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Virulence of *Streptococcus pneumoniae*: PsaA Mutants Are Hypersensitive to Oxidative Stress

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psaA **encodes a 37-kDa pneumococcal lipoprotein which is part of an ABC Mn(II) transport complex.** ***Streptococcus pneumoniae*** **D39** ***psaA*** **mutants have previously been shown to be significantly less virulent than wild-type D39, but the mechanism underlying the attenuation has not been resolved. In this study, we have shown that** ***psaA*** **and** ***psaD*** **mutants are highly sensitive to oxidative stress, i.e., to superoxide and hydrogen peroxide, which might explain why they are less virulent than the wild-type strain. Our investigations revealed altered expression of the key oxidative-stress response enzymes superoxide dismutase and NADH oxidase in** ***psaA*** **and** ***psaD*** **mutants, suggesting that PsaA and PsaD may play important roles in the regulation of expression of oxidative-stress response enzymes and intracellular redox homeostasis.**

Streptococcus pneumoniae is a cause of high morbidity and mortality around the world (10). The emergence of antibiotic-resistant pneumococci and the limitations of current vaccines have led to increased interest in understanding the molecular mechanisms that underpin the pathogenesis of this bacterium (21, 22). A large number of virulence factors in *S. pneumoniae*, many of which are associated with the cell surface, have been described (21). Of particular interest is the pneumococcal surface antigen PsaA (26). Purified PsaA was shown to be a protective immunogen in mice (29). Further studies demonstrated that insertional inactivation of the *psaA* gene in the type 2 strain D39 significantly reduced its virulence in mice in both an intranasal- and an intraperitoneal-challenge model (3). These observations have led to the suggestion that PsaA might be an effective vaccine antigen in humans. Also, mutation of *psaD*, a gene immediately downstream of *psaA*, resulted in a small but significant difference in virulence relative to that of the parental strain, D39, in a low-dose intraperitoneal-challenge model, suggesting that PsaD may also contribute to pathogenesis (3).

psaABC encodes an ATP-binding cassette (ABC) manganese permease; *psaD* is part of the *psa* operon and encodes a putative thiol peroxidase (4). PsaA exhibits amino acid sequence similarity to several streptococcal lipoproteins, including ScaA from *Streptococcus gordonii* (16), SsaB from *Streptococcus sanguinis* (9), and FimA from *Streptococcus parasanguinis* (8). These proteins all appear to have direct or indirect roles in promoting bacterial adhesion to host cells. Sequence analysis of PsaA and related proteins has demonstrated that they form a novel class of bacterial solute binding proteins, the cluster IX family (6). Further experiments have shown that these solute binding proteins are components of ABC-type permeases that transport divalent cations such as Zn(II), Fe(II), and Mn(II) across the cytoplasmic membrane (6). There is strong physiological evidence that PsaA is involved in the transport of Mn(II); a mutation in *psaA* in *S. pneumoniae* led to an absolute requirement of Mn(II) for growth (6). Manganese is increasingly recognized as a key metal in bacterial cellular physiology, particularly in relation to the oxidative-stress response (13). In *Salmonella enterica* serovar Typhimurium, the *mntH* gene has been demonstrated to encode a secondary transporter for Mn(II) (15). Transcription of *mntH* was induced by hydrogen peroxide, and *mntH* mutants were found to be susceptible to peroxide killing. Recently, for *Neisseria gonorrhoeae* a putative ABC permease with a periplasmic binding protein (MntC) that was closely related to PsaA and other proteins of the cluster IX family of solute binding proteins was identified (30). A mutation in *mntC* significantly decreased the uptake of Mn(II) into the gonococcus, a finding consistent with a role for this protein in Mn(II) uptake. However, it was also observed that *mntC* mutants were highly sensitive to killing by superoxide compared to wild-type cells. This observation raised the possibility that the ABC-type Mn(II) permeases might have a key role in the protection of the bacterial cell against oxidative killing.

In this paper, we report the effects of oxidative stress on *psaA* and *psaD* mutants of *S. pneumoniae* and the induction of enzymes involved in the oxidative-stress response.

