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The Effect of Transformation on the Virulence of *Streptococcus pneumoniae*

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Although *pneumococcus* is one of the most frequently encountered opportunistic pathogen in the world, the mechanisms responsible for its infectiveness have not yet been fully understood. In this paper, we have attempted to characterize the effects of pneumococcal transformation on the pathogenesis of the organism. We constructed three transformation-deficient pneumococcal strains, which were designated as Nos. 1d, 2d, and 22d. The construction of these altered strains was achieved via the insertion of the inactivated gene, *comE,* to strains 1, 2 and 22. We then conducted a comparison between the virulence of the transformation-deficient strains and that of the wild-type strains, via an evaluation of the ability of each strain to adhere to endothelial cells, and also assessed *psaA* mRNA expression, and the survival of hosts after bacterial challenge. Compared to what was observed with the wild-type strains, our results indicated that the ability of all of the transformation-deficient strains to adhere to the ECV304 cells had been significantly reduced ($p < 0.05$), the expression of *psaA* mRNA was reduced significantly ($p < 0.05$) in strains 2d and 22d, and the median survival time of mice infected with strains 1d and 2d was increased significantly after intraperitoneal bacterial challenge ($p < 0.05$). The results of our study also clearly indicated that transformation exerts significant effects on the virulence characteristics of *S. pneumoniae,* although the degree to which this effect is noted appears to depend primarily on the genetic background of the bacteria.

Key words: *Streptococcus pneumoniae,* transformation, virulence

Streptococcus pneumoniae is widely considered to be one of the more important pathogenic bacteria known. It has been previously demonstrated to be capable of inducing such varied conditions as meningitis, nasosinusitis, otitis media, and septicemia. The increasing problem of drug-resistant pneumococci, coupled with the suboptimal efficacy demonstrated by polysaccharide vaccines, have been the impetus for a resurgence studies into the mechanisms underlying the pathogenesis of this organism. Although the polysaccharide capsule has been identified to be one of the primary prerequisites with regard to the virulence of the organism (Austrian, 1981), more attention has been paid in recent years to the role played by the pneumococcal proteins in the context of pathogenesis (Paton *et al*., 1993).

S. pneumoniae is naturally capable of genetic transformation, a condition which is normally defined as the physiological state which allows for the binding to, uptake of, and integration of extracellular DNA, which can in many cases culminate in permanent bacterial phenotype changes. Transformation competence in *S. pneumoniae* has been shown to develop abruptly and simultaneously in all of the cells in an exponentially growing culture. Transformational competence is then maintained for about 10-15 min, and then decays nearly as quickly as it emerged (Tomasz, 1965). During this transient competence period, a temporary global switch also occurs in the pattern of protein synthesis in the cells. Since the first report of this protein synthesis shift, it has been further demonstrated that the expression of nearly two dozen specific genes is upregulated during this competence period in *S. pneumoniae* (Morrison and Baker, 1979). It has also been reported that the expression of autolysin is induced after the entrance of *S. pneumoniae* into a competence state (Mortier-Barriere *et al.*, 1998). Autolysin, an important factor in terms of virulence, is capable, once activated, of triggering the release of both pneumolysin and certain cell wall products. Another study has indicated that the mutation of any one of the three competence-inducing proteins induced an attenuation of virulence (Bartilson *et al*., 2001). Transformation is generally thought to perform a primary function in bacterial virulence, from the point of view of the evolution of virulence factors via the homologous recombination of foreign DNA into the chromosomes of hosts (Dowson *et al.*, 1997; Poulson *et al.*, 1998). However, according to the above data, it can also be surmised that transformation

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may also have some direct effects on bacterial virulence. In order to obtain direct data regarding the effects of transformation on bacterial virulence, we have constructed a series of transformation-deficient strains, and have observed the changes in their virulence, on the molecular, cellular, and systemic levels.

