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Nrc of *Streptococcus pneumoniae* suppresses capsule expression and enhances anti-phagocytosis

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ABSTRACT

Streptococcus pneumoniae is a major pathogen of community-acquired pneumonia and one of its major virulence factors is pneumolysin, which functions as a cholesterol-dependent cytolytic pore-forming toxin. In this study, we identified the *ply*-like gene *spd0729* in a BLAST search. Unexpectedly, hemolytic and cytotoxic assays showed no significant differences between a Δ*spd0729* mutant strain and the wild-type strain, whereas the mutant strain exhibited weaker anti-phagocytic activity in human peripheral blood. In addition, real-time RT-PCR analysis revealed that four capsular biosynthesis genes in the mutant strain had expressions 7- to 432-fold greater than those of the wild type, while an enzyme-linked immunoassay showed a mean 3-fold greater amount of total capsular polysaccharide in the mutant strain. These results suggest that Spd0729 is not a cytolysin, though it plays crucial roles in anti-phagocytosis and regulation of capsule expression. Thus, we named Spd0729 as a negative regulator of capsular polysaccharide synthesis (Nrc).

Introduction

Streptococcus pneumoniae is a major cause of human diseases, such as pneumonia, meningitis, otitis, and sepsis. Asymptomatic carriage of pneumococci in biofilm in the throat or the nasopharynx is widespread, with rates especially high in children [1], and millions die every year as a result of pneumonia, bacteremia, and meningitis caused by *S. pneumoniae* [2,3]. The antigenic and biochemical properties of the polysaccharide capsule have been used to characterize *S. pneumoniae* strains into at least 90 distinct serotypes, though *S. pneumoniae* related diseases are most commonly due to 20 of those strains.

S. pneumoniae has a number of virulence factors that contribute to its ability to cause diseases, with the polysaccharide capsule, which is composed of repeating polysaccharide units that help confer resistance to complement-mediated opsonophagocytosis, playing a major role [4,5]. However, expression of the capsule reduces bacterial attachment to respiratory epithelial cells, which may hamper colonization [4,6]. Using electron microscopy, Hammerschmidt et al. found that bacteria in intimate contact with epithelial cells have a thinner capsule layer, i.e., they may down-regulate capsule expression in order to enhance adherence [6]. It has been suggested that, at least in serotype 3, polysaccharide chain length can be modulated by sugar concentration in the environment [7]. Furthermore, the polysaccharide capsule has been reported to play a key role in survival *in vivo* during systemic infection in animal models [4].

The polysaccharide capsule of most *S. pneumoniae* serotypes is encoded by a gene cluster located between *dexB* and *aliA*. Capsule operons of different serotypes show a similar structure with some conservation, particularly within the first four genes, downstream of which are serotype-specific genes [4,8]. The first gene, *cpsA*, is the most conserved and may have a role in regulation of capsule expression [9], which is thought to interfere with biofilm formation, while biofilm development may occur with unencapsulated phenotypic variants [10,11]. Although the ability of pneumococci to regulate capsule expression likely plays an important role in the transition from carriage to invasive disease, the molecular mechanisms involved in the regulation of capsule expression have not been fully elucidated.

Another major virulence factor is the 53-kDa pore-forming toxin pneumolysin (Ply). Ply acts as a cholesterol-dependent cytolysin by binding to cholesterol in the host cell plasma membrane, after which it becomes oligomerized and inserted into the eukaryotic membrane, forming a pore 350–450 Å in diameter [5]. In the present study, we found the *ply*-like gene *spd0729* utilizing the BLAST search algorithms and showed that it encodes a polypeptide with

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a 44% identity to the cholesterol-binding domain of Ply. To detect the function of *spd0729*, a *spd0729* mutant of *S. pneumoniae* was generated by allelic exchange. Interestingly, we found that inactivation of *spd0729* in *S. pneumoniae* did not influence hemolytic activity, while it significantly induced capsular synthesis.