Sensitivity of ***psaA*** **and** ***psaD*** **mutants to oxidative killing.** Construction of *psaA* and *psaD* insertion duplication mutants of *S. pneumoniae* strain D39 has been described previously (3). These cells were grown on blood agar for 16 h and resuspended in Todd-Hewitt broth with 1% yeast extract. A total of 10^4 cells were exposed to 60 mM paraquat (Sigma) over a 2-h period. At time intervals, samples were taken and the number of viable cells was determined by plating onto blood agar. Figure 1 shows that over the 2-h period, there was a slow loss of the viability of wild-type cells. In control experiments without the addition of paraquat, no loss of viability was observed (data not shown). In contrast, the *psaA* mutant was highly

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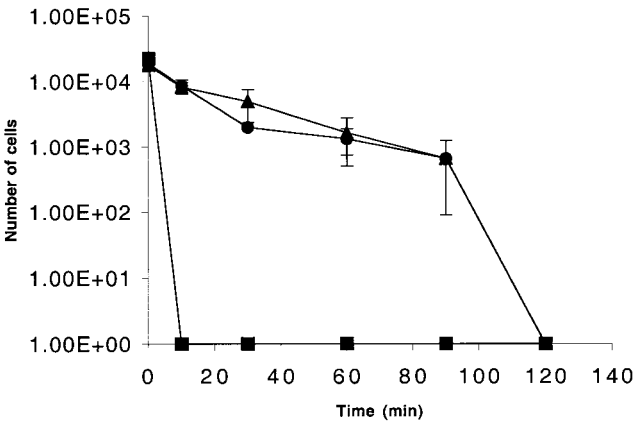

FIG. 1. Paraquat killing of the *S. pneumoniae* D39 wild-type strain (circles) and *psaA* (squares) and *psaD* (triangles) mutants. Experiments were performed in triplicate. Error bars indicate ±1 standard deviation of the mean.

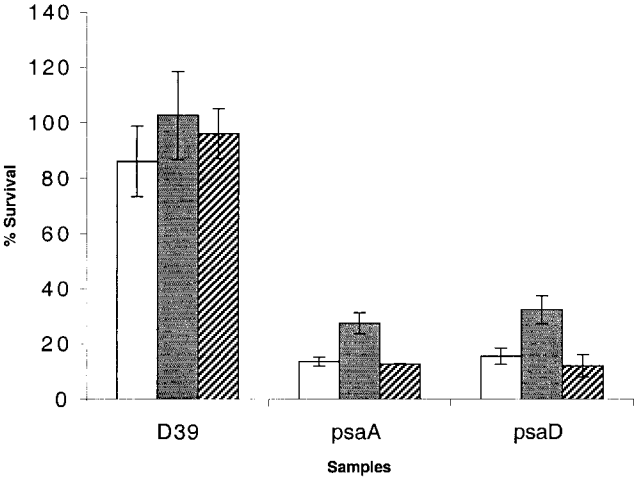

FIG. 2. H_2O_2 survival test of the *S. pneumoniae* D39 wild-type strain and *psaA* and *psaD* mutants. In the assay, cells were grown on blood agar (open columns), blood agar plus 100 μM $MnSO_4$ (filled columns), or blood agar plus 100 μM $FeCl_2$ (hatched columns). Experiments were performed in triplicate. Error bars indicate ±1 standard deviation of the mean. Student's *t* test was performed to determine the statistical significance among different samples. The *psaA* and *psaD* mutants grown on Mn(II) survived at significantly higher rates than did those grown without any supplement ($P = 0.01414$ and 0.01352, respectively).

susceptible to killing by paraquat, and killing was essentially complete after about 10 min of exposure to this reagent (Fig. 1). However, the *psaD* mutants exhibited a level of sensitivity to paraquat that was similar to that of the wild type, D39 (Fig. 1). This may explain why the *psaA* mutant showed a significant reduction in virulence while the *psaD* mutant showed only a small reduction in virulence in the mouse model (3). Paraquat is a redox compound that is reduced by low-potential electron donors inside the bacterial cell and is then oxidized by molecular oxygen, thereby generating superoxide in the cytoplasm (11). The futile redox cycling of paraquat generates a reactive oxygen species but has an additional effect on redox processes in the cell by depleting low-potential reducing agents, such as NADH (11). To determine whether the *psaA* and *psaD* mutants were sensitive to the superoxide anion outside of the cell, we used a different generator, 5 mM xanthine and 350 mU of xanthine oxidase (Sigma)/ml. The wild-type *S. pneumoniae* was highly susceptible to killing by the xanthine-xanthine oxidase system; no viable cells could be recovered after 1 h. However, exposure of cells to superoxide over a period of 10 min resulted in only a small loss in viability. In contrast to the results of experiments using paraquat as a generator of reactive oxygen species, the *psaA* and *psaD* mutants exhibited almost the same sensitivity to externally generated superoxide as wild-type D39 (result not shown). Taken together, these data suggested that the *psaA* and *psaD* mutants were more sensitive to oxidative killing than the wild-type cells but that this sensitivity was not directly associated with the superoxide anion.

