels of wild-type S. pneumoniae extracts. A protein extract of S. pneumoniae D39, CP1015, CP8056, TIGR4 and EF3030 were run natively on a 10% polyacrylamide gel, and the gel was incubated in the presence of NADH and NBT. The presence of bands indicates the ability to oxidize NADH. Recombinant pneumococcal DLDH was used as an enzyme marker. All strains contained DLDH activity, which is indicated by an arrow.
B. Activity gels of DLDH mutant S. pneumoniae strains. Protein extracts of S. pneumoniae D39 and CP8056 were run along with their respective dldh mutant extracts and examined for NADH-oxidizing activity. The mutants lacking bands associated with DLDH activity are indicated by an arrow.
C. Immunoblotting of D39 and CP8056 wild-type and DLDH mutant S. pneumoniae protein extracts. The extracts were run under denaturing conditions on a 12% polyacrylamide gel, transferred to a nitrocellulose membrane and developed using serum from a patient with PBC disease in order to detect lipoylated proteins. Wild-type extracts possessed the 61.4 kDa DLDH protein band as indicated, whereas the DLDH mutant extracts did not.

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confirmed the absence of the 61.4 kDa DLDH protein in the mutant strains (Fig. 2C). These antibodies were used, as patients afflicted with this disease produce autoantibodies that specifically recognize proteins that contain lipoyl domains (Quinn et al., 1993; Morris et al., 1995).

Crude extracts from the CP8056 dldh insertion–duplication mutants and C37 mutants (CP8056-C3701 and C3702) were also tested for their ability to convert NADH and NAD$^+$ or NAD$^+$ to NADH by the colorimetric assay (Table 1). The D39 mutants were not tested in this assay because of the presence of NADH oxidase in these extracts. Both C37 ORF mutants contained LA-dependent DLDH activity similar to that observed for the extract from the wild-type CP8056 strain. This suggested that the protein encoded by the C37 ORF was not a DLDH, which correlated well with the previously mentioned lack of DLDH homology within this sequence (Fig. 1A). On the other hand, both the C08 mutants lacked DLDH activity in both directions of the reaction (Table 1). This suggested that the C08 ORF encodes for the DLDH and that it is the only DLDH present in the pneumococcal genome.

Virulence studies

The role of DLDH in pneumococcal infection was investigated in two different murine model systems: a local lung infection model and a sepsis model. The models were chosen to evaluate the role of the enzyme at different stages of infection and in two different host environments. In each infection model, the two downstream insertion control mutants were used in parallel to control for any polar effects caused by the mutagenesis.

In the sepsis infection model, wild-type D39 and two dldh mutant strains were used to infect CBA/N mice by intravenous injection at a dose of 300 cfu in 100 μl. The survival of the mice was followed to assess virulence. Both the dldh mutant strains were avirulent in this model compared with the wild-type D39 strain (Fig. 3A). Both the downstream insertion mutants killed the mice with similar kinetics to the wild-type D39 strain (Fig. 3A). Both the downstream insertion mutants killed the mice with similar kinetics to the wild-type D39 strain, verifying that the mutation within the dldh gene was responsible for the avirulent phenotype (Fig. 3A). The avirulence of the dldh mutant strains persisted even at 100 times the original infectious dose (Fig. 3A), indicating that the DLDH enzyme is an important factor for bacterial survival or virulence in septic infection.

For the lung infection model, CBA/N mice were anaesthetized and given $1–3 \times 10^6$ cfu intranasally in a volume of 40 μl to facilitate aspiration into the lungs. Half the mice were sacrificed at 48 h after infection, and the bacterial load in the blood and lungs was determined. The other half of the mice were monitored for time to death. After 48 h, the mice infected with the dldh mutants had no detectable bacteria in the lungs or blood. In contrast, around $10^6$ wild-type D39 or downstream insertion mutant D39-C08P1 bacteria were present in the lungs as well as per ml of blood (Fig. 3B). All the mice infected with the wild-type D39 strain and six of seven infected with the downstream insertion mutant strain died within 3–5 days after infection, whereas those infected with the dldh mutants survived the infection (Fig. 3B). Thus, the dldh mutant strains were avirulent in both infection routes.
Dihydrolipoamide dehydrogenase in S. pneumoniae

Activities associated with the pyruvate, acetoin and branched-chain 2-oxo acid dehydrogenase complexes were measured. Measurement of 2-keto-glutarate dehydrogenase activity was excluded, as pneumococci lack the necessary components of the TCA cycle (Baltz et al., 2000), and the glycine cleavage system was excluded based on insufficient sequence homology. A crude extract from E. coli M15 served as an enzyme-containing control for pyruvate and branched-chain 2-oxo acid dehydrogenase activity (Table 2). A pneumococcal extract from THY-grown CP8056 bacteria lacked all three 2-oxo acid dehydrogenase activities, suggesting that the 2-oxo acid dehydrogenase operon was not functional, at least not under these conditions (Table 2). These results were verified by independently measuring the activity for each of the individual components of the dehydrogenase complexes. The CP8056 strain had no E1 (dehydrogenase) activity for either pyruvate, acetoin or 2-keto-isovalerate (Table 2). A low level of E2 (acetyltransferase) activity was present in the pneumococcal extract, which was not surprising, as the assay is not specific for the E2 component of 2-oxo acid dehydrogenase complexes. DLDH activity was present in the CP8056 extract, thus ensuring that these extracts were prepared correctly (Table 2).

