Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung

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The leading cause of mortality in patients with cystic fibrosis (CF) is respiratory failure due in large part to chronic lung infection with *Pseudomonas aeruginosa* strains that undergo mucoid conversion, display a biofilm mode of growth *in vivo* and resist the infiltration of polymorphonuclear leukocytes (PMNs), which release free oxygen radicals such as H₂O₂. The mucoid phenotype among the strains infecting CF patients indicates overproduction of a linear polysaccharide called alginate. To mimic the inflammatory environment of the CF lung, *P. aeruginosa* PAO1, a typical non-mucoid strain, was grown in a biofilm. This was treated with low levels of H₂O₂, as if released by the PMNs, and the formation of mucoid variants was observed. These mucoid variants had mutations in *mucA*, which encodes an anti-σ factor; this leads to the deregulation of an alternative σ factor (σ²², AlgT or AlgU) required for expression of the alginate biosynthetic operon. All of the mucoid variants tested showed the same mutation, the mucA²² allele, a common allele seen in CF isolates. The mucoid mucA²² variants, when compared to the smooth parent strain PAO1, (i) produced 2-6-fold higher levels of alginate, (ii) exhibited no detectable differences in growth rate, (iii) showed an unaltered LPS profile, (iv) were ~72% reduced in the amount of inducible-β-lactamase and (v) secreted little or no LasA protease and only showed 44% elastase activity. A characteristic ~54 kDa protein associated with alginate overproducing strains was identified as AlgE (Alg76) by N-terminal sequence analysis. Thus, the common phenotype of the mucoid variants, which included a genetically engineered mucA²² mutant, suggested that the only mutation incurred as a result of H₂O₂ treatment was in mucA. When a *P. aeruginosa* biofilm was repeatedly exposed to activated PMNs *in vitro*, mucoid variants were also observed, mimicking *in vivo* observations. Thus, PMNs and their oxygen by-products may cause *P. aeruginosa* to undergo the typical adaptation to the intractable mucoid form in the CF lung. These findings indicate that gene activation in bacteria by toxic oxygen radicals, similar to that found in plants and mammalian cells, may serve as a defence mechanism for the bacteria. This suggests that mucoid conversion is a response to oxygen radical exposure and that this response is a mechanism of defence by the bacteria. This is the first report to show that PMNs and their oxygen radicals can cause this phenotypic and genotypic change which is so typical of the intractable form of *P. aeruginosa* in the CF lung. These findings may provide a basis for the development of anti-oxidant and anti-inflammatory therapy for the early stages of infection in CF patients.

Keywords: *Pseudomonas aeruginosa*, alginate, biofilm, PMN, oxygen radicals, cystic fibrosis

Abbreviations: CF, cystic fibrosis; OMP, outer-membrane protein; PMN, polymorphonuclear leukocyte.
The GenBank accession number for the sequence reported in this paper is L14762.
INTRODUCTION

Cystic fibrosis (CF) is a common autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a phosphorylation-regulated Cl− channel in the apical membrane of involved epithelia (Hilman, 1997; Sierra & Collins, 1993). The disease is characterized by bacterial colonization and chronic airway infection that progressively destroys the lung and often leads to respiratory failure. The most common pathogen responsible for the morbidity and mortality seen in these patients is Pseudomonas aeruginosa (Koch & Høiby, 1993). There is evidence that the CFTR contributes to host defence by acting as a bacterial ligand and thus the CF defect may prevent normal clearance of P. aeruginosa from the respiratory tract (Pier et al., 1996). Bactericidal activity against P. aeruginosa has been shown to be reduced in the CF lung environment due to elevated levels of NaCl (Smith et al., 1996). A feature of the complex CF respiratory environment that is not well understood is the selection pressure for mucoid conversion common to P. aeruginosa strains that thrive in this environment. The initial and intermittent colonization of the CF lungs by P. aeruginosa can be eradicated by early aggressive antibiotic therapy (Frederiksen et al., 1997). However, when the colony morphology of bacteria isolated from sputum samples is observed to convert to the mucoid form, the organisms can no longer be eliminated from the lungs, despite aggressive antibiotic therapy (Frederiksen et al., 1997). This mucoid conversion, readily observed in the colony morphology of the organisms found in sputum, is indicative of the overproduction of a capsule-like polysaccharide called alginate (Evans & Linker, 1973). The potential roles of this exopolysaccharide in pathogenesis include a mechanism for bacterial adherence, a barrier to phagocytosis and a mechanism to neutralize oxygen radicals (for a review, see Govan & Deretic, 1996). Alginate also affects leukocyte functions, such as the oxidative burst and interference with opsonization, and plays an immunomodulatory role via induction of proinflammatory cytokines and suppression of lymphocyte transformation (Pedersen, 1992; Pedersen et al., 1992).