***PsaA* and *psaD* mutants are sensitive to killing by hydrogen peroxide.** We reasoned that the difference in levels of susceptibility to paraquat between the two *psa* mutants and the wild-type cells might be associated with a failure of the mutants to cope with one of the products of superoxide dismutation, hydrogen peroxide (H_2O_2). In order to determine whether the *psaA* and *psaD* mutants were sensitive to H_2O_2, a survival test was carried out according to the method of Johnson et al. (14). Cells were grown on blood agar, blood agar plus 100 μM $MnSO_4$, or blood agar plus 100 μM $FeCl_2$ before being assayed. Then, cells were exposed to 40 mM H_2O_2 at room temperature for 15 min. Compared to the wild type (D39), the *psaA* and *psaD* mutants were very sensitive to H_2O_2 (Fig. 2). Since PsaA is a component of a potential Mn(II) permease, we tested whether Mn(II) could provide protection against killing by H_2O_2. We found that Mn(II) supplementation in the growth medium led to a twofold greater rate of survival. The survival rates of the *psaA* and *psaD* mutants grown on Mn(II) are significantly different from those observed without any supplement ($P = 0.01414$ and 0.01352, respectively; Student's *t* test). In contrast, Fe(II) did not provide any protection and possibly caused cells to be slightly more susceptible to killing.

Sensitivity of *psaA* mutants to oxidative killing is independent of the Mn(II) ion and SodA. Recently, it has been demonstrated that Mn(II) limitation lowers manganese superoxide dismutase (SodA) activity in *Streptococcus suis*, a finding consistent with the role of this ion in enzyme activity (19). Furthermore, a *sodA* mutant of *S. pneumoniae* is susceptible to paraquat killing (32). The possibility that hypersensitivity to oxidative killing in the *psaA* mutant was a consequence of Mn(II) limitation was therefore examined. Superoxide dismutase activity was measured by the method of Crapo et al. (5). Figure 3A shows that Mn(II) supplementation did not greatly alter the level of Sod activity in wild-type cells ($P = 0.4466$). However, in the *psaA* mutant, there was a 30% decrease in Sod activity in cells cultured without supplemented Mn(II) and there was a 40% decrease in Sod activity in the *psaD* mutant. This is consistent with the role of the PsaABC transport system in Mn(II) uptake.

An additional experiment was carried out to determine whether the reduced activity of SodA in Mn(II)-limited cells was correlated with increased sensitivity to oxidative killing. We grew *S. pneumoniae* on blood agar in the presence of 100