Measurement of 2-oxo acid dehydrogenase activities in crude extracts from S. pneumoniae

In other bacterial systems, the DLDH enzyme is generally involved in central metabolism as part of various 2-oxo acid dehydrogenase complexes (Perham et al., 1987; Kruger et al., 1994; Oppermann and Steinbuchel, 1994; de Kok et al., 1998). When analysing the known sequence surrounding the pneumococcal dldh gene, it was observed that the dldh gene was located within a locus that contained the components of a putative 2-oxo acid dehydrogenase complex (for details, see Fig. 4). A BLAST search showed these genes to be most homologous to those of the acetoin dehydrogenase complex of C. magnum, although homology to pyruvate or branched-chain 2-oxo acid dehydrogenase complexes could not be excluded. The acetyltransferase component of the pneumococcal locus lacked the conserved N-terminal lipoyl domain, suggesting that this component might not be functional. As the activity of the pneumococcal DLDH enzyme seemed to be critical for virulence in the mouse infection models, the role of the pneumococcal enzyme as part of a 2-oxo acid dehydrogenase complex was investigated.

Table 2. 2-Oxo acid dehydrogenase complex activities.

<table>
<thead>
<tr>
<th>Enzyme activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Substrate</th>
<th>E. coli</th>
<th>CP8056 THY&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CP8056 SH glu&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CP8056 SH val&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CP8056 SH ace&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>Full complex</td>
<td>Pyruvate</td>
<td>136.80</td>
<td>0.00</td>
<td>0.46</td>
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<td></td>
<td>2-oxo valerate</td>
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<td>0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetoin</td>
<td>0.00</td>
<td>0.00</td>
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</tr>
<tr>
<td>E1</td>
<td>Pyruvate</td>
<td>23.68</td>
<td>0.07</td>
<td>0.00</td>
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<td>0.00</td>
</tr>
<tr>
<td></td>
<td>2-oxo valerate</td>
<td>5.85</td>
<td>0.33</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Acetoin</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>Acetyl-CoA</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E3</td>
<td>NAD + DLA</td>
<td>865.65</td>
<td>66.53</td>
<td>57.41</td>
<td>95.04</td>
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<td></td>
<td>NADH + LA</td>
<td>139.45</td>
<td>11.76</td>
<td>14.11</td>
<td>16.77</td>
<td>16.13</td>
</tr>
</tbody>
</table>

<sup>a</sup> Full complex denotes the transformation of the substrate through all three components of the 2-oxo acid dehydrogenase complex. E1 denotes the measurement of the dehydrogenase activity. E2 denotes acetyltransferase activity, and E3 denotes DLDH activity.

<sup>b</sup> THY, grown in Todd–Hewitt medium; SH glu, SH val, SH ace, grown in SH media with glucose, valine or acetoin as sole carbon sources respectively.

**Fig. 4.** A depiction of the putative 2-oxo acid dehydrogenase complex within S. pneumoniae. Upstream of the DLDH gene (3.3 kb) is the start of a putative α-subunit of a dehydrogenase-homologous sequence, which is followed by a β-subunit, and an acetyltransferase homologous sequence. The acetyltransferase ORF lacks an N-terminal lipoyl domain that is required for activity in the 2-oxo acid dehydrogenase complex. Downstream of the DLDH gene is a lipoyte ligase homologous ORF, which is usually involved in attaching lipoic acid to the lipoylation site. An angled arrow before the gene sequence indicates promoter-like sequences, and a vertical line with a filled circle on top indicates the presence of terminator sequences for RNA polymerase.
In some model systems, the components of the 2-oxo acid dehydrogenase complexes are not expressed unless the appropriate carbon source is present in the surrounding media (Grundy et al., 1993; Lorenzi et al., 1993; Kruger et al., 1994; Oppermann and Steinbuchel, 1994). To address this, we first attempted to grow pneumococci in a defined media with glucose, acetoin or valine as sole carbon sources. The CP8056 pneumococcal strain grew well in the presence of glucose, but could not grow in the presence of acetoin or valine.

Secondly, we grew CP8056 bacteria to stationary phase in THY media, washed them and incubated them for various times in SH media containing glucose, acetoin or valine as sole carbon sources. Activities from the 2-oxo acid dehydrogenase complexes and their individual components were determined thereafter from crude extracts. No 2-oxo acid dehydrogenase activity was observed under these growth conditions (Table 2). Furthermore, results for the individual E1, E2 and E3 components of the dehydrogenase complex were indistinguishable from those of the THY-grown bacteria (Table 2).

Cumulatively, these data indicate that pneumococci do not express any of the reported functional 2-oxo acid dehydrogenase complexes, either when grown in THY broth or when incubated in the presence of carbon sources that would promote such activities.

Carbon source utilization of dldh mutant strains of S. pneumoniae

Previous reports from Richarme’s laboratory (Richarme and Heine, 1986; Richarme, 1987; 1989) have described how one of two DLDHs in E. coli is involved in the transport of maltose and galactose into these cells. As our data suggested that the pneumococcal DLDH was not involved in a typical 2-oxo acid dehydrogenase complex, we decided to investigate whether or not this enzyme affects the ability of the pneumococcus to use different carbon sources. The wild-type D39 strain and the two dldh mutants were screened for the ability to oxidize 95 different carbon sources using GP2 microplates (Biolog). The dldh mutant strains oxidized most of the carbon sources to the same degree as the wild-type strain, but showed an impaired ability to oxidize galactose- and α-galactoside-linked sugars (data not shown).