The CF pulmonary tract is typically clogged with a viscid sputum containing a large number of polymorphonuclear leukocytes (PMNs) (Høiby et al., 1993) and it has been widely speculated that this environment of dehydration and high osmolarity may contribute to the emergence of mucoid Pseudomonas (Berry et al., 1980; DeVault et al., 1989; Jensen et al., 1992; Hull et al., 1997). During phagocytosis of the bacteria, free oxygen radicals are produced which generate oxidative stress and lead to further inflammation (Brown et al., 1995; Hull et al., 1997).

In this study, we explored the role of activated PMNs and their release of toxic oxygen by-products in the CF lung environment, in the generation and/or selection of mucoid variants during the inflammatory response to P. aeruginosa that exhibits a biofilm mode of growth.

METHODS

Bacterial strains, plasmids and media. P. aeruginosa strains used in this study were the prototypic non-mucoid strain PA01 (Holloway & Morgan, 1986) and an alginate-producing CF isolate FRD1 (DeVries & Ohman, 1994). Escherichia coli strain HB101 [F Δ(gpt-proA)62 leuB6 supE44 ara-14 galK2 lacY1 Δ(mcrC-mrr) rpsL2O (StrR) xyl-5 mtl-1 recA13] (Boyce & Rouillard-Dussoix, 1969) was used. E. coli HB101 harbouring pRK2013 [ColElori-Tra (RK2)O, KmR] was used as a conjugative helper plasmid strain in triparental matings (Gurviss & Helinski, 1979). Both E. coli and P. aeruginosa were routinely cultured in L broth (1 g tryptone, 5 g yeast extract, 5 g NaCl). LA/PIA, used in triparental matings, was a 1:1 mix of Pseudomonas Isolation Agar (Difco) and L agar. A defined AB medium (15 mM (NH4)2SO4, 34 mM Na2HPO4·2H2O, 22 mM KH2PO4, 51 mM NaCl, 4 mM sodium citrate, 1 mM MgCl2, 0.1 mM CaCl2, 0.01 mM FeEDTA, pH 7.0) was used to grow cells in flow chambers. To provide an early indication of the mucoid phenotype of P. aeruginosa, blue agar plates were used (Høiby, 1975). Sucrose sensitivity was tested on L agar plates containing 5% sucrose. Antibiotics were used at the following concentrations unless otherwise indicated (µg ml−1): ampicillin, 50; kanamycin, 30; carbenicillin, 300.

Isolation of PMNs. PMNs were isolated from citrated human peripheral blood from normal healthy individuals by dextran sedimentation and Lymphoprep (Nyegaard) gradient centrifugation as described previously (Jensen & Kharazmi, 1991). Erythrocytes were removed by hypotonic lysis and PMNs were resuspended in Kreb-Ringer solution (130 mM NaCl, 5 mM KCl, 0.9 mM CaCl2, 1.2 mM MgCl2, 15 mM NaH2PO4, pH 7.2) containing 1% human serum albumin at a cell concentration of 2×106 cells ml−1.

