Influence of the \textit{spxB} Gene on Competence in \textit{Streptococcus pneumoniae}^\textsuperscript{\textregistered}

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In \textit{Streptococcus pneumoniae} expression of pyruvate oxidase (SpxB) peaks during the early growth phase, coincident with the time of natural competence. This study investigated whether SpxB influences parameters of competence, such as spontaneous transformation frequency, expression of competence genes, and DNA release. Knockout of the \textit{spxB} gene in strain D39 abolished spontaneous transformation (compared to a frequency of \(6.3 \times 10^{-6}\) in the parent strain [\(P < 0.01\)]). It also reduced expression levels of \textit{comC} and \textit{recA} as well as DNA release from bacterial cells significantly during the early growth phase, coincident with the time of spontaneous competence in the parent strain. In the \textit{spxB} mutant, supplementation with competence-stimulating peptide 1 (CSP-1) restored transformation (rate, \(1.8 \times 10^{-5}\)). This speaks against the role of SpxB as a necessary source of energy for competence. Neither supplementation with CSP-1 nor supplementation with the SpxB products H2O2 and acetate altered DNA release. Supplementation of the parent strain with catalase did not reduce DNA release significantly. In conclusion, the pneumococcal \textit{spxB} gene influences competence; however, the mechanism remains elusive.

The human nasopharynx is the natural habitat of \textit{Streptococcus pneumoniae}. Colonization is the prerequisite for invasion, transmission, and genetic evolution of pneumococci. An increasing number of adherence factors that support colonization are being discovered (13). In addition, production of hydrogen peroxide (H2O2) is thought to inhibit competitive nasopharyngeal flora (23). In the pneumococcus, H2O2 is produced under rich and aerobic conditions by the enzyme pyruvate decarboxylates pyruvate to acetyl phosphate plus CO2 (24). Spellerberg et al. (29) showed that an \textit{spxB}-deficient mutant exhibits reduced virulence for nasopharyngeal colonization, pneumonia, and sepsis. Expression of SpxB peaks during the early growth phase (18). This coincides with the time of pneumococcal competence.

\textit{S. pneumoniae} shares with at least 40 bacterial species the property of natural transformation (19). The importance of transformation for genetic evolution has been illustrated by the emergence of penicillin-resistant pneumococcal isolates and pneumococcal strains undergoing capsule switch (8, 9, 22). In \textit{S. pneumoniae}, competence is induced by the competence-stimulating peptide (CSP) (7). Induction of competence-specific (\textit{com}) genes leads to DNA uptake and processing. Competence also triggers cell lysis and DNA release from a fraction of bacterial cells.

This study investigated whether the \textit{spxB} gene or the products of \textit{spxB} have any influence on competence in \textit{S. pneumoniae}. It is shown that in strain D39, deletion of \textit{spxB} abolished spontaneous transformation, reduced expression levels of an early competence gene (\textit{comC}) and a late competence gene (\textit{recA}), and reduced competence-associated DNA release. There was, however, no evidence for a role of the products of \textit{spxB}, i.e., H2O2 and acetyl phosphate. The mechanisms connecting \textit{spxB} gene with competence remain to be unraveled.

**MATERIALS AND METHODS**

**Bacterial strains and culture.** \textit{Streptococcus pneumoniae} strain D39 (serotype 2) (2) was kindly provided by Jeffrey Weiser (University of Pennsylvania, Philadelphia, PA) and strain R6 (a spontaneous nonencapsulated derivative of D39) by Philippe Moreillon (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland).

Bacteria were grown on Columbia sheep blood agar (CSBA) plates at 37°C in a 5% CO2-enriched atmosphere. Liquid culture was performed in brain heart infusion (BHI) broth, pH 7.4 to 7.5 (Becton Dickinson and Company, le Pont de Claix, France), containing 5% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany) in a water bath at 37°C without shaking. Transformation experiments were performed either in BHI broth supplemented with 5% FCS or in TSB competence medium (pH 8) (27). Where stated, 100 ng/ml CSP-1 (NeoMPS S.A., Strasbourg, France), 0.1% sodium acetate (Merck, Darmstadt, Germany), or 5,000 U/ml catalase (Sigma-Aldrich, Buchs, Switzerland) was added to the BHI broth with 5% FCS. Bacteria were stored at −80°C using Protex bacterial preservers (TSC, Heywood, United Kingdom). Optical density at 600 nm (\textit{OD}_{600}) was measured using a Perkin-Elmer Lambda-2 spectrometer (Perkin-Elmer [Schweiz] AG, Schwerzenbach, Switzerland).