Transformation competence in *S. pneumoniae* is known to be regulated by *com* locus genes. The *com* operon has been determined to consist of three genes, *comC*, *comD*, and *comE*, which encode for a competence-simulating peptide (CSP), histidine kinase, and a response regulator, respectively (Havarstein *et al.*, 1995; Havarstein *et al.*, 1996; Pestova *et al.*, 1996; Cheng *et al.*, 1997). Extracellular signals, including certain environmental factors, such as Ca^{2+} and O_2 concentrations, growth stage, bacterial density, and others may also exert effects on transformation characteristics, by virtue of this unique regulator (Havarstein *et al.*, 1996; Pestova *et al.*, 1996; Pozzi *et al.*, 1996). Upon the inactivation of any of the three aforementioned genes in *S. pneumoniae*, the strain has been shown to abrogate all of its transformational competence. In this study, then, we constructed a series of transformation-deficient pneumococcal strains, via the insertion of the *comE* inactivating gene. We then conducted a comparison of the virulence of the deficient strains with the wild-type strains, via the *in vivo* bacterial challenge of mice, via the intraperitoneal route. We also compared the cell variants with regard to their ability to adhere to ECV-304 cells *in vitro*. We determined, as the result of these trials, that pneumococcal surface adhesin A (PsaA) constitutes a protective immunogen in mice, and is clearly involved to some degree in the ability of the cell to adhere to mucosal cells or other cells, during the pathogenic process of *S. pneumoniae* (Sampson *et al.*, 1994; Berry and Paton, 1996; Talkington *et al.*1996). We then assessed the expressions of *psaA* mRNA via RT-PCR, in order to ascertain whether or not transformation affects the expression of this mRNA during the competence period. Our results in this regard indicate that transformation capacity is crucial to pneumococcal virulence.

Materials and Methods

Bacterial strains and growth condition

The *S. pneumoniae* strains used in this study were virulent type 2 strains. Strains 1 and 2 were obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China, and strain 22 was isolated clinically at the Medical Department of Tottori University, in Japan. The *Pneumococci* were routinely plated on tryptic soy agar (TSA) supplemented with sheep's blood, at a final concentration of 3% (v/v). We also grew some of the cultures in a liquid semi-synthetic casein hydrolysate medium, which had been supplemented with yeast extract (C+Y medium, pH 8.0)) (Lacks and Hotchkiss, 1960). Whenever appropriate, we added erythromycin (*erm*) to the media, at a concentration of 0.25 μg/ml.

Construction of transformation-deficient S. pneumoniae mutant

The transformation deletion derivatives of strains 1, 2, and 22, which referred to as 1d, 2d, and 22d, respectively, were constructed according to the following procedure: Firstly, using *S. pneumoniae* CPM17 (supported by Morrison DA) as the template, we amplified the comEup-ermcomEdw fragments using the following primers: 5'-TGCTCAGTCAATTGTCTATGCTCG-3'and 5'-ACCAACGGACCTTCTATCTGTAGC-3' (MSL52 and MSL54, Lee and Morrison, 1999). The fragment was then transformed into strains 1, 2, and 22, via the following procedure. The bacteria were allowed to grow in C+Y medium (pH 8.0) at 37°C, and the samples were removed when their optical density at 550 nm reached a measurement of 0.1. The PCR fragment (final concentration, 1 μg/ml) and CSP (supported by Morrison DA, final concentration, 20 ng/ml) were then added to the culture, followed by 90 min of further incubation at 37°C. The transformants were then identified via selection on TSA which harbored erythromycin, and these identifications were further confirmed via PCR and transformation experiments. The primers of *comE* which were used in the PCR identifications were the same as those listed above, and the primers employed in the amplification of the *erm* fragments were as follows: 5'-CCGGGCCCAAAATTTGTTTGAT-3' and 5'-AGTCGGCAGCGACTCATAGAAT-3' (DAM212 and DAM213, Lee and Morrison, 1999). The transformation experiments were conducted using the chromosomal DNA of CP1250 (supported by Morrison DA, containing streptomycin resistant gene) as a donor, and the transformants were selected on TSA harboring streptomycin.