Biofilm mode of growth. Cultures of P. aeruginosa strain PA01 were grown in an apparatus containing dual continuous-flow chambers with a glass surface for bacterial adherence (Fig. 1). Procedures used were as described by Wolfardt et al. (1994). AB medium was passed through flow cells at 0.2 mm s−1. P. aeruginosa was inoculated upstream of the growth chamber as a single 0.5 ml pulse (~5×106 cells in stationary phase), filling the chamber while the medium pump was off. The flow was resumed 1 h after inoculation. After 24 h incubation at 37 °C, the growth of the biofilm was...
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Monitored using a phase-contrast microscope and a single-layer biofilm was observed. One biofilm of *P. aeruginosa* in the apparatus was subjected either to human peripheral blood PMNs or to sublethal concentrations of \( \text{H}_2\text{O}_2 \) as described in Methods. The other chamber in the apparatus served as an untreated control.

**Construction of a mucA22 mutant of *P. aeruginosa* PAO1.** Plasmid pKM180 (Fig. 2) was formed by cloning a 2.0 kb EcoRI–HindIII DNA fragment containing the mucA22 allele from the alginate-producing CF isolate FRD1 (DeVries & Ohman, 1994) into the blunt-ended SmaI site of pEX100T (Schweizer & Hoang, 1995), a mobilizable suicide plasmid containing a sacB gene that confers sucrose sensitivity in *P. aeruginosa*. This plasmid was transferred into strain PAO1 from *E. coli* and the merodiploids that formed by homologous recombination with the chromosome were selected with carbencillin (i.e., 300 \( \mu \)g ml\(^{-1} \)) resistance conferred by *bla*). Such strains were then grown without selection to permit a second cross-over to occur, which excised the plasmid and left the mutant allele in the chromosome. Colonies appearing on agar plates containing sucrose to select for loss of the plasmid-encoded *sacB* were screened for the mucoid phenotype and carbencillin sensitivity. The presence of the mucA22 mutation in one mucoid derivative (PDO300) was confirmed by DNA sequence analysis.

**Measurement of \( \beta \)-lactamase activity.** Strains were grown in induction in the presence of benzylpenicillin (500 mg l\(^{-1} \) in L broth) for 2.5 h, lysed by sonication and assayed for \( \beta \)-lactamase activity using a spectrophotometric assay as previously described by Campbell et al. (1997) and Giwercman et al. (1991).

**Protease activity.** Strains were incubated at 37 °C in L broth with rapid aeration for 18 h under standardized conditions and cells were removed by centrifugation. LasA protease (staphylolytic) activity was determined in a spectrophotometric assay, essentially as described by Kessler et al. (1993) by measuring the rate of lysis of a suspension of *Staphylococcus aureus* cells. LasB protease (elastase) activity was determined in a spectrophotometric assay using elastin-Congo red (Sigma) as a substrate as described by Ohman et al. (1980).

**RESULTS**

**Isolation of Alg \(^{+} \)** variant after repeated exposure of *P. aeruginosa* PAO1 biofilm to sublethal concentrations of \( \text{H}_2\text{O}_2 \)

We hypothesized that activated PMNs and their release of toxic oxygen by-products in the CF lung environment could play a role in the generation and/or selection of mucoid variants during the inflammatory response to *P. aeruginosa*. The biofilm mode of growth by *P. aeruginosa* in the CF lung may play an important role in enhancing this response to reactive oxygen intermediates released by activated PMNs. To mimic the biofilm mode of growth seen in vivo, *P. aeruginosa* PAO1 (a commonly used wild-type strain) was grown in continuous-flow culture chambers (i.e., flow-cells; Fig. 1) as described by Wolfaedt et al. (1994). Responses to oxidative stress, leading to the expression of specific genes, is known to occur by a variety of mechanisms in prokaryotic and
variants obtained by gene replacement (see Fig. 2). 'H₂O₂-induced' represents the eight mucA22 derivatives obtained by treatment of PA01 in a biofilm (see Fig. 1), which all produced approximately the same results in these tests.

† One milliunit of β-lactamase activity is defined as the amount of enzyme required to hydrolyse 1 nmol nitrocefin min⁻¹ (mg protein)⁻¹. All strains showed basal uninduced β-lactamase levels of 12-16.5 μU.