**Construction of the D39 \textit{spxB} mutant.** Transformation of \textit{Escherichia coli} and \textit{S. pneumoniae} was performed as described previously (14, 20). A 1.3-kb fragment (\textit{spxB}_{S2_F}, 5′-GAATTTGATGGCGCTCAAC-3′; \textit{spxB}_{S1_B}, 5′-CGCGTTTGTGAAGTCTACACC-3′) of \textit{spxB} was amplified and cloned into pGEM-T Easy Vector (Promega, Wallisellen, Switzerland). An erythromycin cassette (\textit{ermB}) (5) was inserted at the HindIII restriction site of the amplified \textit{spxB} fragment. Strain D39 Smr was transformed with the whole plasmid, and recombinants were selected on CSBA plates containing erythromycin (2 μg/ml). Knockout of \textit{spxB} was confirmed by PCR and phenotypically by a 4-fold-decreased \textit{OD}_{600} release per cell and a 10-fold-decreased \textit{H2O2} concentration in the supernatant at an \textit{OD}_{600} of 0.75 compared to the wild type (data not shown).

**Assay for transformation frequency.** Transformations were performed as described below with the following adaptations. Rifampin-susceptible strain D39 Smr or its \textit{spxB}-deficient mutant were grown in BHI broth with 5% FCS to an \textit{OD}_{600} of 0.05, 0.15, 0.25, 0.35, or 0.45 and transformed with a total of 1 μg DNA consisting of the \textit{spxB} rifampin resistance (\textit{Rf}) gene (20). Transformation was performed with or without addition of CSP-1; 100 μl of culture was spread on CSBA plates containing 0.5 μg/ml rifampin. Transformants were counted after 48 h. Total cell counts were obtained by plating serial dilutions of culture onto CSBA plates.

DNA isolation for reverse transcription-PCR (RT-PCR). Bacteria were prepared as previously described (15), transferred to a 1.5-mL tube containing 0.05 g
of 100-μm acid-washed glass beads (Sigma-Aldrich, Buchs, Switzerland), and vibrated for 10 min at half-maximum speed using a Mickle vibratory tissue disintegrator (Mickle Laboratory Engineering, Gomshall, United Kingdom). The mixture was then centrifuged and RNA extracted from the supernatant using the Qiagen RNeasy minkit (Qiagen AG, Hombrechtikon, Switzerland) according to the manufacturer’s instructions. The RNA recovered was treated with DNase I (Stratagene Europe, Amsterdam, Netherlands) according to the manufacturer’s instructions to remove any contaminating DNA. RNA concentration and purity were determined by measuring absorbance at both 260 nm and 280 nm (Lambda-2 spectrometer; Perkin-Elmer [Schweiz], Schwerzenbach, Switzerland).

Quantitative gene expression using a cRNA standard curve. Quantitation of absolute mRNA copy numbers by real-time RT-PCR was performed by using a standard curve generated based on in vitro-transcribed RNA (cRNA) as previously described (12, 16). For the in vitro transcription of the spxB, comC, and recA genes, the following primers were used: spxB_F1/T1 (5′-TAATACGACTCACTATAGGGAGAGTACGTCACAT-3′ and 5′-GGATCTTAAAAAGTCTCCTGATTG-3′), comC_Start1/T1 (5′-TAATACGACTCACTATAGGGAGAGTACGTCACAT-3′ and 5′-GTCCCAAATCCAAATAAATCCAT-3′), and recA_Start1/T1 (5′-TAATACGACTCACTATAGGGAGAGTACGTCACAT-3′ and 5′-GAATAAAXACTCCTGAAAATGT-3′), respectively. Each cDNA mixture was then centrifuged and RNA extracted from the supernatant using the Qiagen RNeasy minikit (Qiagen AG, Hombrechtikon, Switzerland) according to the supplier’s protocol. For the cultures of each OD<sub>600</sub> from which we extracted RNA, serial dilutions were spread onto CSBA plates to determine the cell count (CFU).