Materials and methods

Bacterial strains, and reagents. *S. pneumoniae* strain D39 (NCTC 7466) was obtained from the National Collection of Type Cultures. *S. pneumoniae* strain R6, which was unencapsulated and derived from D39, was kindly provided by Dr. Shin-ichi Yokota (Sapporo Medical University, Japan) and grown in Tryptic-Soy (TS) broth (Difco), with spectinomycin (500 μg/ml) added to the medium for mutant strain selection. *Escherichia coli* strains XL-10 Gold (Stratagene) and BL21 (DE3) pLysE (Merck) were grown in Luria–Bertani broth (Sigma) or on Luria–Bertani agar plates, supplemented with 100 μg/ml of ampicillin and spectinomycin. Growth curves were determined by serial measurements of absorbance at 600 nm (A_{600}) and/or counts of viable colony forming units (CFU).

Human neutrophil and red blood cell isolation. Neutrophils were prepared as previously described [12,13]. Briefly, 10 ml of heparinized blood was obtained from healthy donors and mixed 1:1 with phosphate buffered saline (PBS) containing 3% dextran T500. After incubation at room temperature for 60 min, the supernatant was layered on Ficoll-Paque (GE Healthcare). After centrifugation at 450 *g* for 20 min, layers containing red blood cells and neutrophils were collected. Residual red blood cells were lysed by hypotonic shock, then the cells were suspended in RPMI 1640. Cell viability was monitored using the Trypan blue exclusion technique and the cells were counted in a hemocytometer. Fresh normal red blood cells (RBC) were obtained by drawing heparinized blood from volunteer donors, then washed three times in RPMI 1640 to remove the buffy coat and used as required.

Mutant construction. Inactivation of the *spd0729* and *ply* genes in *S. pneumoniae* was performed as described previously [14]. To construct mutant strains, PCR products from the upstream and downstream regions of *spd0729* and *ply* were separately ligated into pYT339 vector [14], then the resultant plasmids were digested with EcoRI and HindIII, and used to transform competent cells of *S. pneumoniae* strain D39. To prepare competent cells, 0.5 ml of exponential-phase organisms in TS broth were added to prewarmed TS broth (9.5 ml) and incubated at 37 °C for 30 min. A portion (1 ml) of the culture was then removed and placed in a tube containing 100 ng of competence-stimulating peptide [15]. After further incubation at 37 °C for 15 min, 0.2-ml portions were removed and placed in fresh tubes containing approximately 0.1 μg of linearized plasmid (10 μl) and incubated at 37 °C for 2 h. Thereafter, each culture was plated on TS blood agar and incubated at 37 °C for 24 h. Inactivation of the *spd0729* gene in mutant strain, which was designated MCY-1, was confirmed by reverse transcription-PCR (RT-PCR) amplification using *spd0729* primer pairs (Table 1). Inactivation of the *ply* gene in the mutant strain was confirmed by site-specific PCR and hemolytic assays.

Cytotoxic assay. To quantify dead neutrophils, a classical Trypan blue exclusion assay was employed. Briefly, neutrophils at a concentration of 10^6 cells/ml were incubated with *S. pneumoniae* at a multiplicity of infection of 1:1 at 37 °C for 90 min, after which viable neutrophil cells were counted in a hemocytometer.

Bactericidal assays. Lancefield bactericidal assays were performed as described previously [16,17]. Strains D39 and MCY-1 were grown, washed, and resuspended in 1 ml of PBS, as previously described. Diluted cultures (10 μl) were combined with fresh human blood (90 μl), then the mixtures were rotated at 37 °C for 1, 2, or 3 h. Viable bacterial cell counts were determined by plating diluted samples on blood agar.

Table 1
PCR primers used in this study.