In order to investigate these results further, we developed an alternative microtitre plate assay for the assessment of carbon oxidation. Bacteria were added to wells containing a carbon source and p-iodonitrotetrazolium violet (INT), and oxidation was measured as a purple coloration of the bacterial suspension. Four different α-galactosides were tested (D-galactose, melibiose, raffinose and stachyose). In addition, lactose was included as a comparison of a β-galactoside and maltose and maltotriose were included to compare our results with those seen in E. coli (Richarme, 1989). DLDH-negative bacteria of both CP8056 and D39 origin were impaired in their ability to oxidize all four α-galactosides, with the highest reduction seen for D-galactose and stachyose (85% and 81% reduction for D39-C0832:1 and 81% and 77% reduction for CP8056-C0805 respectively) (Table 3). No difference in oxidation was seen with glucose, maltose, maltotriose or lactose. The two downstream insertion mutants, D39-C08P1 and D39-C08P2, displayed results indistinguishable from those of the wild-type D39 strain (Table 3).

As this assay only addresses the ability of the pneumococcus to oxidize these carbon sources, we also investigated the ability of the bacteria to use each of them for growth. D39 and CP8056 wild-type strains grew well on all carbon sources; however, the growth on galactose was slower than that observed for the remaining carbohydrates. Melibiose could not support growth of either of the wild-type strains. The same results were seen for both downstream insertion mutants (Table 4).

The growth of the dldh mutants was indistinguishable from that of the wild-type bacteria on glucose, maltose,
maltotriose and lactose. The mutants did not grow on D-galactose or melibiose as sole carbon sources. The *dldh* mutants also showed reduced growth in the presence of raffinose and stachyose (Table 4). These results suggest that the pneumococcal DLDH enzyme might play an important role in the ability of the bacteria to metabolize α-galactosides.

### α-Galactosidase activity and galactose transport

The common characteristic of all α-galactosides is the presence of a galactose-α-1-6-glucose bond that must be cleaved by α-galactosidase before being used in the central metabolism of the organism. The α-galactosidase of *S. pneumoniae* was recently identified as part of the raffinose utilization system (Rosenow *et al*., 1999).

To evaluate the role of DLDH in the expression of α-galactosidase activity, wild-type and DLDH-negative strains of *S. pneumoniae* D39 were grown in THY medium, and bacterial extracts were assayed for α-galactosidase activity using p-nitrophenyl-α-D-galactopyranosyl as a substrate in a colorimetric assay. Wild-type and downstream insertion mutant bacteria showed α-galactosidase activity ranging from 34 to 56 mU mg⁻¹ extract, whereas both DLDH-negative strains showed a drastic reduction in α-galactosidase activity with only 15% and 7.5% of wild-type levels for D39 C0832:1 and C0832:2 respectively, (Fig. 5A). The decreased α-galactosidase activity would explain why the DLDH-negative bacteria showed a reduced ability to grow on α-galactoside-based sugars, but does not explain why they cannot use galactose alone.

To address this, transport of galactose into the cells was assessed. Wild-type and mutant cells were grown in THY medium, washed extensively in PBS and mixed with 14C-labelled D-galactose for various times. 14C-labelled D-glucose was used for comparison, as glucose uses a different transport system from galactose (Tettelin *et al*., 2001). The bacteria were placed on a filter, washed and radioactivity associated with the bacteria was measured. Heat-killed bacteria were included as a control for background radioactivity, and transport was assessed as the amounts of radioactivity above the baseline level of heat-killed cells.

Initial studies using the wild-type D39 strain indicated that galactose transport was slower than glucose transport, with near saturating levels of transport occurring after between 5 and 10 min. For glucose transport, saturation was reached within the first minute (data not shown). For analysis of transport in our mutant strains, we therefore chose to look at galactose transport after 1 and 5 min and glucose transport after 30 s and 1 min.

Wild-type bacteria showed a strong accumulation of galactose that increased from 0.54 (± 0.18) nmol/10⁹ bacteria at 1 min to 0.89 (± 0.30) nmol/10⁹ bacteria at 5 min (Fig. 5B). Similar kinetics and galactose accumulation were observed in the two control mutants D39-C08P1 and -C08P2. The DLDH-negative bacteria, on the other hand, showed an accumulation of galactose very close to the baseline interaction with heat-killed cells with an accumulation of only 0.01 (± 0.00) and 0.03 (± 0.02) nmol/10⁹ bacteria (Fig. 5B). This difference was not observed when the transport of glucose was investigated. Both the wild-type and DLDH-negative bacteria accumulated glucose to a similar degree (Fig. 5C).

These results suggest that DLDH is important not only for the induction of α-galactosidase activity, enabling efficient use of α-galactoside-based sugars, but also for a functional transport of galactose to occur.

### Effect of the *dldh* mutation on capsule production in *S. pneumoniae*

The somewhat smaller colony morphology of bacteria grown on blood agar, and the pellet size after centrifugation of broth-grown bacteria, indicated that the
DLDH-negative bacteria might produce less capsular polysaccharide. Given that the D39 dldh mutant strains were avirulent in mouse infection models, we wanted to investigate whether or not these phenotypes were linked to capsule production. Results obtained by enzyme-linked immunosorbent assay (ELISA), after correcting for background absorbance using the unencapsulated strain R36A, showed that the two D39 dldh mutants (C0832:1 and C0832:2) expressed 47% ($\pm$ 6.2%) and 51% ($\pm$ 2.9%) of the amount of the capsule expressed by wild-type bacteria, whereas the downstream insertion mutants produced levels of capsule (97 $\pm$ 7.7% and 103 $\pm$ 11.3%) equal to that of the wild-type strain. Even though these results suggest that a dldh mutation results in a reduction in the amount of capsule, the level of capsule that is made is still higher than that needed to retain virulence for an S. pneumoniae type 3 strain (Magee and Yother, 2001).