FITC-Labeling of bacteria

The *S. pneumoniae* strains from the fresh overnight blood agar cultures were then inoculated in C+Y medium (pH 8.0) at 37°C, until they had grown to an OD_{620} of between 0.1 and 0.15 (approximately 1×10^8 CFU/ml). The bacteria were mixed with fluorescein isothiacyanate (FITC) (Sigma, USA; 1 mg/ml) dissolved in a buffer containing 0.05 M Na_2CO_3 and 0.1 M NaCl, for 1 h at 4°C (Geelen *et al.*, 1993).Then, the bacteria were washed four times in phosphate-buffered saline (PBS, pH 7.5) and resuspended in DMEM–Ham's F-12 medium, with 5% fetal calf serum (without antibiotics, pH 7.5), to a final concentration of 2.5×10^8 CFU/ml. In our preliminary experiments, we detected identical numbers of *S. pneumoniae* by Gram staining and fluorescent labeling. We also determined, in our preliminary experiments, that *S. pneumoniae* labeled with FITC grew rather robustly in the growth medium described previously.

Adherence of the wild-type and mutant strains to ECV-304 cells

The ability of the mutant strains to adhere to the human vascular endothelial cell line, ECV 304 (Shanghai Institute of Cell Biology, China) was then assessed in 6-well tissue culture plates, as was previously described (Geelen *et al.*, 1993; Cundell *et al.*, 1995). The ECV 304 cells were maintained in 10% fetal calf serum containing 5% DMSO, and then cultured in DMEM/Ham's F-12 medium (pH 7.5) for 2-3 days, using 5% fetal calf serum. The ECV-304 cell monolayers were then rinsed twice in PBS, and the FITC-labeled *S. pneumoniae* (2.5×10^8 CFU/ml; 2 ml/well) which had been suspended in DMEM–Ham's F-12 medium were added to the cells on 6-well plates. After 0.5 h of incubation at 37°C, the culture fluid was extracted from each of the wells, and the cells were washed five times in PBS (pH 7.5) in order to remove any remaining nonadherent bacteria. The numbers of adherent bacteria were then determined under a fluorescence microscope (BH-2; Olympus, Japan) equipped with an IF DM-510 filter (Olympus, Japan). Adherence was expressed as the number of adherent bacteria per 100 cells counted in a 200× field. The values expressed reflect averages of three or four wells during separate experiments. The results shown are expressed as means ± SD. We conducted statistical analysis using unpaired Student's tests (two-tailed). ($P < 0.05$ was considered to be significant.)

Expression of psaA mRNA

In order to assess the contribution lent by f transformation to the expression of *psaA*, both the wild and mutant pneumococcal strains, both of which had been grown at a pH of 8.0, were induced to the point of competence by CSP (20 ng/ml), at their respective optimal bacterial densities (OD_{550} of 0.1 for No.1 and 1d, 0.08 for No.2 and 2d, 0.07 for No.22 and 22d). In our RT-PCR analysis, the bacteria were collected at 0, 10, and 20 min intervals after the addition of CSP to the culture. Our transformation experiments were simultaneously conducted in order to determine whether or not the bacteria had indeed been compelled to enter the competence period.

In order to determine the transcription levels of *psaA* via RT-PCR, we extracted the total RNA from mid-log phase *S. pneumoniae* cultures using a RnEasy mini kit (Qiagen, USA). The integrity of the RNA was confirmed via gel electrophoresis, and the quantities of RNA were determined via optical density. The cDNA was obtained using reverse-transcribed RNA with random 6-oligonucleotide employed as primers. The 16S rRNA was then amplified from the cDNA as a standard, using the following primers: 5'-ATAGCCGACCTGAGAGGGTGA-3'and 5'-TACAAGCCAGAGAGCCGCTTT-3' (in 16S rRNA; 258-279 and 700-721; GenBank accession no. AJ617796). The sense and antisense primer sequences used for *psaA* were as follows: 5'-GACCCTCACGCTTGGCTCAATCTC-3' and 5'-ATAGGCAGATGGGACGCGTAGGC-3', respectively. For quantification, we subjected the PCR products to electrophoresis on agarose gel. Gene Ruler™ Ladder 0.1 kb was employed as a DNA Marker (Marker2, supported by MBI Company, Lithuania). The proportions of *psaA* to 16S rRNA were estimated via the measurement of the ethidium bromide intensity. The level of *psaA* gene expression was determined via Quantity-one version 3.0 software (supported by the Bio-Rad, USA).