Designation	Sequence (5′–3′)	Reference
For deletional mutagenesis		
aad9 BamF	CGGGATCCTTGATTTTCGTTCGTGAATAC	[14]
aad9 XbaR	GCTCTAGATTATAATTTTTTTAATCTGTTATTTAAATAG	[14]
spd729KOu/ EcoF	GAATTCGAATTAGCCAAGGATATAAG	This study
spd729KOu/ XmaR	CCCGGGATCTTTAGGAGATGTTATACG	This study
spd729KOd/ XbaF	TCTAGATCTGATGGTACAATGATAGTTAGTTTGTC	This study
spd729KOd/ HindR	AAGCTTGAATTTTATCAGCAATTTCACC	This study
PlyKOu/ EcoF	GAATTCGTAGCTCTTTATTTGCCTTTTCC	This study
PlyKOu/ BamR	GGATCCTCGATAACAACAAACTCATCGG	This study
PlyKOd/ XbaF	TCTAGAGGACAATACAGAAGTGAAGGC	This study
PlyKOd/ HindR	AAGCTTCTAGTCATTTTCTACCTTA	This study
aad9 F2	GGAGGATGATTCCACGGTACCATT	This study
aad9 R2	GGGAGAGAATTTTGTTAGCAGTTCGT	This study
For RT-PCR and real-time RT-PCR		
spd729KOu/ EcoF	CATATGTCAGCACAAATTACGATTAACCA	This study
spd729KOu/ XmaR	CCCGGGCAAGACATCATCGTCACTCACC	This study
D39 Cps2A Fw	TGCGGGCATTTATGGAGTTG	This study
D39 Cps2A Rv	ATCGGCTAGTGAGTAGCGTT	This study
D39 Cps2E Fw	GGTTCCTTTGATTCGAAAGGATG	This study
D39 Cps2E Rv	GAACATTATAAACCTGTGGTAGCTC	This study
D39 Cps2K Fw	CTTCCTAAAGATACCAAGCAACT	This study
D39 Cps2K Rv	CCTTGAATAGAGCTTTGACGA	This study
D39 RfbC Fw	TTGATCTACGCGAGGGTGAA	This study
D39 RfbC Rv	TGTTTATCTGCTTCTGAAACCTCT	This study
GapA/BamF	CGGGATCCAAATCACTAATGGTAGTTAAAGTTGG	This study
GapA/HindR	CCCAAGCTTTTATTTAGCAATCTTTGCGAAGTATTC	This study

RNA isolation and real-time reverse transcription (RT)-PCR. RNA isolation was performed as described previously [14,18]. Pneumococcal RNA was extracted from exponentially growing cultures in TS broth. Total RNA was prepared from cells using a TRIzol Max Bacterial RNA Isolation Kit (Life Technologies), then 0.8 μg of total RNA was reverse transcribed in the presence of random hexamers using the SuperScript III first-strand synthesis system (Life Technologies), according to the manufacturer's instructions. DNA contamination was assessed by PCR in non-RT samples. Real-time RT-PCR was performed using the SYBR Green system with the Step One plus system (Life Technologies). The level of the *gapA* gene transcription was used for normalization.

Determination of hemolytic activity of mutant strain. Hemolytic titer assays were performed using cultures grown in TS broth to an A_{600} value of approximately 0.3–0.5. The cultures were separated into cell and supernatant fractions by centrifuging, with each supernatant used as a pneumococcal supernatant fraction. The cells were washed in PBS, then cell suspensions were sonicated and debris removed by centrifugation, and the supernatant was

used as a pneumococcal cell fraction. For each assay, 40 μl of erythrocytes was added to 160 μl of lysate and incubated for 1 h at 37 °C. Cell debris and unlysed erythrocytes were removed by centrifugation, and the degree of hemolysis was determined spectrophotometrically by measuring released heme at 405 nm.

Quantitation of capsular polysaccharide. *S. pneumoniae* strain D39 and mutant strains were grown to mid-log phase and harvested by centrifugation for 8 min at 3000*g* to separate the supernatant and the whole cell fractions. The cells were resuspended in PBS at the original culture volume, then sonicated three times for 10 s each on ice and stored at −20 °C.

The amount of capsular polysaccharide was analyzed using antigen-specific serum with a modification of a specific ELISA method [19]. Briefly, 96-well ELISA plates (Sumitomo Bakelite) were coated with sonicated *S. pneumoniae* in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4 °C. The wells were then blocked with PBS containing 1% Block Ace solution (DS Pharma Biomedical) and 0.1% Tween 20, and incubated overnight at 4 °C. Duplicate 2-fold serial dilutions of capsule specific rabbit serum (Denka Seiken) were added to the wells and incubated for 1 h at room temperature. The wells were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Southern Biotechnology Associates) for 1 h at room temperature, then washed and developed with 3,3′,5,5′-tetramethylbenzidine solution (Moss Inc.). After incubation for 15 min, the reaction was stopped by adding 0.5 N HCl and absorbance was read at 450 nm (A_{450}) using a microplate reader (Titertek MK11; Titertek). Capsule specific antiserum was absorbed with a cell extract from the capsule negative strain R6 obtained by sonicating pneumococcal cells. Blocked antiserum stock was obtained after adding the cell extract to 100 ml of 1:100 diluted antiserum, incubation for 1 h at 37 °C, and final centrifugation for 30 min at 16,000 *g* at 4 °C.