Quantification of gene expression was achieved by real-time RT-PCR using TaqMan primers and probes created by the Assay-by-Design service of Applied Biosystems (Rotkreuz, Switzerland) based on the most conserved regions of the spxB, comC, and recA genes in <i>S. pneumoniae</i> strains TIGR4 (AE005672), R6 (AE007317, NC003098), D39 (NC008533, AY254852), and AB15 (AY254854) and was amplified (with primers hexA_f11 [5′-AAGGAGAAATATGCGATAGAAA-3′] and hexA_b2641 [5′-ATAGACAAAGGGGAGCAGATG-3′]) and cloned into pGEM-T Easy vector (Promega, Wallisellen, Switzerland). A 1,100-bp fragment of hexA was cut out with HindIII and was replaced with an erythromycin cassette (ermB of pJDC9). <i>S. pneumoniae</i> strain R6 was transformed with the whole plasmid, and recombinants were selected on CSBA plates containing erythromycin (2 μg/ml). Positive recombinants were confirmed by Southern blotting, PCR, and an increased mutation rate to rifampin resistance (data not shown).

An overnight culture of strain D39 Smr or its spxB mutant was prepared with 3 to 10 colonies in 5 ml BHI broth containing 5% FCS. One hundred microliters of culture was subcultured in 5 ml BHI broth with 5% FCS and grown to mid-log phase (OD<sub>600</sub>, 0.5). Two milliliters of culture was then pelleted at 2,500 × g and 4°C for 10 min and washed twice with 5 ml Hanks medium to remove extracellular DNA. The pellet was diluted 100-fold in BHI broth with 5% FCS, and 20 ml was incubated at 37°C.

Samples of 0.2 ml were taken at OD<sub>600</sub> of 0.05, 0.15, 0.25, 0.35, 0.45, 0.55, or 0.65 and were reversed transcribed to cDNA using Superscript II (Amersham, Buckinghamshire, United Kingdom) and random hexamer primers (Promega, Wallisellen, Switzerland) according to the supplier’s protocol. The cultured cells were incubated for 15 min. CSP-1 (Neosystems, Strasbourg, France) was added to a final concentration of 0.2 U/ml and incubated for 15 min at 37°C. Aliquots of 750 μl were withdrawn at different OD<sub>600</sub> phases. H<sub>2</sub>O<sub>2</sub> concentration was determined by direct measurement using the Amplex Red hydrogen peroxide/peroxidase assay kit (Molecular Probes, Eugene, OR). Fifty microliters of bacterial culture was applied to a 96-well plate (Nunclon Nalge Nunc, Roskilde, Denmark), and 50 μl Hanks medium containing 0.2 U/ml (nonencapsulated <i>S. pneumoniae</i>) was amplified (with primers hexA_f11 [5′-AAGGAGAAATATGCGATAGAAA-3′] and hexA_b2641 [5′-ATAGACAAAGGGGAGCAGATG-3′]) and cloned into pGEM-T Easy vector (Promega, Wallisellen, Switzerland). A 1,100-bp fragment of hexA was cut out with HindIII and was replaced with an erythromycin cassette (ermB of pJDC9). <i>S. pneumoniae</i> strain R6 was transformed with the whole plasmid, and recombinants were selected on CSBA plates containing erythromycin (2 μg/ml). Positive recombinants were confirmed by Southern blotting, PCR, and an increased mutation rate to rifampin resistance (data not shown).

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Samples of 0.2 ml were taken at OD<sub>600</sub> of 0.05, 0.15, 0.25, 0.35, 0.45, 0.55, and 0.75. Cells were removed by filtration and centrifugation for 60 s using Micrope-EZ enzyme removers (Millipore AG, Volketswil, Switzerland). To maximize DNA recovery, 50 μl 50 μl Tris-EDTA buffer (pH 8.0) was added to the Micrope-EZ reservoir and spun for 30 s. For each experiment, three independent measurements were performed on different days.