**Discussion**

The pneumococcal dldh sequence was identified from the genome sequence of a type 4 encapsulated strain of S. pneumoniae, based upon its homology to DLDH sequences from other bacterial species. The pneumococcal dldh sequence was highly conserved between three different strains of S. pneumoniae, at both nucleotide and amino acid sequence levels, suggesting that this enzyme is important for and therefore ubiquitous in pneumococci. The pneumococcal DLDH was most similar to that of S. pyogenes but, interestingly, more closely associated with DLDHs from intracellular parasites such as Chlamydia pneumoniae and Rickettsia prowazekii than to other Gram-positive bacteria, including the closely related Lactococcus lactis. These distinct groupings suggest that the pneumococcal DLDH, as well as the DLDH from S. pyogenes, have possibly evolved in a different manner from those of the other Gram-positive bacteria. Alternatively, it is possible that this locus has been acquired by the pneumococcus through horizontal gene transfer.

Mutation of the dldh gene resulted in loss of enzymatic activity in cell extracts, as well as loss of virulence in both septic and lung infection models in mice. Both these effects were specific to the disruption of the dldh gene, as downstream insertion mutants showed the wild-type phenotypes in both models. The D39 strain used for these studies is known to be highly virulent in CBA/N mice infected by the intravenous route. The fact that the dldh mutant strains were unable to kill mice even at a dose 100 times that of the LD$_{100}$ for D39 strongly indicates that the DLDH enzyme is critical for survival in the bloodstream of mice. When the respiratory route was used for infection, the DLDH-negative but not the wild-type bacteria were cleared from the lungs and were not present in blood.
by 48 h after infection. This indicated that the impairment caused by the DLDH mutation prevented pneumococcal survival in two very different host environments. It is important to note that this attenuation in virulence is not the result of an intrinsic growth deficiency, as the DLDH-negative bacteria and the wild-type bacteria grew equally well in broth.

It is not surprising that an impairment of steps in the metabolism of an organism would cause a decrease in its virulence. Mutations of two other metabolic enzymes of *S. pneumoniae*, the pyruvate oxidase and the NADH oxidase, were shown to cause less virulent phenotypes (Spellerberg *et al.*, 1996; Auzat *et al.*, 1999). For the pyruvate oxidase (SpxB), Spellerberg *et al.* (1996) found that a mutation of *spxB* led to a reduction of virulence in both pneumonia and sepsis models in mice. The decreased virulence was attributed to a decrease in acetyl phosphate levels, resulting in downregulation of adhesive proteins.

For NADH oxidase, Auzat *et al.* (1999) showed that mutation of the gene resulted in decreased virulence when bacteria were injected intraperitoneally. The attenuation of virulence was thought to involve the change in the NADH/NAD⁺ ratio or the increased sensitivity to oxidative stress. The involvement of these enzyme systems in virulence suggests that the *in vivo* metabolism of the pneumococcus is more complicated than the homolactate fermentation seen in broth-grown cultures (Hewitt, 1932; Kandler, 1983).

As the DLDH enzyme is usually part of 2-oxo acid dehydrogenase complexes (Wieland, 1983; Dietrichs and Andreesen, 1990; Kruger *et al.*, 1994; Oppermann and Steinbuchel, 1994; Berg and de Kok, 1997; de Kok *et al.*, 1998; Aevarsson *et al.*, 1999; Huang *et al.*, 1999), it would not be surprising if impairment of these enzyme systems affected the nutritional fitness of the organism in their environmental niche, thus reducing virulence. Our study, however, indicated that the bacteria lacked all known 2-oxo acid dehydrogenase systems. Even though a lack of measurable activity does not prove that such enzyme systems are not expressed under conditions other than those addressed in this study, our results still suggest that this activity is not expressed as readily as in other organisms, including *E. coli*. A lack of enzyme activity was also suggested based on sequence analysis of the components of the putative 2-oxo acid dehydrogenase complex operon in the TIGR4 genome. This analysis indicated that the E2 (acyetyltransferase) component lacked the N-terminal lipoyl domain, which would make this component and therefore the enzyme complex non-functional. Even though a previous report has suggested that *S. pneumoniae* lack pyruvate dehydrogenase activity (Spellerberg *et al.*, 1996), this is the first report that indicates that pneumococci lack all known functional 2-oxo acid dehydrogenase complexes, thus proposing that the pneumococcal DLDH might have an alternative function in this organism.

The DLDH-negative pneumococci showed an impaired ability to use galactose and α-galactoside-based sugars (raffinose, melibiose and stachyose) as substrates for oxidation and as carbon sources for growth. This effect was not seen for the utilization of the β-galactoside lactose as well as a number of other carbon sources. In accordance with earlier studies (Rosenow *et al.*, 1999), neither wild-type nor DLDH-negative bacteria could grow on melibiose as a sole carbon source. However, the wild-type strains and, to a lesser extent, the mutant strains could still oxidize melibiose. The reason for this discrepancy is not known.

The α-galactosidase (*Aga*) in *S. pneumoniae* was recently identified by Rosenow *et al.* (1999) as part of the raffinose utilization system, and mutations of the *aga* gene resulted in loss of fermentation of raffinose. Wild-type bacteria grown in THY medium displayed an α-galactosidase activity in the same range as that seen by Rosenow *et al.* (1999). In the present study, the DLDH-negative bacteria, on the other hand, showed approximately 90% reduction in α-galactosidase activity. This decrease in α-galactosidase activity explains why the DLDH-negative bacteria do not grow well on melibiose, raffinose or stachyose, which all require cleavage into sucrose/glucose and galactose for efficient fermentation. The expression of α-galactosidase is known to be upregulated in the presence of raffinose and downregulated by sucrose, which is a product of α-galactosidase activity on raffinose and stachyose (Rosenow *et al.*, 1999). Our data suggest that DLDH is also involved in the regulation of this system, either directly or indirectly. This regulation will be addressed in a future study.