Results and discussion

Sequence analysis of spd0729

To determine whether homologs of *ply* were present in *S. pneumoniae*, a BLAST search was performed using the *ply* gene as a probe. The results indicated that *S. pneumoniae* D39 contained a sequence similar to that of *ply*. The identified gene was *spd0729*, which encodes a polypeptide 39.4% identical to Ply (DDBJ/EMBL/GenBank Accession No. AB517950). Furthermore, the *spd0729* gene was shown to be 513 bp and encode a 170-amino acid residue protein with a predicted molecular mass of 19,457 Da and pl of 9.61. SignalP3.0 analysis (http://www.cbs.dtu.dk/services/SignalP/) indicated that Spd0729 did not have characteristic signal sequence pattern. In addition, Spd0729 was shown to be located in the bacterial cytoplasm by PSORT prediction (http://psort.ims.u-tokyo.ac.jp/), which was equivalent to the prediction results for Ply. The Spd0729 amino acid sequence was found to be 95–100% identical to the homologs in the finished genome sequences of 10 *S. pneumoniae* strains (D39, R6, P1031, G54, 70585, Taiwan19F-14, CGSP14, TIGR4, JJA, Hungary19A-6). Also, the 4–107 amino acid region of Spd0729 was shown to be 45% identical to the cholesterol-binding domain of Ply.

Spd0729 did not have cytolytic activity

To investigate the role of Spd0729 in the virulence of *S. pneumoniae*, we constructed an Δ*spd0729* mutant strain, MCY-1, and Δ*ply*