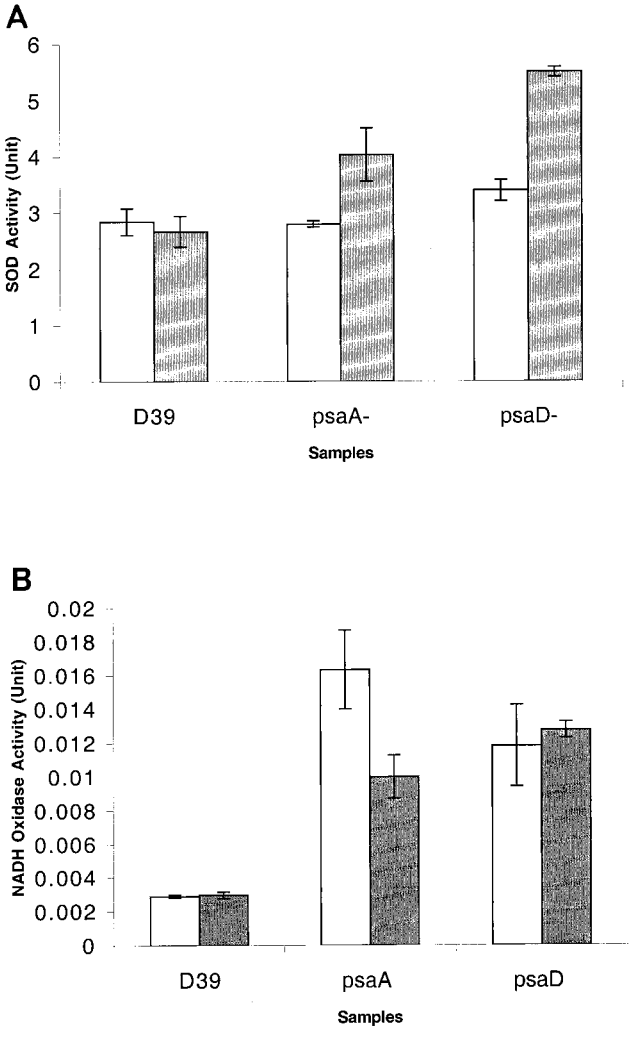

FIG. 3. Superoxide dismutase (SOD) (A) and Nox (B) activities in the *S. pneumoniae* D39 wild-type strain and *psaA* and *psaD* mutants. In this assay, cells were grown on blood agar (open columns) or blood agar plus 100 μM $MnSO_4$ (filled columns). Experiments were performed in triplicate. Error bars indicate ±1 standard deviation of the mean. Student's *t* test was performed to determine the statistical significance among different samples. Mn(II) supplementation did not significantly increase Sod activity in the wild-type cells ($P = 0.4466$). In the *psaA* and *psaD* mutants, Mn(II) significantly increased Sod activities ($P = 0.0444$ and 0.0006, respectively). The addition of Mn(II) did not significantly alter the levels of Nox production in the wild type or the *psaD* mutant ($P = 0.5925$ and 0.5705, respectively). In contrast, the *psaA* mutant had lower Nox activity when it was grown on Mn(II) than when it was grown without the supplement ($P = 0.0248$).

μM $MnSO_4$. Growth in the presence of Mn(II) did not affect resistance to oxidative killing in wild-type cells or the *psaA* or *psaD* mutants as measured by the paraquat or xanthine-xanthine oxidase killing assay; the results (not shown) were essentially the same as those shown in Fig. 1 for growth in the absence of exogenous Mn(II). Since the *psaA* mutant supplemented with Mn(II) had essentially the same level of sensitivity to paraquat challenge as the mutant without Mn(II), lowered SodA activity cannot explain the hypersensitivity of the *psaA* mutant to oxidative killing.

***psaA* and *psaD* mutants have elevated Nox activity.** Although streptococci are not able to respire by using oxygen as an electron acceptor (17), they are aerotolerant and possess enzymes that can reduce oxygen in non-energy-conserving reactions (12). These reactions help dissipate excess reducing power generated during fermentation reactions. Of particular importance is NADH oxidase (Nox), a flavoprotein that reduces oxygen to H_2O (33). Nox activities were measured by the method of Ahmed and Claiborne (1). We observed that the *psaA* mutant had a level of Nox activity approximately six times higher than that of the wild-type cells when both were grown in the absence of Mn(II) and that the *psaD* mutant had about fourfold-higher Nox activity than the wild type (Fig. 3B). Mn did not affect the amounts of Nox produced in the wild type and the *psaD* mutant ($P = 0.5925$ and 0.5705, respectively). In contrast, the *psaA* mutant had a lower level of Nox activity when it was grown on Mn than when it was grown without the supplement (Fig. 3B).

Discussion. The surface antigen PsaA has attracted much attention in pneumococcal research as a consequence of the observation that a *psaA* mutant is avirulent in a mouse model system (3) and that PsaA can act as a protective immunogen in mice (29). As a consequence, PsaA has been discussed as a potential vaccine candidate or drug target (21). However, the effect of a *psaA* mutation on pneumococcal physiology appears to be complex, and previous studies have not been in agreement (4, 20). In this paper, we have shown that *psaA* mutants are hypersensitive to killing by the oxidative-stress mediator paraquat. This sensitivity to oxidative killing may be a major contributor to the avirulence phenotype of *psaA* mutants, since such cells would be less likely to survive attacks by innate host defense systems.