Animal studies

For these studies, we obtained 6- to 8-week-old inbred male BALB/c mice (weight 18-22g) from the Animal Center of the Third Military Medical University. The inoculation of the mice with the pneumococci was conducted according to the previously described procedure (Polissi *et al.*, 1998). The wild-type and mutant strains were tested in two separate experiments, and each of these experiments involved 12 to 21 mice. In order to assess virulence in the blood, we infected the BALB/c mice intraperitoneally with 0.1 ml of 5×10^1 or 5×10^6 CFU/ml of a suspension of pneumococcal cells (the doses were determined by virulence pretesting). The numbers of surviving animals were then counted daily for 14 days. Differences in the median survival time between the groups were analyzed via Mann-Whitney U tests (two-tailed). Differences in the overall survival rates between the groups were analyzed via χ^2 tests (two-tailed).

Results

Identification of the transformation-deficient strain

Using 1.0% agarose gel electrophoresis (Fig. 1), we determined that only one PCR product of wild type *comE* (1229 bp) was amplified in the wild-type strains, whereas two products were generated in the mutant strains, and these products were 1515 bp and 726 bp in size. These

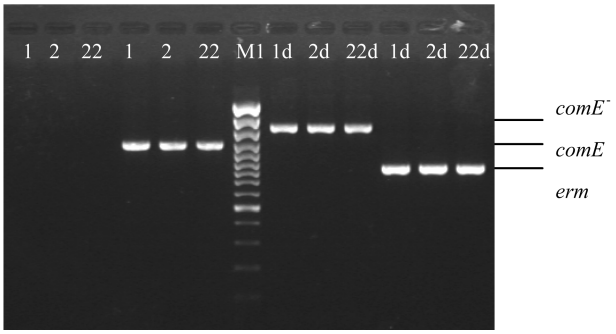

Fig. 1. Transformation-deficient *S. pneumoniae* strains 1d, 2d, and 22d, which were identified via PCR. 1.5% agarose gel electrophoresis contrasted with Marker1. Two PCR products were generated with *S. pneumoniae* strains 1d, 2d, and 22d: *comE*dw-*erm*-*comE*up (*comE*-, 1515 bp) and *erm* (726 bp). Only one PCR product was generated with *S. pneumoniae* strains 1, 2, and 22: *comE* (1229 bp).

sizes are consistent with the length of the fragments *comE*up-*erm*-*comE*dw and *erm*, thereby indicating that the mutation strains were correctly constructed. Our transformation experiment demonstrated clearly that the wild strains had been transformed with as much efficiency as before, but we detected no transformant on the TSA selecting different plates of mutated strains. We performed these experiments three times, and each of the trials met with similar results. These results indicated that the mutation strains had undergone deletions in the regions responsible for transformation.

The effect of transformation deletions on pneumococcal adherence

We utilized three different strains of pnemococcus to determine the effects of transformation mutagenesis on adherence to ECV-304 cells *in vitro*. After 30 min of incubation with ECV-304, we counted the attached pneumococci with a fluorescence microscope. At a dose of 2.5×10^8 CFU, the total adherence evidenced by *S. pneumoniae* No.1 was 36.0±4.1 CFU per 100 cells, whereas the adherence of the No.1d strain was determined to be 10.5±1.0 CFU per 100 cells ($P < 0.001$). The total adherence of *S. pneumoniae* strain 2 was 37.2±3.3 CFU per 100 cells, whereas strain 2d was 11.5±4.3 CFU per 100 cells ($P < 0.001$). Similarly, the total adherence of *S. pneumoniae* No.22 was shown to be 72.5±3.4 CFU per 100 cells, as compared with 55.8±4.5 CFU per 100 cells for strain 22d ($P < 0.01$) (Fig. 2). Clearly, the adherence to ECV-304 evidenced by the transformation-deficient strains 1d, 2d, and 22d differed significantly from that of the wild-type strains, and the adherence percentages had been reduced by 71%, 69%, and 23%, respectively.