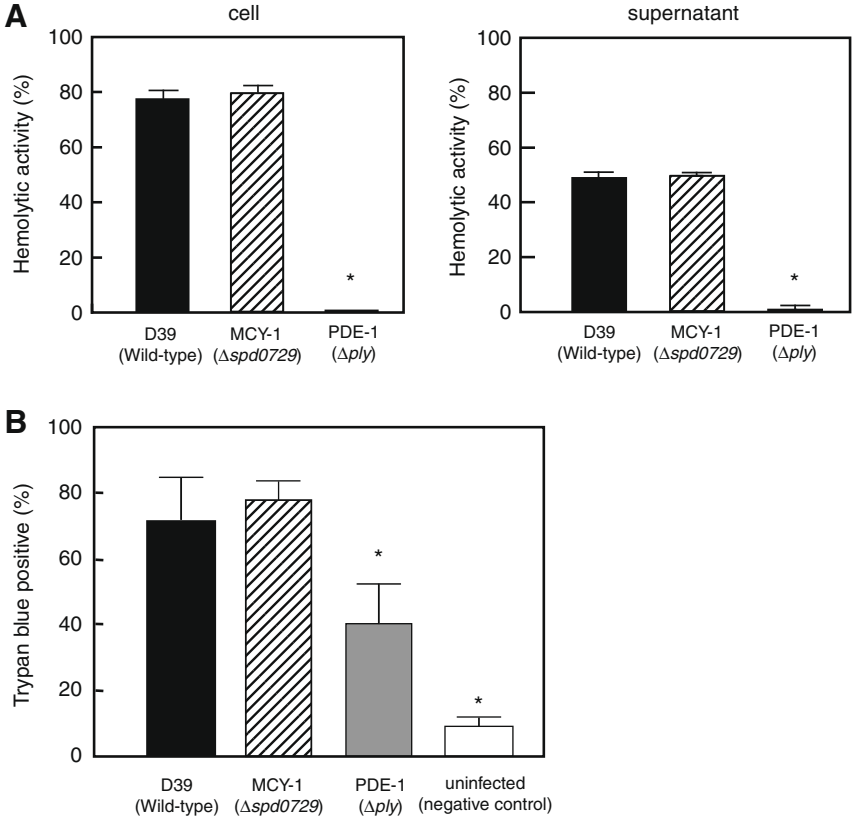

Fig. 1. Pneumolysin-like Spd0729 does not work as a cytolysin. (A) Hemolytic activities of culture supernatant and cell fractions from wild-type strain D39 and *spd0729* mutant strain MCY-1. Data shown are from representative experiments. Significant differences are indicated by vertical lines. $^{*}P < 0.05$, as compared with wild-type strain D39 ($n = 3$). (B) Trypan blue positivity induced by *S. pneumoniae* in neutrophils. *S. pneumoniae* D39, MCY-1, and PDE-1 strains were incubated for 90 min with neutrophils (10^6/ml), then the proportions of Trypan blue-positive cells were counted ($n = 3$).

mutant strain, PDE-1, by insertional inactivation and used a differential display approach to demonstrate the cytolytic activity of Spd0729 against human hemocytes. In addition, we fractionated mid-exponential-phase cultures of strains D39, MCY-1, and PDE-1 into culture supernatant and cell fractions, and assayed each fraction for hemolytic activity. Those results showed that strain PDE-1 had no hemolytic activity, whereas strain MCY-1 was not significantly different from strain D39 (Fig. 1A).

Next, to determine if Spd0729 killed neutrophils as effectively as Ply, we infected cells with strains D39, MCY-1, strain PDE-1 and determined neutrophil viability using a Trypan blue exclusion assay. After 90 min of incubation, 73% of the neutrophils incubated with strain D39 had lost the ability to exclude Trypan blue, while 48% of neutrophils incubated with strain PDE-1 lost that ability at the same time point. However, there was no significant difference between the numbers of dead neutrophils following incubation with strain D39 and strain MCY-1 (Fig. 1B). These results indicate that Spd0729 does not function as a cytolysin.

Growth of Δspd0729 mutant strain

When strains MCY-1 and D39 were tested to determine their growth kinetics profiles, similar growth rates were shown until 4 h of culture, though the A_{600} values at the stationary phase differed between them (MCY-1, $A_{600} = 0.9$; D39, $A_{600} = 0.5$) (Fig. 2A). Thereafter MCY-1 showed significantly lower cell viability after 24 and 44 h of incubation (Fig. 2B). A previous study demonstrated that *S. pneumoniae* died via a mechanism independent of LytA, but dependent on SpxB and its by-product H_2O_2 in the stationary phase [20]. We speculated that strain MCY-1 produces a higher concentration of H_2O_2, thus killing itself, as compared to strain D39, as MCY-1 showed greater growth than D39 during the stationary phase.

Reduced anti-phagocytotic activity of Δspd0729 mutant strain

For a bactericidal assay to investigate the function of Spd0729, we incubated 2.5×10^2 CFU of bacteria with 100 μl of human whole blood, and then determined anti-phagocytotic activities based on the viability of strains D39 and MCY-1 in human blood. The growth activity of strain MCY-1 in human whole blood was approximately 65%, 43%, and 23% after 1, 2, and 3 h, respectively, as compared to strain D39 (Fig. 3). Furthermore, the two strains did not have significant differences in regard to their influence on neutrophil viability (Fig. 1B). These results indicate that Spd0729 is involved in phagocytosis resistance.

spd0729 deficiency has no effect on capsule synthesis

The ability of *S. pneumoniae* to exist in diverse host environments is dependent on its capability to undergo spontaneous phase variation between transparent and opaque colony phenotypes [6,21]. In comparison to the opaque colony variant, cells that display a transparent phenotype have increased amounts of cell wall carbohydrate (C polysaccharide or teichoic acid) and reduced capsular polysaccharide. Our observations with oblique transmitted light showed that the colony morphology of strain MCY-1 was similar to the opaque phenotype (Fig. 4A). Therefore, we examined the transcription of capsule synthesis genes in *S. pneumoniae* strain MCY-1 and strain D39 using real-time RT-PCR. Expression profiling was performed with the genes *cps2A*, *cps2E*, *cps2K*, *rfbC*, and *gapdh*. These genes responsible for capsule biosynthesis in most serotypes are located between *dexB* and *aliA* (Fig. 4B). The first gene, *cps2A*, is the most conserved and has a role in the regulation of capsule expression. In addition, *cps2E* is one of the glycosyltransferase genes and *cps2K* codes a UDP-glucose dehydrogenase, while *rfbC*