The decrease in α-galactosidase activity does not, however, explain why the bacteria failed to grow with galactose as sole carbon source. Galactose does not require any processing to be transported or used by the metabolic machinery of the cells. The lack of galactose utilization was therefore studied by assessing the transport of galactose into the cells. Wild-type bacteria accumulated galactose over time, with saturating transport attained after 5 min, whereas the DLDH mutant bacteria showed almost no accumulation of galactose. The transport of glucose, which uses separate transport pathways from galactose (Tettelin *et al.*, 2001), occurred irrespective of the presence of DLDH.

The galactose transport system of *S. pneumoniae* has so far not been studied, and no information on its regulation is available. The annotated transport system for galactose in *S. pneumoniae* (Tettelin *et al.*, 2001) is highly homologous (data not shown) to the binding protein-dependent galactose transport system in *E. coli*, studied by Richarme’s laboratory (Richarme and Heine, 1986;
Richarme, 1987; 1989). In *E. coli*, it was shown that the presence of dihydrolipoic acid in the culture medium increased the transport of maltose and galactose through the *E. coli* membrane and that DLDH activity was important for functional transport (Richarme and Heine, 1986). It was also shown that one of two DLDH enzymes in *E. coli* co-purified with one of the components of the galactose transport system (Richarme, 1989). The authors speculated that this interaction might indicate a direct regulation of DLDH on the transport. Our studies indicate a similar role for DLDH in the transport of galactose into *S. pneumoniae*. In the case of the pneumococcal DLDH, the enzyme has the dihydrolipoic acid already attached to its N-terminal lipoyl domain. This arrangement may lead to a more efficient regulation of transport, as both the regulatory components (the dihydrolipoic acid and the DLDH enzyme) are present within the same entity. The regulation of carbohydrate transport and metabolism may be the reason why some species have obtained lipoyl domains in their DLDH sequences. Richarme and Heine (1986) also showed evidence that the homologous binding protein-dependent maltose transport system was influenced by dihydrolipoic acid and DLDH. This was not addressed in this study, as DLDH-negative strains grew equally well on maltose and maltotriose as the wild-type bacteria. It is still conceivable that the equivalent transport system in *S. pneumoniae* is also regulated by DLDH, even though the bacteria efficiently ferment the carbohydrate. Maltose may be transported into the cell via other transport mechanisms or may be cleaved extracellularly before transport.

The impairment of α-galactoside breakdown and galactose uptake resulting from DLDH mutation could be important during infection, either through the requirement of this carbon source for survival in the *in vivo* environment or by affecting other systems more directly involved in virulence, as seen for pyruvate oxidase (Spellerberg *et al.*, 1996).

Besides their effect on α-galactoside metabolism, the DLDH-negative bacteria also displayed reduced capsule production, with about 50% of wild-type capsule levels. The capsule is an important factor for bacterial survival in the blood, as *S. pneumoniae* lacking capsule are readily cleared from the blood of infected mice (Avery and Dubose, 1931; Magee and Yother, 2001). The type 2 capsule does not contain any galactose-derived sugars, indicating that the reduction in capsule production is probably not directly related to the impairment of α-galactoside and galactose metabolism. Still, as the production of capsular material takes up much of the carbohydrate utilization of the cell, a shift in metabolism may very well have secondary effects on capsule production.

It was reported that a type 3 pneumococcal strain producing only 20% of the wild-type capsule still remained highly virulent in both intraperitoneal and intravenous mouse infection models. If the result for the type 3 strain also holds true for the type 2 strain used here, then this suggests that the 50% reduction of capsule in the DLDH-negative cells would not be expected to account for the loss of virulence. Still, the causes of decreased capsule levels are different. In the study by Magee and Yother (2001), the capsule production was targeted specifically, whereas in this study, the reduced capsule production resulted from a mutation unrelated to the capsule genes. Therefore, a reduction in capsule production from DLDH mutations may be more serious for the organism than if DLDH is intact. This needs to be addressed in future studies.

Further investigations will be necessary to define better the relationships between the DLDH enzyme and its effects on virulence, and to determine how the DLDH regulates α-galactosidase expression and galactose transport and metabolism. Gaining a more accurate understanding of basic bacterial metabolism, as it functions in the environment of the host, may lead to alternative therapeutic methods for the treatment of pneumococcal disease in the future.

**Experimental procedures**

**Reagents**

Protein markers were from Amersham Pharmacia Biotech. NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) were from Fisher Scientific. Alkaline phosphatase-conjugated streptavidin, biotin-conjugated goat anti-mouse and goat anti-human antibodies were from Southern Biotechnology Associates. Bacterial growth media were from Difco Laboratories. Lactated Ringer’s solution was from Abbott Industries. Acetoin was from Aldrich. PCR reagents and restriction endonucleases were from Fisher Promega. All other reagents were from Sigma Chemicals.

Dihydrolipoamide was produced through reduction of lipoamide with sodium borohydride as described previously (Reed *et al.*, 1958).

Serum from a patient with primary biliary cirrhosis (anti-PBC serum), was kindly provided by Dr J. Palmer (University of Newcastle, School of Biochemistry and Genetics, Newcastle upon Tyne, UK).