Expression of psaA mRNA during competence

The results of our transformation experiment indicated that the wild-type strains (No. 1, 2, and 22) all entered transformation competence phases after the addition of CSP, and the transformation ratios of these variant were uniformly in excess of 0.7%. Conversely, we obtained no transformants in the transformation-deficient strains (No. 1d, 2d, and 22d). These results implied that those strains could not be induced to enter the transformation phase by the addition of CSP. This finding was consistent with the characteristic of the mutant strains. Our RT-PCR results revealed that in strains 1 and 1d, although the expression of *psaA* mRNA had increased 20 min after the addition of CSP, this increase occurred in a competence-independent manner (Fig. 3). In strains 2 and 2d, 10 min after the addition of CSP, the expression of *psaA* mRNA was shown to differ significantly between the two strains ($p < 0.05$); Compared at a timepoint of 0 min, mRNA expression in strain 2 had clearly increased substantially, while mRNA expression in strain 2d persisted unchanged ($p < 0.05$) (Fig. 4). The expression of *psaA* mRNA changed in a similar fashion in strain 22 and 22d (Fig. 5). It is worthy of notice that the expression in strain 22 at the 20 min timepoint was determined to be significantly lower than that observed at the 10-min timepoint ($p < 0.05$). At the 20 min timepoint, mRNA expression was almost identical to that observed at the 0 min timepoint. This finding is consistent with our data regarding the emergence and rapid shutoff of bacterial competence. Our results suggest that *psaA* expression level would increase in cases in which bacteria were induced into competence in strains 2 and 22.

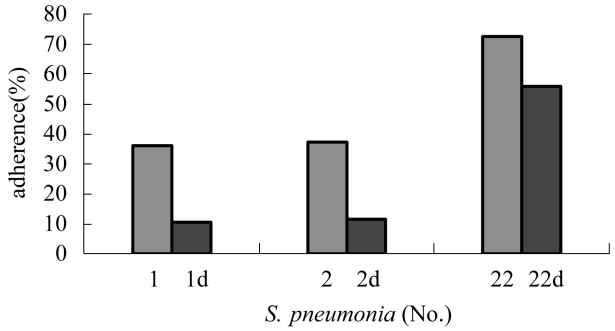

Fig. 2. Adherence of *S. pneumoniae* strains 1, 1d, 2, 2d, 22, and 22d to ECV304. The ECV-304 cells were incubated with 2.5×10^8 CFU/ml of *S. pneumoniae* for 30 min. Adherence was expressed as the number of pneumococci attached per 100 EC. The results were expressed as the means ± SD for triplicate wells, in at least three independent experiments.

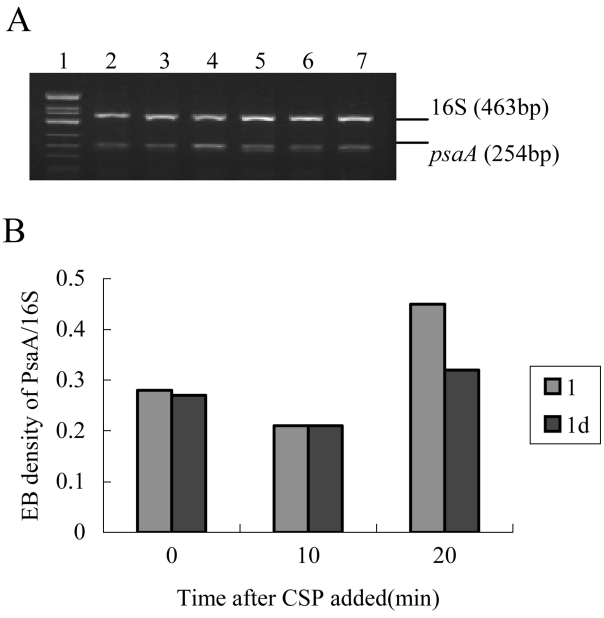

Fig. 3. (A) RT-PCR *psaA* of *S. pneumoniae* strains 1 and 1d on 2% agarose gel electrophoresis. Lane 1, Marker2; lanes 2-4, 0 min, 10 min, 20 min after CSP was added into *S. pneumoniae* No.1; lanes 5-7: 0 min, 10 min, 20 min after the addition of CSP to *S. pneumoniae* strain 1d. (B) *psaA* mRNA expression of *S. pneumoniae* strains 1 and 1d, at different times. *psaA* mRNA expression was compared with that of 16S rRNA after the addition of CSP to bacteria at 0 min, 10 min or 20 min timepoints in strains 1 and 1d, using Quantity-one software.