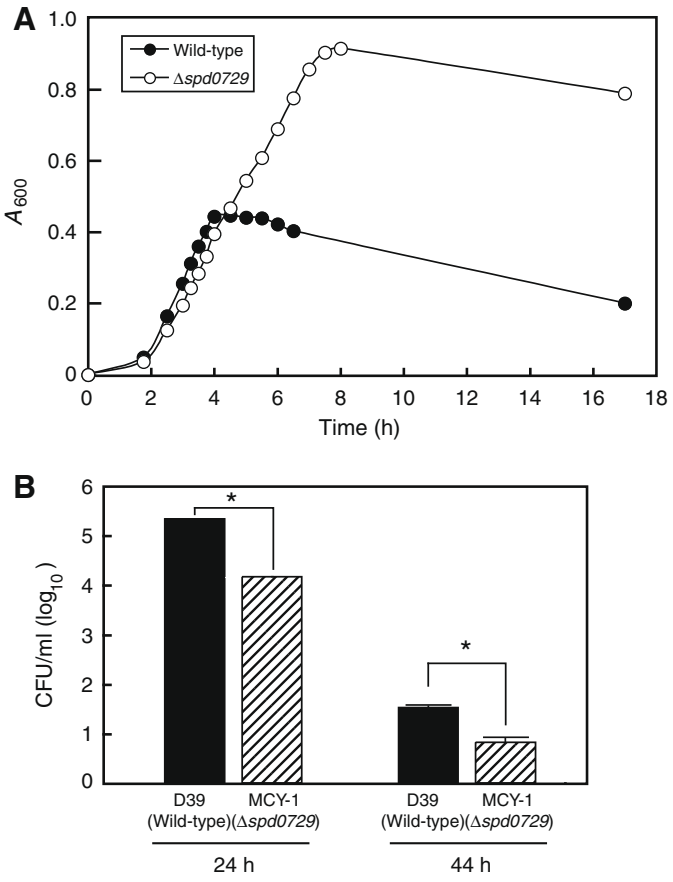

Fig. 2. The growth of strain MCY-1 *in vitro*. (A) Growth curves of wild-type strain D39 and *spd0729* mutant strain MCY-1. The strains were grown at 37 °C in TS medium and absorbance at 600 nm was measured at the indicated time points ($n = 3$). (B) Viability of *S. pneumoniae* at late-stationary phase. The number of CFU was determined at 24 and 44 h after plating aliquots on TS blood agar plates at 37 °C. Each bar represents $\log_{10}$ CFU. Representative data are shown. Significant differences are indicated by vertical lines. $^*P < 0.05$, as compared with wild-type strain D39 ($n = 3$).

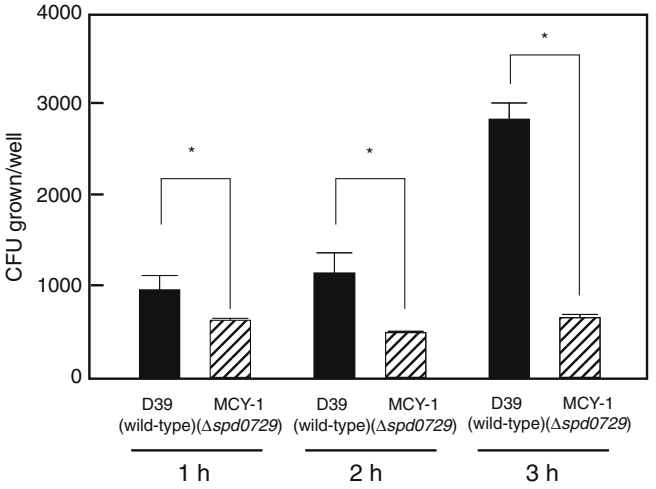

Fig. 3. Role of Spd0729 in anti-phagocytosis. Bacteria (10 μl, ~100 CFU) were added to heparinized whole blood (90 μl), then gently mixed for 1, 2, or 3 h at 37 °C. Next, each mixture was serially diluted and plated on TS agar. Following incubation, the number of CFU was determined. Three experiments were performed, with representative data shown. Significant differences are indicated by vertical lines. $^*P < 0.05$, as compared with wild-type strain D39 ($n = 3$).