Understanding the mechanisms of resistance to oxidative stress in bacteria is a well-developed field for which a variety of strategies have been described. With regard to the removal of superoxide, it appears that *S. pneumoniae* relies primarily on MnSod (SodA) (32). It has already been established that a *sodA* mutant of this bacterium has reduced intranasal virulence in mice (32). In *Streptococcus pyogenes*, MnSod is also secreted to the cell surface, where it can dismutate exogenous superoxide. In the xanthine-xanthine oxidase killing assay, superoxide is produced outside the cell, and it is interesting that wild-type *S. pneumoniae* and the *psaA* mutant showed essentially identical killing curves when superoxide was generated exogenously. This result contrasts with our observations for the gram-negative bacterium *N. gonorrhoeae*, in which a mutation in *mntC*, which is equivalent to *psaA*, caused cells to be far more sensitive to killing by superoxide produced by the xanthine-xanthine oxidase system than wild-type cells were (30). An additional difference between gonococcus and pneumococcus is that the latter has a thick peptidoglycan cell wall, and this may also help quench exogenous superoxide. Whatever the mechanism of protection against superoxide, it does not appear that a change in the level of activity of MnSod can account for the reduced level of resistance to oxidative stress seen in the *psaA* mutant challenged with paraquat. Sod activity levels were lower in the *psaA* and *psaD* mutants grown without Mn supplementation, indicating that the PsaABC transport system probably does have a key role in providing Mn(II) for the cell. However, since Mn supplementation did not provide protec-

tion against paraquat killing for the *psaA* mutant, it can be concluded that failure to protect against superoxide is not the primary cause of the sensitivity of these mutants to oxidative stress.

Hydrogen peroxide is another reactive oxygen species that can cause damage to DNA and other cell components. Hydrogen peroxide is produced by *S. pneumoniae* by means of superoxide dismutase and via the action of pyruvate oxidase (SpxB), an enzyme that is known to have an important role in virulence (27). There is evidence that hydrogen peroxide produced by *S. pneumoniae* is cytotoxic towards alveolar epithelial cells (in culture) and that this oxidizing agent may also limit the growth of competitive floras such as *Haemophilus influenzae* (23). Since *S. pneumoniae* lacks catalase, the question of how it defends itself against hydrogen peroxide is of critical importance in relation to oxidative stress for this bacterium. Ferrous iron [Fe(II)] potentiates the effects of hydrogen peroxide by reacting with it to form hydroxyl radical, a far more toxic and damaging reactive oxygen species (24). *S. pneumoniae* lacks a respiratory chain and is one of a small group of bacteria that may have no need of iron for growth (25). The presence of Mn(II) may help protect against hydrogen peroxide, since this ion has been shown to quench this molecule (28). The presence of Mn(II) did improve the survival of the *psaA* and *psaD* mutants in the hydrogen peroxide killing assay. However, even in the presence of Mn(II), both mutants were much more sensitive to hydrogen peroxide killing than were wild-type cells. Thus, the phenotype of *psaA* and *psaD* mutants is not simply a consequence of a loss of the ability to transport Mn(II) via a high-affinity transporter.

The reason why *psaA* and *psaD* mutants are so sensitive to oxidative killing is still not understood, but the availability of microarrays may very soon help define the cell components that are regulated via this system. One candidate is Dps-like peroxide resistance (Dpr), a protein that is recognized to be of central importance in the defense against hydrogen peroxide in *Streptococcus mutans* (31). In view of the profound effects of the *psaA* and *psaD* mutations on the defense against oxidative stress for *S. pneumoniae*, it seems likely that PsaA and PsaD are components of a signal transduction system involved in the response to oxidative stress. It is very interesting that Nox levels increased sixfold in the *psaA* mutant. Nox is required for optimal competence expression in aerobic cultures (2), since it seems to interact with the regulators of competence development *ciaRH* and *comCDE* at the transcriptional and posttranscriptional levels (7). The observation that Nox levels increase in a *psaA* mutant suggests that the avirulence phenotype of the *psaA* mutant is associated with a defect in redox homeostasis. Nox catalyzes the oxidation of two NADH molecules per oxygen molecule reduced (33). Excess Nox activity may result in a low ratio of NADH to NAD^+ that may have serious consequences for carbon metabolism in the cell. In *Lactobacillus lactis*, overexpression of Nox results in a low NADH/NAD^+ ratio, and this diverts pyruvate away from its conversion to lactate via lactate dehydrogenase and instead to other pathways (18). In the *S. pneumoniae psaA* mutant, the elevated Nox activity probably has a similar effect, and this will lead to excessive production of hydrogen peroxide by pyruvate oxidase, with its attendant killing effects (23).

In summary, the avirulence of the *psaA* mutant of *S. pneumoniae* appears to be linked to lowered resistance to oxidative stress. We suggest that PsaA and, possibly, PsaD are components of a signal transduction pathway that regulates redox homeostasis in *S. pneumoniae* and as a consequence influences the response of this bacterium to oxidative stress.

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