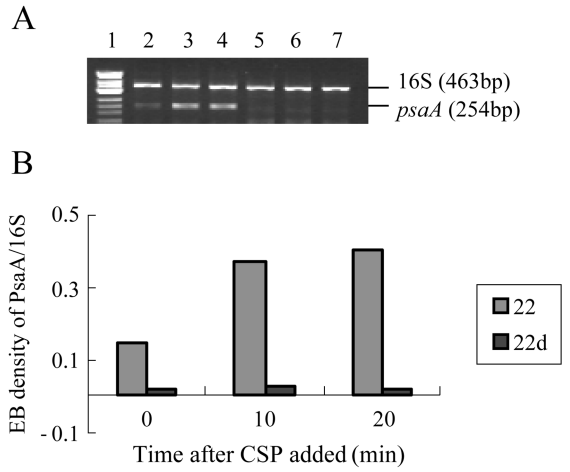

Fig. 4. (A) RT-PCR of the *psaA* gene of *S. pneumoniae* strains 2 and 2d on 2% agarose gel electrophoresis. Lane 1, Marker2; lanes 2-4, 0 min, 10 min, 20 min after the addition of CSP to *S. pneumoniae* strain 2; lanes 5-7, 0 min, 10 min, and 20 min after the addition of CSP to *S. pneumoniae* strain 2d. (B) *psaA* mRNA expression of *S. pneumoniae* strains 2 and 2d, at different times. *psaA* mRNA expression was compared with that of 16S rRNA when CSP was added to the bacteria at 0 min, 10 min, or 20 min timepoints in strains 2 and 2d, using Quantity-one software.

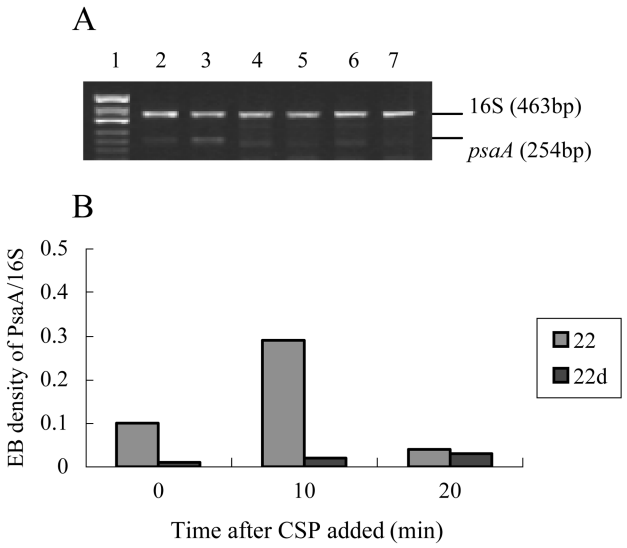

Fig. 5. (A) RT-PCR *psaA* of *S. pneumoniae* strains 22 and 22d, on 2% agarose gel electrophoresis. Lane 1, Marker2; lanes 2-4, 0 min, 10 min, 20 min after CSP was added to *S. pneumoniae* strain 22; lanes 5-7, 0 min, 10 min, 20 min after CSP was added to *S. pneumoniae* strain 22d. (B) *psaA* mRNA expression of *S. pneumoniae* strains 22 and 22d, at different times. *psaA* mRNA expression was compared with that of 16S rRNA when CSP was added to the bacteria at 0 min, 10 min, or 20 min timepoints in strains 22 and 22d, as assessed by Quantity-one software.