To delete hexA in R6, a 2.6-kb fragment of the hexA gene of strain 108.21 (nonencapsulated <i>S. pneumoniae</i>) was amplified (with primers hexA_f11 [5′-AAGGAGAAATATGCGATAGAAA-3′] and hexA_b2641 [5′-ATAGACAAAGGGGAGCAGATG-3′]) and cloned into pGEM-T Easy vector (Promega, Wallisellen, Switzerland). A 1,100-bp fragment of hexA was cut out with HindIII and was replaced with an erythromycin cassette (ermB of pJDC9). <i>S. pneumoniae</i> strain R6 was transformed with the whole plasmid, and recombinants were selected on CSBA plates containing erythromycin (2 μg/ml). Positive recombinants were confirmed by Southern blotting, PCR, and an increased mutation rate to rifampin resistance (data not shown).

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horseradish peroxidase and 100 µM Amplex Red reagent were added. The absorbance was read at a wavelength of 563 nm (SpectraMax GeminiXS; Molecular Devices, Sunnyvale, CA). Concentrations were determined based on a standard. The concentrations of H₂O₂ in the supernatant of D39 Sm r at OD₆₀₀s of 0.005, 0.05, 0.15, 0.25, 0.45, and 0.75 were 1.5, 150, 270, 320, 525, and 480 µM, respectively.

To mimic H₂O₂ concentrations in the growth medium, the spxB-deficient mutant was grown in 20 ml BHI broth with 5% FCS. At the start, 0.05 µmol H₂O₂ (Merck, Darmstadt, Germany) was added to reach an initial concentration of 2.5 µM. Thereafter, H₂O₂ was added at intervals of 10 min for a total of 300 min. The amount of H₂O₂ added was incrementally increased by 0.05 µmol for each subsequent addition.

**Statistical analyses.** Statistical analyses were done in StatView version 5.0 (SAS Institute Inc., Cary, NC). Proportions were compared with the chi square test or Fisher’s exact test, and mean differences were assessed by Student’s t test. A cutoff P value of 0.05 (two tailed) was used for all statistical analyses.

**RESULTS**

**Kinetics of spxB gene expression.** Figure 1 confirms that transcription levels of the spxB gene in strain D39 were highest at an OD₆₀₀ of 0.150 (P < 0.05 compared to values at all other ODs).

**Influence of spxB gene on transformation frequency.** The frequencies of spontaneous transformation in the parent strain D39 Smr and its spxB-deficient mutant were compared at different ODs (Fig. 2 and 3A and B). No transformants were obtained for the spxB mutant at OD₆₀₀s of 0.05, 0.15, 0.25, and 0.45. A few transformants were obtained at an OD₆₀₀ of 0.35, for a mean transformation frequency of 4.9 × 10⁻⁶. Spontaneous transformation in the parent strain was highest at an OD₆₀₀ of 0.150.

**FIG. 2.** Transformation frequency of strain D39 Smr and its spxB mutant at an OD₆₀₀ of 0.15. Strain D39 Smr and its spxB mutant were grown to an OD₆₀₀ of 0.15 in BHI broth with 5% FCS. The transformation frequency was measured in TSB competence 8.0 medium with and without the addition of CSP-1, and the number of Rifr transformants per CFU was calculated. Mean values of triplicates from three independent experiments (±SE) are presented.

**FIG. 3.** comC and recA gene expression and spontaneous transformation frequencies of strain D39 Smr and its spxB mutant during the time of competence. Strain D39 Smr and its spxB mutant were grown to OD₆₀₀ of 0.05 to 0.45 in BHI broth with 5% FCS. (A and B) Left Y axes, the comC (A) and recA (B) gene transcription levels (gray and white bars) were determined by real-time RT-PCR and expressed as the copy number per 100 CFU in strain D39 Smr compared to its spxB-deficient mutant at each OD. Mean values of triplicates from three independent experiments (±SE) are presented. *, P < 0.05; the P values were calculated by comparing the comC or recA gene expression in D39 Smr with the comC or recA gene expression in D39 Smr ΔspxB at each OD. Right y axes, the spontaneous transformation frequencies were measured in TSB competence 8.0 medium, and the number of Rifr transformants per CFU was calculated. Mean values of duplicates from two independent experiments (±SE) are presented. Gray squares and solid lines, D39 Smr; white squares and broken lines, D39 Smr spxB knockout mutant. (C) Growth curves in BHI broth supplemented with 5% FCS of strain D39 Smr and its spxB knockout mutant. (D) CFU per ml of each OD presented in panel C for strain D39 Smr and its spxB knockout mutant.
0.05 and decreased thereafter (OD$_{600}$ of 0.05, 5.9 $\times$ 10$^{-4}$ [standard error (SE), 2.3 $\times$ 10$^{-4}$; OD$_{600}$ of 0.45, 3.5 $\times$ 10$^{-5}$ [SE, 2.6 $\times$ 10$^{-5}$ at) (Fig. 3A and B).