**Bacteria**

The bacterial strains and plasmids used in this study are described in Table 5. The pneumococcal strains were stored at –80°C in 15% glycerol, transferred to blood agar plates (with or without the addition of 0.3 μg ml−1 erythromycin) and incubated at 37°C in a 5% CO2 atmosphere overnight. Colonies from blood agar were used to inoculate liquid growth medium [Todd–Hewitt medium containing 0.5% yeast extract (THY) or SH medium, containing 10 g l−1 bactopeptone, 40 g l−1 neopeptone, 5 g l−1 NaCl, 2 g l−1 K2HPO4, 5 g l−1 NaHCO3, 0.2 g l−1 thioglycolic acid and 20 mM of each
Dihydrolipoamide dehydrogenase in *S. pneumoniae* 443 carbon source; modified from O’Meara and Brown, 1936]. The bacteria were harvested by centrifugation at 1500 g for 15 min and suspended in appropriate buffers or media. The bacterial concentration was estimated by optical density at 600 nm (Ultrospec III spectrophotometer; Pharmacia-LKB) and confirmed by viable counts.

*Escherichia coli* M15 cells were grown on Luria agar, transferred to Luria–Bertani medium and grown to mid-logarithmic phase before being used to make bacterial extracts.

**Bacterial extracts**

The *E. coli* M15 cells and the pneumococcal strains were grown to mid-logarithmic phase in LB medium (*E. coli*) or THY medium (pneumococci) and pelleted. The bacteria were washed once in cold 50 mM potassium phosphate buffer, pH 8.0, and the pellet was resuspended in fresh 50 mM potassium phosphate buffer containing 1 mM EDTA. The bacteria were sonicated on ice using four pulses of 20 s each with a Sonic Dismembrator model 300 (Fisher), and the insoluble fraction was pelleted at 10,000 g for 20 min. The remaining supernatant was used for enzyme assays as described below. Protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad).

**Sequencing and sequence alignment of the dldh gene**

The *dldh* gene was sequenced from concentrated PCR products generated from the Dldh-seq-F and Dldh-seq-R primer pair (Table 6). The PCR was run with a 55 °C annealing temperature for 35 cycles. Automated sequencing reactions used dye terminator chemistry and were run on a

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Reference</th>
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</tr>
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<td>C08-R</td>
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<td>Dldh-seq-R</td>
<td>5’-TTGACTGAGGATGTCG-3’</td>
<td>This study</td>
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*a.* Lower case letters refer to restriction sites added to the primers. Upper case letters refer to actual genomic sequence.

model ABI Prism 377 sequencer (Applied Biosystems). Sequencing runs for the three different strains used four primers made in both sense and antisense directions (Table 6). Sequences were assembled and edited using the SEQUENCER software (GeneCodes). The sequences were submitted to GenBank and have accession numbers AY038330, AY038331, and AY038332.

Sequences from GenBank as well as the predicted amino acid sequence for the pneumococcal DLDH were aligned and analysed with MACVECTOR DNA sequence analysis software (Oxford Molecular). The protein alignments were generated by CLUSTALW algorithm using BLOSUM30 amino acid scoring matrix. The homology trees were generated using the nearest neighbour joining method with bootstrap values determined for 1000 resamplings of the data.

**Mutational inactivation of the dldh gene**

An internal fragment of the gene (nucleotides 512–990 of the ORF) was amplified using PCR with EcoRI and XbaI restriction sites added to the 5’ ends of the forward and reverse primers respectively (Table 6). Additionally, a fragment homologous to the intergenic region downstream of the gene (nucleotides 1144–1710 after the beginning of the ORF) was amplified using the C08P-F/C08P-R primer pair, and an internal fragment of the homologous sequence (C37; nucleotides 241–816 in the ORF) was amplified using the C37-F/C37-R primer pair. The PCR reactions were run in 35 cycles with a 62°C annealing temperature using S. pneumoniae D39 chromosomal DNA as the template. The reactions resulted in amplicons of the expected sizes. The fragments were digested with EcoRI and XbaI, ligated into the EcoRI–XbaI-digested plasmid pJY4164 (Yother et al., 1992) and electroporated (Electroporator 2510; Eppendorf Vertrieb Deutschland) into E. coli JM109. Erythromycin-resistant clones were screened by restriction digest for the appropriate size. Two independent clones harbouring each of the C08, C08P and C37 inserts were verified by sequencing. These clones (pSH0805 and pSH0832 for C08 dldh, pSH08P1 and pSH08P2 for the dldh intergenic region and pSH3701 and pSH3702 for the C37 dldh homologous ORF) were used for insertion–duplication mutagenesis.

**Transformation of S. pneumoniae**

Plasmid preparations were used to transform S. pneumoniae strains D39 and CP8056 essentially as described previously (Hardy et al., 2000). All strains were grown in competence media (THY with the addition of 0.2% BSA, 0.2% glucose and 0.02% CaCl₂, made fresh) until just turbid. Bacteria were then diluted 1:30 in fresh competence media, and 200 ng ml⁻¹ of competence stimulating peptide 1 (Kohoutova et al., 1968; Havarstein et al., 1995) was added together with 100 ng of plasmid DNA. The bacteria were grown at 37°C for an additional 2h, after which the culture was plated on blood agar containing 0.3 μg ml⁻¹ erythromycin. Resistant transformants were saved and analysed for insertional mutagenesis of the genes.

To verify that plasmid had crossed over and become inserted into the correct places in the chromosome, bacterial DNA from each of the S. pneumoniae transformants was used as a template for a PCR. The primer pairs for the PCR included verification primers located upstream of the site of each insertion (C08-ver or C37-ver; Table 3) and a primer corresponding to the inserted plasmid pJY4164 (Cat-1). The amplified PCR products from each of the transformants were sequenced to ensure that the plasmid insertions had occurred at the correct site. The mutations were also verified by Southern blot analysis. Once confirmed, two independent mutants for the C08 (C0805 and C0832) and C37 (C3701 and C3702) sequences in the D39 and CP8056 background and of the downstream insertion (C08P1 and C08P2) in the CP8056 background were used for further assays (Table 5).