Virulence studies in mice

In order to assess the contribution of transformation to the virulence of the pneumococcal strains, groups of 19 to 21 BALB/c mice were bacterially challenged intraperitoneally, with approximately 5×10^0 CFU of either the wild-type strains 1 or 2, or with the mutant strains 1d or 2d. Other groups, consisting of 12 to 13 BALB/c mice, were bacterially challenged using approximately 5×10^5 CFU of either wild-type strain 22 or mutation-type strain 22d, as is described in the Materials and Methods section. Survival time was determined in all cases (Fig. 6). Mice which were challenged with No.1 evidenced a median survival time of 1.5 days. Mice challenged with strain 1d were demonstrated to survive for a significantly longer time (median survival time, 1.8 days; $p < 0.01$). Although we determined there to be no significant differences with regard to overall survival rates between the groups of mice challenged with strains 2 and those challenged with strain 2d, the median survival times (1.8 and 3.0 days for No. 2, and 2d, respectively) differed to a significant degree ($P < 0.05$). The median survival times of strains 22 and 22d were indistinguishable ($p > 0.05$).

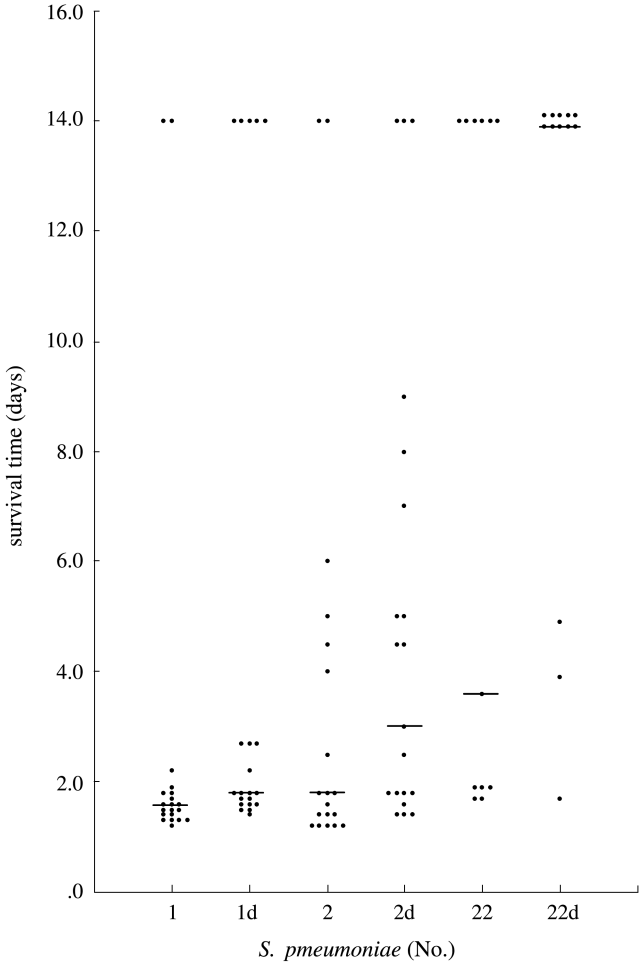

Fig. 6. Intraperitoneal challenge. Groups of BALB/c mice were intraperitoneally injected with 5×10^0 CFU of strains 1, 1d, 2, and 2d, or 5×10^5 CFU of strains 22 and 22d. The survival time of each of the mice is indicated. Transverse lines denote median survival times for each of the groups.