Table 1. Phenotypic comparison of P. aeruginosa wild-type PA01 and its mucA22 variants obtained by gene replacement (PD0300) or H₂O₂ treatment of a biofilm

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Genotype</th>
<th>Alginate [μg (ml culture supernatant)⁻¹]</th>
<th>Induced β-lactamase levels (mU)†</th>
<th>LasA protease [A₄₅₀ min⁻¹ (mg protein)⁻¹]</th>
<th>LasB protease [A₄₅₀ min⁻¹ (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA01</td>
<td>Wild-type</td>
<td>ND</td>
<td>234.5±40.3</td>
<td>128.1±7.4</td>
<td>15.7±7.3</td>
</tr>
<tr>
<td>PD0300</td>
<td>mucA22</td>
<td>170.5±9.4</td>
<td>66.0±20.2</td>
<td>160.6±16</td>
<td>5.7±2.4</td>
</tr>
<tr>
<td>H₂O₂-induced</td>
<td>mucA22</td>
<td>229.1±59.5</td>
<td>63.2±12.8</td>
<td>11.9±4.7</td>
<td>5.1±1.7</td>
</tr>
</tbody>
</table>

ND, Not detected.

* P. aeruginosa PA01 is a non-mucoid wild-type strain. PD0300 is a mucA22 derivative of PA01 constructed by gene replacement (see Fig. 2). 'H₂O₂-induced' represents the eight mucA22 derivatives obtained by treatment of PA01 in a biofilm (see Fig. 1), which all produced approximately the same results in these tests.

† One milliunit of β-lactamase activity is defined as the amount of enzyme required to hydrolyse 1 nmol nitrocefin min⁻¹ (mg protein)⁻¹. All strains showed basal uninduced β-lactamase levels of 12-16.5 μU.

Mucoid conversion is due to a mutation in mucA encoding an anti-σ factor

When P. aeruginosa strains infecting CF patients convert to the mucoid phenotype in vivo, they frequently display a mutation in the gene encoding MucA (Martin et al., 1993). MucA is an anti-σ factor that controls the activity of σ²², a 22 kDa alternative σ factor encoded by algT (also known as algU) (DeVries & Ohman, 1994; Hershberger et al., 1995; Martin et al., 1994). To examine whether the mucoid variants of P. aeruginosa obtained by H₂O₂ treatment also had mucA defects, the chromosomal DNA from this region of these strains was amplified by PCR and subjected to sequence analysis. The results showed that in eight mucoid variants examined (obtained in two independent experiments), all had the same mutation; this was a deletion of a G residue in a string of five G residues located at 426–430 bp in the mucA ORF (Fig. 2). This gives rise to premature termination of translation, resulting in the production of a truncated MucA protein.

Construction of a PA01 derivative with a mucA22 allele

To provide an isogenic control strain for comparison between PA01 and the mucoid variants isolated in the biofilm experiment, a gene replacement technique was used to generate a mucA22 derivative of PA01 called PD0300 (Fig. 3). This was accomplished by cloning a fragment containing the mucA22 allele from the alginate-producing CF isolate FRD1 (DeVries & Ohman, 1994) into pEX100T (Schweizer & Hoang, 1995), a mobilizable suicide plasmid containing sacB that confers sucrose sensitivity in P. aeruginosa. This plasmid was transferred into strain PA01 from E. coli and the merodiploids that formed by homologous recombination with the chromosome were selected with carbenicillin. A second cross-over occurred when these strains were grown without selection, which excised the plasmid and left the mutant allele in the chromosome. Colonies exhibiting a mucoid phenotype, sucrose resistance and carbenicillin sensitivity were isolated. DNA sequence analysis of one mucoid derivative, PD0300, confirmed the presence of the mucA22 mutation.
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Alginate was overproduced by PDO300 (170 μg alginate ml⁻¹) whereas the parental PAO1 made virtually none (Table 1).