We investigated the effect of CSP addition on the transformation frequency in the spxB mutant compared to its parent strain D39 Sm$^+$ at an OD$_{600}$ of 0.15 (Fig. 2). Addition of CSP-1 increased the transformation frequency in the spxB mutant significantly to 1.8 $\times$ 10$^{-2}$ (SE, 7 $\times$ 10$^{-2}$) (comparison between frequencies with or without CSP-1, $P = 0.025$). The comparison with the parent strain (mean transformation frequency, 2.3 $\times$ 10$^{-2}$; SE, 6 $\times$ 10$^{-2}$) no longer showed a significant difference ($P = 0.5$). In order to better understand at which stage spxB interfered with spontaneous transformation, expression of an early and a late competence gene was analyzed.

Expression of comC and recA genes in D39 Sm$^+$ and the spxB knockout mutant. In strain D39 Sm$^+$, transcription levels of comC (an early competence gene) and recA (a late competence gene) were highest between OD$_{600}$ of 0.05 and 0.15, which is consistent with spontaneous competence. Deletion of the spxB gene significantly reduced comC and recA expression at an OD$_{600}$ of 0.05 ($P = 0.004$ and $P = 0.040$, respectively) and at an OD$_{600}$ of 0.15 ($P = 0.028$ and $P = 0.033$, respectively) (Fig. 3A and B). Growth of the spxB mutant in BHI broth supplemented with 5% FCS was slightly delayed compared to that of its parent strain D39 Sm$^+$ and required 30 to 45 min longer to reach an OD$_{600}$ of 0.75 (Fig. 3C).

Influence of the spxB gene on DNA release. DNA release has been shown to be associated with competence (21). Therefore, we investigated whether deletion of spxB also influenced DNA release.

**FIG. 4.** DNA release of strain D39 Sm$^+$ and its spxB mutant during the lag and log phases. Bacteria were grown to different ODs in BHI broth with 5% FCS. DNA release into the supernatant was measured with and without the addition of CSP-1 by real-time PCR (A) or as the number of transformants as described by Moscoso and Claverys (21) (B). (A) The DNA quantity in the supernatant is expressed as the fold difference between DNA in the supernatant and that for D39 Sm$^+$ without the addition of CSP-1 ($\pm$SE). (B) Values for DNA release represent streptomycin-resistant transformants per ml. Mean values of duplicates from three independent experiments ($\pm$SE) are presented. * and **, $P < 0.05$; the $P$ values were calculated by comparing the DNA release of D39 Sm$^+$ with the DNA release of D39 Sm$^+$ΔspxB at each OD without (*) or with (**) the addition of CSP-1.
release. Amounts of DNA in the supernatants of D39 Sm

strain and its spxB mutant were determined by real-time PCR at OD_{600} of 0.05, 0.15, 0.25, and 0.35 with and without the addition of CSP-1. Deletion of the spxB gene lowered the DNA content in the supernatant up to 3.5-fold at an OD_{600} of 0.15.

Addition of CSP-1 increased the DNA quantity in the supernatants of both the D39 Sm

strain and the spxB mutant. However, addition of CSP-1 could not fully restore DNA release in the supernatant of the spxB mutant (Fig. 4A).

DNA release was also measured using the method described by Moscoso and Claverys (21), using the strain R6 hexA mutant with the supernatants of D39 Sm

strain and its spxB mutant. The results were in line with those obtained by measuring DNA in the supernatant by real-time PCR (Fig. 4B). In strain D39 Sm

strain, release of DNA peaked at an OD_{600} of 0.15. The spxB mutant showed significantly reduced DNA release between OD_{600} of 0.05 and 0.35 (P ≤ 0.025). Addition of CSP-1 increased DNA release significantly between OD_{600} of 0.05 and 0.55 in strain D39 Sm

(P ≤ 0.003) and in the spxB mutant (P ≤ 0.0392). However, DNA levels in the mutants never reached those in the parent strain (P ≤ 0.0023).