Discussion

In this study, our results indicated that the adherence of the three pneumococcal strains to ECV 304 could be attenuated significantly after mutation at the *comE* gene. Our RT-PCR results indicated an increase in the expression of the *pasA* gene in strains 2 and 22 during the competence period. PsaA is one of the most important known adhesin factors in *S. pneumoniae* (Mortier-Barriere *et al.*, 1998). In a mouse infection model, PsaA and PspC were determined to be the most effective vaccine proteins. Between these two, PsaA was shown to be superior with regard to its ability to exert protective effects against carriage (Briles *et al.*, 2000). There is no available data from any previous studies regarding the relationship between the *psaA* gene and transformation. According to our results, it appears that pneumococcal transformation may effect an increase in bacterial adherence to ECV-304, via the induction of increased *psaA* expression in strains 2 and 22. However, *psaA* expression was determined to be independent of transformation in strain 1, thereby indicating that the expression of some other adhesin factor might be regulated via transformation in pneumococcal strain 1. The LacI/GalR family regulator, RegR, has been previously determined to regulate competence, and has also been shown to activate pneumococcal adherence to A_{549} cells (Chapuy-Regaud *et al.*, 2003). In that study, RegR was determined to activate the adherence of pneumococci grown at neutral pH 7.0, independent of pneumococcal competence. However, the cell strains used in this adherence experiment were different from the ones we used in this study. They used A_{549} cells, whereas we employed ECV-304 cells. Therefore, RegR might still possibly be one of the adhesin factors which facilitates the increased adherence associated with transformation in strain 1. However, further studies will be required in order to precisely determine the accuracy of this hypothesis. There are also some other proteins which are known to be associated with bacterial adherence to host cells. These cells include PspA, PspC, CbpA (Hammerschmidt *et al.*, 1997; Rosenow *et al.*, 1997; Balachandran *et al.*, 2002). However, there has yet to be a report regarding the relationship between these cells and bacterial transformation characteristics. Also, it remains possible that there are some unknown adhesin factors which are related to transformation. Further study in this regard remains a necessity.

In experiments involving a mouse infection model, our results indicated that the virulence of pneumococcal strains 1 and 2, upon the induction of mutations in the *comE* gene, had been significantly attenuated. However, the virulence of pneumococcal strains 22, and 22d did not significantly differ. This would appear to suggest that the competence and transformation characteristics in the bacterial cells may constitute a contributory factor in the pathogenic process of *S. pneumoniae*. However, this also appears to rely to some degree on the genetic background of the bacteria. Bartilson determined that the virulence of a pneumococcal strain was reduced by approximately 8000 fold in systemic infection, after the induction of mutation in the *comD* gene (Bartilson *et al.*, 2001). It seems that some factors, the expression of which could be induced by *comD,* but not *comE*, performed an important function in pneumococcal virulence. Whether or not these factors are involved in competence and transformation is a matter which requires further investigation in the future.

In studies involving the effects of O_2 on pneumococcal transformation, Trombe (Auzat *et al.*, 1999; Echenique and Trombe, 2001) determined that O_2 was able to indirectly regulate pneumococci transformation via the modulation of the activity of protein ComE, through the regulation of nox gene expression. It was found that the virulence was attenuated by a nox insertion mutation. This group also found the nox-deficient strain (nox-) appeared weak transformation. And the ComE protein was demonstrated to exhibit a low degree of activity in this strain. Our results indicated that the deletion of the *comE* gene resulted in weaker virulence in the mutant strain. We surmise that the weakened virulence of the nox- strain might be attributable to the low degree of activity witnessed in conjunction with the ComE protein. These findings also showed that O_2 might be indirectly regulating pneumococcal transformation and virulence, in the same manner.

Other studies performed by our research group previously have shown that the expressions of three pneumococcal virulence factors were significantly lower during the transformation-deficient phases of the bacteria (Wang *et al.*, 2003). These factors are autolysin, a choline binding protein which exhibits autolytic activity, neuraminidase A, which performs a vital function in the course of seeding on and invasion into the host cell, and hyaluronidase, which is known to function as a spreading factor in pneumococcal species (Berry *et al.*, 1996; Zwijnenburg *et al.*, 2001).

Overall, we are ready to advance a hypothesis regarding the mechanism of pneumococcal opportunistic infection: as certain circumstances changed, including O_2 and Ca^{2+} concentrations, temperature, pH, etc., the bacteria could be pushed into competence phase. Simultaneously, some proteins associated with adherence, invasion, and transfer were induced, and were expressed with increasing abundance. Therefore, bacterial virulence was enhanced, and the bacteria might parasitically infect the host. However, it remains unclear as to whether any other proteins are involved in this pathogenesis. A large-scale selection study may be required and, using those proteins, we might be able to determine the best proteins for the development of a vaccine product.

Acknowledgments

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