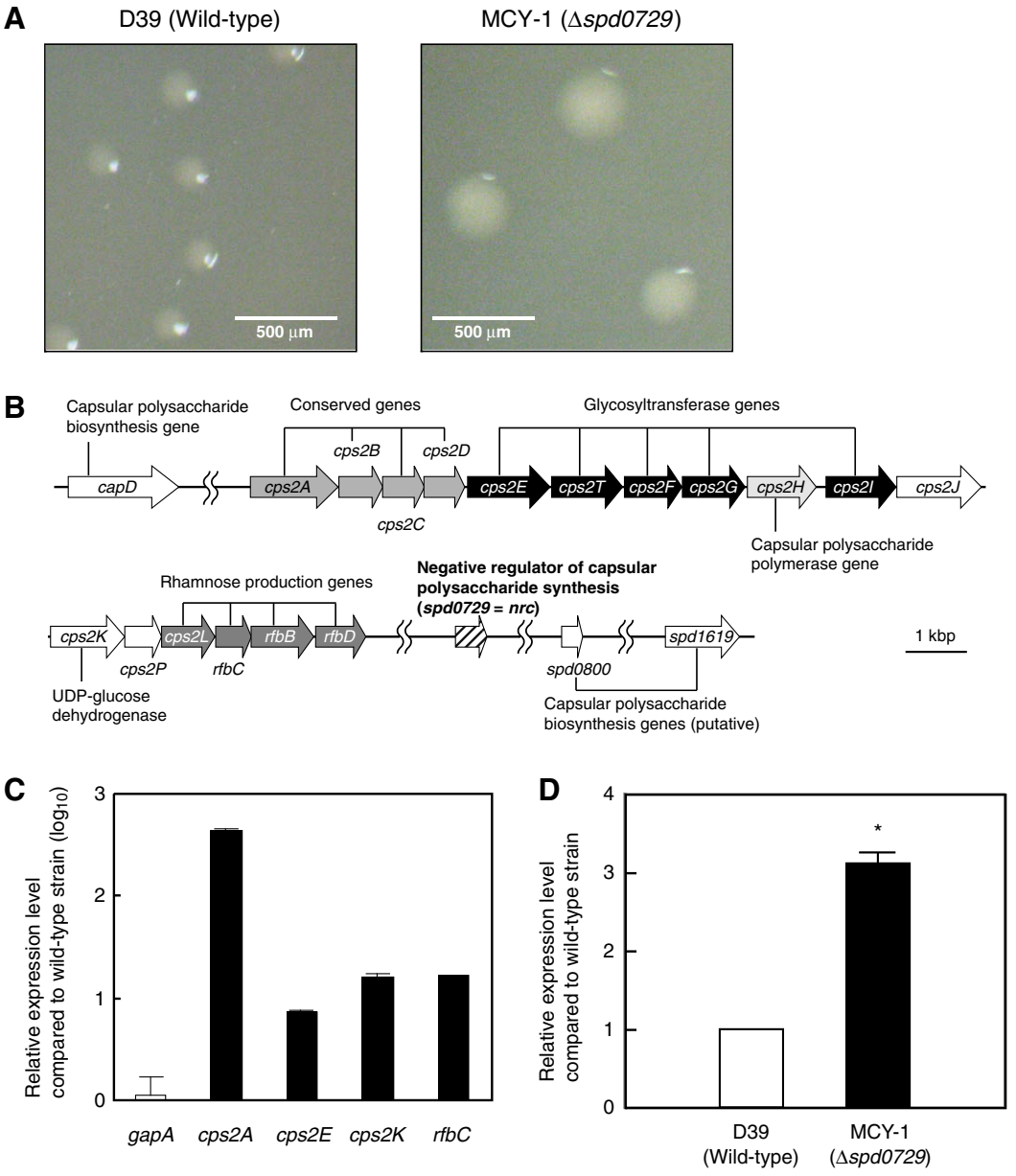

Fig. 4. Spd0729 suppresses capsule expression. (A) Photographs of colonies on TS agar obtained with oblique transmitted light. Differences in colony capacity are shown. (B) Genetic locus of capsule gene cluster and *spd0729* in the genome sequence of *S. pneumoniae* strain D39. Arrows represent open reading flames. (C) The expression profiles of known and putative *Streptococcus pneumoniae* capsule synthesis genes of *S. pneumoniae* mutant strain MCY-1 and wild-type strain D39 were analyzed by qRT-PCR *in vitro*. Bars represent expression profiling of the *cps2A*, *cps2E*, *cps2K*, *rfbC*, and *gapA* genes of strain MCY-1 relative to those of strain D39. The *gapA* gene was used as a control. All fold changes were significant ($n = 3$). (D) Quantification of capsule polysaccharide by ELISA. The capsule amount of strain D39 was considered to be 1-fold. Representative data are shown. Significant differences are indicated by vertical lines. $^*P < 0.05$, as compared with wild-type strain D39 ($n = 3$).

is involved in rhamnose production [22]. Equal amounts of total RNA from exponential-phase cultures ($A_{600} = 0.4–0.5$) of the MCY-1 and D39 strains were subjected to reverse transcription, and the transcript levels of the above-mentioned genes were determined using real-time RT-PCR, then the data obtained were normalized to the expression of the *gapA* gene in both strains. The expression levels of *cps2A*, *cps2E*, *cps2K*, and *rfbC* of MCY-1 were at least 7-fold greater than those of D39. Notably, *cps2A* of MCY-1 was increased by 432-fold as compared to that of D39 (Fig. 4C). Next, we examined the level of capsule expression by ELISA. Our results showed a mean 3-fold greater capsular polysaccharide in strain MCY-1 as compared to strain D39 (Fig. 4D). In a previous report, Hammershimidt et al. noted that it seemed obvious that genes outside the type 3 capsule locus were essential for capsule biosynthesis and regulation [6]. Interestingly, it has become apparent that down-regulation of pneumococcal capsule production enhances host-cell invasion in the asymptomatic carrier state, though the capsular phase must be restored for the bacteria to survive after invasion [6,23]. Spd0729 might be involved in the adaptability of *S. pneumoniae* to respond to selection pressures. Although the exact molecular mechanism is not yet understood, deletion of the *spd0729* gene leads to up-regulation of capsule expression. Thus, Spd0729 has a potential to suppress capsular expression.