**Phenotypic characteristics of the alginate-producing variants**

Compared to *P. aeruginosa* strains from environmental and non-CF clinical sources, the strains responsible for chronic pulmonary disease in CF patients often display a number of interesting phenotypic abnormalities [e.g. alginate overproduction, altered LPS, new outer-membrane proteins (OMPs), altered antibiotic sensitivities, etc.]. We examined whether the *mucA*₂² mucoid variants derived from exposure to H₂O₂ might show similar phenotypic changes. The rates of growth in L broth of all the *mucA*₂² mucoid strains were virtually identical and a PFGE analysis of chromosomal DNA revealed no obvious genetic rearrangement (data not shown). The LPS ladders observed after SDS-PAGE were unchanged in the *mucA*₂² variants of both H₂O₂ treatment and genetic manipulation when compared to the smooth strain, PAO1 (data not shown). The antibiotic resistance profile of *mucA*₂² variants was identical to that observed with PAO1 (antibiotics tested: piperacillin, aztreonam, ceftazidime, imipenem, meropenem, colistin, ciprofloxacin, ofloxacin, tobramycin and netilmicin; data not shown).

The amount of inducible-β-lactamase is reduced in the alginate-producing variants

*P. aeruginosa* isolates from CF patients typically produce high levels of chromosomal β-lactamase, a group 1 cephalosporinase encoded by *ampC* (Campbell et al., 1997; Giwercman et al., 1991). Since most of the mucoid CF isolates also harbour a mutation in *mucA*, we wanted to determine if high-level β-lactamase expression is associated with this particular mutation. However, all of the *mucA*₂² variants produced a low basal level of β-lactamase that was inducible. In addition, the induced level of β-lactamase (following treatment with 500 μg benzylpenicillin ml⁻¹) was reduced to ~72% in all the alginate-producing variants (Table 1).

Protease production is reduced in the alginate-producing variants

*P. aeruginosa* secretes a number of proteases, including elastase, LasA (staphyloytic) protease and alkaline protease (Kharazmi, 1989; Morihara & Homma, 1985). The *mucA*₂² variants were characterized for elastolytic and LasA protease activities relative to the wild-type
PAO1. Optimal culture conditions for protease production and measurement were used to evaluate extracellular levels of elastolytic and LasA protease activities. Analysis of the mucoid mucA22 variants of PAO1 showed that elastolytic activity was reduced by ~66% as compared to parental PAO1 and the presence of LasA protease was barely detectable (Table 1).

**Outer-membrane profiles of the alginate-producing variants showed the presence of a ~54 kDa protein**

Alginate-overproducing strains have been shown to have OMP profiles with a prominent ~54 kDa protein which is not observed in non-mucoid strains (Goldberg & Ohman, 1987; Grabert et al., 1990). This characteristic protein was also observed in all of the mucA22 mucoid variants and absent in non-mucoid PAO1 (Fig. 4). We performed an N-terminal sequence analysis on the purified protein which produced the sequence: Xaa-Asn-Ser-Gly-Glu-Ala-Pro-Lys-Xaa-Phe-Gly-Leu-Asp-Val-Lys-Ile-Thr. Apart from Asn at position 2, this sequence corresponds to the N terminus of AlgE (Alg76) previously identified in *P. aeruginosa* (Chu et al., 1991). The discrepancy in the amino acid composition may be due to strain differences. The comparison of OMP profiles between the mucA22 mucoid variants and the non-mucoid parent PAO1 also showed that the expression of several OMPs is up-regulated, although the expression of some is down-regulated.