**Infection models**

Six- to 8-week-old female CBA/N mice (Jackson Laboratory) were injected intravenously through the tail vein with 100 μl of D39 wild-type (300 cfu) or mutant bacteria (300–300 000 cfu). Mice were bled after 24 h to monitor the infection, and survival was recorded to assess virulence.

For local lung infection, CBA/N mice were anaesthetized with Metofane (Pitman-Moore), and 40 μl of bacterial suspension (1–3 × 10⁶ cfu) was instilled through the nose resulting in aspiration into the lungs. Blood from the mice was collected retroorbital 48 h after infection, and dilutions were plated for viable counts to determine the bacterial load in the blood. After bleeding, the mice were sacrificed, and both lungs were excised, homogenized in 1 ml of lactated Ringer’s solution and plated for viable counts to assess the amount of bacteria present in the lungs. Additionally, in a parallel lung infection experiment, the mice were followed to record days until death.

**Enzyme assays**

DLDH activity was measured as the oxidation of NADH in the presence of 6,8-thioctic acid amide (lipoamide) or the reduction of NAD⁺ in the presence of dihydrolipoamide, visualized as decreased absorbance at 365 nm or increased absorbance at 340 nm, respectively (Dietrichs and Andreessen, 1990; Engels et al., 1997). Briefly, enzyme source was added to 50 mM potassium phosphate buffer containing 1 mM EDTA, 0.4 mM NADH or NAD⁺ with and without 1.5 mM LA or DLA in a 1 ml final volume. Enzymatic activity was defined as the oxidation of NADH or reduction of NAD⁺ in μmol min⁻¹ at 25°C. Calculations were performed using the extinction coefficients for NADH at 265 nm (3.29 mM⁻¹ cm⁻¹) and 340 nm (6.22 mM⁻¹ cm⁻¹).

DLDH activity was also detected in a native gel assay. Enzyme sources were run on 10% polyacrylamide gels (Bio-Rad ready gels) under native conditions, and the gel was then incubated at 37°C in 50 mM potassium phosphate buffer containing 1 mM EDTA, 1 mM NADH and 0.75 mg ml⁻¹ NBT as an electron acceptor. Activity was detected as purple-coloured protein bands visualized in the gel.

The activities of the 2-oxo acid dehydrogenases were
measured according to the method of Hinman and Blass (1981). Briefly, enzyme source was added to 50 mM potassium phosphate buffer (pH 8.0) containing 0.1 mM coenzyme A, 0.3 mM dithiothreitol (DTT), 0.2 mM thiamine pyrophosphate, 2.5 mM NAD\(^+\), 0.6 mM \(\text{p-iodonitrotetrazolium violet}\) (INT) and 5 mM pyruvate (pyruvate dehydrogenase complex activity), 5 mM acetoin (acetoin dehydrogenase activity) or 5 mM 2-keto-isovalerate (branch-chain 2-oxo acid dehydrogenase activity). The reduction of NAD\(^+\) was measured by the change in INT absorbance at 500 nm. Activity of the enzyme complex was determined as the reduction of INT in \(\mu\text{mol min}^{-1}\) at 25°C using the extinction coefficient for INT of 12.4 \(\text{mM}^{-1} \text{cm}^{-1}\) at 500 nm.

The activity of the E1 dehydrogenase component was measured according to the method of Schwartz and Reed (1970). Briefly, enzyme source was added to 20 mM Tris-HCl, pH 7.8, 4 mM MgCl\(_2\), 0.1 mM thiamine pyrophosphate, 1.8 mM potassium ferricyanide and 5 mM of pyruvate, acetoin or 2-keto-isovalerate. Reduction of ferricyanide was monitored at 420 nm, and activity was measured based on the extinction coefficient for ferricyanide of 1.04 \(\text{mM}^{-1} \text{cm}^{-1}\) at 420 nm.

Acetyltransferase (E2) activity was measured according to the method of Reed and Wills (1966) and is based on the colorimetric determination of \(\text{S-acetylthiolipolipoamide}\) within a ferric acetyhydrolase complex. Briefly, enzyme source was added to 100 mM Tris-HCl buffer (pH 6.8) containing 0.1 mM coenzyme A, 10 mM acetyl phosphate, 10 mM DLA and 5 units \(\text{ml}^{-1}\) phosphotransacetylase to a final volume of 200 \(\mu\)l. The tubes were incubated for 30 min at room temperature, the reaction mixture was stopped by the addition of 0.1 N HCl, and the mixture was then boiled for 10 min to destroy any unreacted acetyl phosphate. After boiling, 200 \(\mu\)l of 0.1 M potassium citrate (pH 5.4) and 200 \(\mu\)l of 2 M hydroxylamine-NaOH solution (pH 6.4) were added, and the solution was allowed to stand for 10 min at room temperature. Finally, 600 \(\mu\)l of ferric chloride solution (equal volumes of 3 N HCl, 12% trichloroacetic acid and 5% FeCl\(_3\) in 0.1 N HCl) was added, the sample was centrifuged, and the optical density of the supernatant was determined at 540 nm. The acetyltransferase activity was measured in optical density units using a buffer-only control reaction to assess background absorbance.