It was previously shown that pneumococcal capsule gene products influence bacterial growth *in vitro* of strain D39 [22]. Those findings raise the possibility that a change in MCY-1 growth is not caused by deletion of Spd0729, but rather up-regulation of capsule gene products. Capsule production is strongly anti-phagocytic in a non-immune host. Therefore, it seems that the present bactericidal data conflicts with the up-regulation of capsule

expression. However, it was reported the mRNA level of *cps2A* of *S. pneumoniae* in mouse whole blood was higher than that in THY broth [24]. Thus, the capsule amounts *in vitro* may not reflect the capsule amounts *ex vivo* (in the human blood). An additional simple explanation is that Spd0729 is more important than the up-regulation of capsule expression in regard to anti-phagocytic activity.

Conclusion

In the present study, we found that deficiency of the *ply*-like gene *spd0729* did not influence pneumococcal hemolytic activity, though it caused up-regulation of capsule expression. As a result, we deposited to DDBJ/EMBL/GenBank the *spd0729* gene as a negative regulator of capsular polysaccharide synthesis (*nrc*). In previous studies, we reported unexpected findings that GAPDH of *Streptococcus pyogenes* functioned as a C5a-binding protein, and that the pectinase-like protein of *S. pneumoniae* functioned as an adhesin and invasin [14,25]. There is no doubt that bioinformatic predictions play an important role in functional analysis, however, there is a risk of overlooking other possibilities when computational analysis is the only method used. Our findings provide insight into the mechanism of capsule regulation. Additional studies are needed to understand the mechanism involved in regulation of the expression of the capsule polysaccharide of *S. pneumoniae*.

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