**Influence of supplementation with acetate and H_{2}O_{2} on DNA release in the spxB mutant.** In order to investigate whether the end products of SpxB, i.e., H_{2}O_{2} and/or acetyl phosphate, played a role, DNA release was measured in the spxB mutant with H_{2}O_{2} supplementation and/or the addition of acetate. However, the compounds alone or in combination did not influence DNA release (Fig. 5).

**Influence of supplementation with catalase on DNA release in strain D39 Sm

.** In order to test whether the reduction of DNA release in the spxB mutant was due to the lack of a toxic effect of H_{2}O_{2}, DNA release in parental strain D39 Sm

was measured in the presence of catalase. Catalase reduced the H_{2}O_{2} concentration in the supernatant of strain D39 Sm

to an undetectable level (data not shown). DNA release was not significantly reduced upon addition of catalase compared to that in cultures without catalase at ODs ranging from 0.15 to 0.75. Catalase tended to reduce DNA release slightly in the early growth phase (reduction factor of 1.8 at OD_{600} of 0.025 and 0.05 [P = 0.016 if values for both time points were pooled] [data not shown]). However, overall these results were not sufficiently convincing for indicating a direct toxic effect of H_{2}O_{2} in the early growth phase.

**DISCUSSION**

This study investigated whether pyruvate oxidase (SpxB) plays a role in competence of *S. pneumoniae*. Such a role was suggested by the coincidence between competence and peak expression of spxB gene during the early growth phase as shown in this and earlier reports (18). Deletion of the spxB gene in strain D39 influenced three competence-associated parameters: transformation frequency, expression of the comC and recA genes, and DNA release. Therefore, a connection between spxB and the competence machinery seems to exist, but the mechanisms are still unknown.

Supplementation with CSP restored spontaneous transformability in the spxB mutant. Therefore, deletion of spxB did not affect the response to CSP, DNA uptake, or recombination. Others have shown that expression of spxB is not controlled by CSP (25, 26).

It may be that the role of SpxB in competence is energy supply and is similar to the role of NADH oxidase. Competence in *S. pneumoniae* depends on the availability of oxygen (1) and high levels of ATP (6). Energy supply is required for pneumococci to enter competence and for the uptake of transforming DNA (6). Most of the ATP supply in *S. pneumoniae* is derived from the glycolytic breakdown of glucose, which is more efficient in the presence of NADH oxidase. NADH accumulated during glycolysis is reoxidized by the NADH oxidase using O_{2} (1, 4, 10, 11). SpxB also utilizes O_{2} and produces the energy-rich acetyl phosphate, a potential source of ATP (24). In addition, knockout of the spxB gene reduces the ATP level, as shown by Pericone et al. (24). However, addition of CSP restored transformability, which speaks against the energy hypothesis for the effect of SpxB on competence.

SpxB generates H_{2}O_{2} and acetyl phosphate. Therefore, we investigated whether either of the two compounds may mediate the observed effects of spxB deletion on DNA release. Supplementation of the spxB mutant with H_{2}O_{2} and/or acetate (restores the acetyl phosphate level [29]) also revealed no effect on DNA release. Therefore, we found no indication of an intracellular effect for either H_{2}O_{2} or acetate. We cannot exclude the possibility that more sophisticated ways of supplementation would have shown an effect. The hypothesis seemed attractive, because low intracellular concentrations of H_{2}O_{2} may not be toxic but may induce oxidative stress and trigger competence (7, 28). Also, Kim et al. demonstrated that acetyl phosphate can act as an intracellular messenger (17), and Spellerberg et al. (29) showed that reduced adherence in an spxB mutant can be restored by addition of 0.1% sodium acetate.

Lastly, we investigated whether a direct toxic effect of H_{2}O_{2} may have contributed to DNA release (3). Supplementation of the parent strain with catalase did not reduce DNA release significantly. The limited effect of catalase supplementation on DNA release was not due to an insufficient amount of catalase addition, since the H_{2}O_{2} concentration in the supernatant was
at an undetectable level during the whole experiment (data not shown). However, catalase supplementation may not be appropriate to study the effect of endogenously produced \( \text{H}_2\text{O}_2 \), since catalase cannot penetrate cell membranes.

In conclusion, this study provides evidence for a role of the strain D39 \( \text{spxB} \) gene in competence; however, the mechanism remains elusive.

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