**Western blot**

Enzyme sources (1–10 \(\mu\)g) were run on 10% polyacrylamide gels (Bio-Rad) under denaturing conditions, and the gels were electrophoretically transferred to a 0.45 \(\mu\)m nitrocellulose membrane (Bio-Rad) in Tris–glycine buffer (20% methanol, 25 mM Tris and 192 mM glycine, pH 8.1–8.4) at 100 V for 1 h. Blotted membranes were blocked in PBS containing 0.05% Tween-20 (PBS-T) with 1% BSA for 45 min at room temperature and washed three times (5 min each) with PBS-T. The membrane was overlaid with serum from a patient with PBC (1:2000 dilution in PBS-T) for 1 h at room temperature and washed three times in PBS-T. The membrane was incubated further with a mix of biotinylated goat anti-human antibodies (1:1000 in PBS-T) and alkaline phosphatase-conjugated streptavidin (1:500 dilution in PBS-T) for 1 h at room temperature. After washing, the membrane was developed using 0.1 mg ml\(^{-1}\) NBT and 0.5 mg ml\(^{-1}\) BCIP in 0.15 M Tris-HCl, pH 8.8.

**Carbon utilization**

Carbon source utilization of *S. pneumoniae* strains was first measured as the oxidation of different carbon sources in GP2 Microplates (Biolog). For a more detailed examination, a similar assay was developed. Briefly, 10 \(\mu\)l of 6 mM INT and 10 \(\mu\)l of a 0.5 M carbon source were added to each well of a microtitre plate. Bacterial suspension (80 \(\mu\)l in PBS of bacteria grown to an OD\(_{600}\) of 0.4) was added, the plate was incubated at 37°C, and the optical density was measured after 2 h and 24 h at 550 nm. All samples were compared with a control well without any carbon source added. Carbon utilization was considered positive if the optical density exceeded the non-carbon control by 0.1 OD units.

In addition to the oxidation of carbon sources, wild-type and DLDH mutant strains were also tested for growth on specific carbon sources. Bacteria grown in THY medium were washed and resuspended in SH medium and used to inoculate SH media containing different individual carbon sources at a concentration of 20 mM. The OD\(_{600}\) was determined after 24, 48 and 72 h as a measure of growth and compared with an inoculated control tube of SH media containing no carbon source. No growth was ever detected in the no carbon source control.

**\(\alpha\)-Galactosidase activity**

\(\alpha\)-Galactosidase activity was measured essentially as described by Rosenow et al. (1999). Cells were grown in THY medium to an OD\(_{600}\) of 0.5. The cells were pelleted and resuspended in 0.1 M potassium phosphate buffer (pH 7.5) containing 0.25% Triton X-100 and incubated at room temperature until lysed. Twenty microtitre plates of extract was added to 980 \(\mu\)l of potassium phosphate buffer containing 1 mM MgCl\(_2\), 4.5 mM \(\beta\)-mercaptoethanol and 50 \(\mu\)g of \(p\)-nitrophenyl-\(\alpha\)-D-galactopyranoside. Activity was followed as an increased absorbance at 405 nm from the release of \(p\)-nitrophenol after cleavage of the substrate. Enzymatic activity in units was defined as the accumulation of \(p\)-nitrophenol in \(\mu\)mol min\(^{-1}\) at 25°C. Calculations of activity were performed using the extinction coefficient for \(p\)-nitrophenol at 405 nm (18.4 \(\text{mM}^{-1} \text{cm}^{-1}\)). Protein concentrations were determined using the Bio-Rad DC protein assay.

**Galactose and glucose transport measurements**

For transport experiments, bacteria were grown in THY medium to an OD\(_{600}\) of 0.5. The bacteria were pelleted and washed three times in PBS and resuspended in PBS to the original volume. The transport of galactose was measured according to the method of Richarme and Heine (1986). Transport of glucose was determined for comparison. Bacteria (500 \(\mu\)l) were mixed with \(^{14}\text{C}\)-labelled galactose (55 Ci mol\(^{-1}\)) or glucose (258 Ci mol\(^{-1}\)) at concentrations of 2 \(\mu\)M and 0.4 \(\mu\)M respectively. After various times, the mixture was filtered through a cellulose ester filter (HAWP, 0.45 \(\mu\)m; Millipore), washed with 3 ml of PBS, and the filter was assayed for radioactivity. For each time point, heat-killed bacteria served as a control for background association of the radiolabelled carbohydrate with the bacteria. The carbohydrate transport is presented as the association of
$[^{14}C]$-galactose or -glucose with the bacteria in nmol/10$^9$ bacteria after subtracting the results for heat-killed bacteria.

Capsule production
Quantification of capsule was performed by ELISA according to the method of Bender and Yother (2001). Briefly, pneumococci were grown to an OD$\text{$_{600}$}$ of 0.5, washed once and resuspended in PBS and heat killed at 56°C. Once cooled, the OD was adjusted to 0.2 with PBS, and 100$\mu$l of the heat-killed cells was added to a twofold dilution series to ELISA plates and left at 4°C overnight. After washing the plates three times with PBS-T, a capsule type 2-specific rabbit anti-serum (Statens Serum Institute, Copenhagen, Denmark) was added as the primary antibody (1:10 000 dilution in PBS). After 1 h of incubation at room temperature, the plates were washed three times with PBS-T, and alkaline phosphatase-conjugated goat anti-rabbit antibodies (1:1000 in PBS-T) were added for 1 h. The plates were washed and developed using $p$-nitrophenyl phosphate substrate, and the absorbance was read at 405 nm in a Multiskan MS microtitre plate reader (Labsystems). Results are expressed as the absorbance was read at 405 nm in a Multiskan MS microtitre plate reader (Labsystems). Results are expressed as the percentage of capsule produced compared with that of the wild-type pneumococcal strain after subtracting for the percentage of capsule produced compared with that of the wild-type pneumococcal strain after subtracting for the absorbance of the unencapsulated D39 derivative R36A.

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