**Isolation of Alg⁺ variants after repeated exposure of a *P. aeruginosa* PAO1 biofilm to activated PMNs**

A direct approach was employed to test our hypothesis that activated PMNs, by release of toxic oxygen by-products, are responsible in the CF lung environment for the generation and/or selection of mucoid variants during the inflammatory response to *P. aeruginosa*. A PAO1 biofilm was then repeatedly exposed to activated human peripheral blood PMNs. Bacteria from the flow cell effluent were plated onto agar, incubated and screened for mucoid colony formation. Several mucoid variants were isolated in two independent experiments in the culture treated with PMNs. No mucoid colonies appeared from control chambers where biofilms were not challenged. These PMN-derived mucoid variants exhibited the same phenotype as the mucA22 mucoid variants derived from exposure to H₂O₂ (data not shown). This further supports the hypothesis that mucoid conversion of *P. aeruginosa* in the CF lung may result from interaction with PMNs.

**DISCUSSION**

In this study, we provide evidence that activated PMNs and their release of toxic oxygen by-products in the CF lung environment may play a role in the generation and/or selection of mucoid variants during the inflammatory response to *P. aeruginosa* which is typically present in a biofilm. We were able to mimic the biofilm mode of growth seen in vivo by growing *P. aeruginosa* PAO1 in continuous-flow culture chambers (Fig. 1). Mucoid variants of PAO1 were isolated from a biofilm that was repeatedly exposed to activated human peripheral blood PMNs and sublethal concentrations of H₂O₂.

*P. aeruginosa* strains infecting CF patients often convert to the mucoid phenotype in vivo and these strains frequently have mutations in the gene encoding MucA (Martin et al., 1993). MucA is an anti-σ factor, located in the inner membrane, which is part of a protein complex that controls the activity of σ²², a 22 kDa alternative σ factor encoded by algT (algU) (Mathee et al., 1997). σ²² is a member of the extracytoplasmic function subfamily of eubacterial RNA polymerase σ factors involved in the regulation of extracytoplasmic functions (DeVries & Ohman, 1994; Hershberger et al., 1995; Lonetto et al., 1994; Martin et al., 1994). The loss of MucA protein results in the deregulation of σ²² and leads to the activation of σ²²-dependent promoters, including those for the alginate biosynthetic genes (Mathee et al., 1997; Schurr et al., 1996; Xie et al., 1996). The mucoid variants of *P. aeruginosa* obtained by H₂O₂ treatment also had a mucA defect – deletion of a G residue in a string of five G residues located at 426–430 bp in the mucA ORF. Even though other mutations in mucA could theoretically produce the same effect, this particular region seems to be a hot-spot for mutations, probably due to slip-strand mispairing in this string of G residues during DNA replication. A premature termination codon in mucA was thus formed as a result of the deletion, leading to the formation of a MucA protein truncated in the C-terminal domain. This results in the loss of the periplasmic domain of this transmembrane protein, and thus the anti-σ factor activity of MucA on σ²² is lost (Mathee et al., 1997; Schurr et al., 1996; Xie et al., 1996). Interestingly, 25% of mucoid clinical isolates from CF patients also show this same G residue deletion, which is known as the mucA22 allele (Boucher et al., 1997).

To compare the phenotypic differences of the isolated mucoid variants with a PAO1 derivative that only differed in the mucA allele, an isogenic strain was constructed by homologous recombination between a DNA fragment containing the mucA22 allele from alginate-producing CF isolate FRD1 and PAO1 (DeVries & Ohman, 1994). The presence of the mucA22 mutation in the Alg⁺ strain (PDO300) was confirmed by DNA sequencing. Inactivation of mucA in non-mucoid *P. aeruginosa* PAO1 was sufficient to cause constitutive alginate production; this result and data from others (Martin et al., 1993), confirm that MucA functions as a negative regulator of alginate production.

All investigations of the isolated Alg⁺ variants isolated in the flow-cell experiments were run in parallel with analysis of the genetically engineered mutant strain, PDO300. The Alg⁺ variants made 2–6-fold higher levels of alginate than the parent strain (Table 1). Alginate production has been associated with altered rates of
growth and altered LPS. The Alg\(^{+}\) variants exhibited no detectable differences in growth rate. Most mucoid strains of CF origin are rough or semi-rough due to an altered LPS with shortened O side-chains (Haiby et al., 1993). However, the LPS profile was unchanged in the mucA22 variants when compared to the smooth parent strain, PAO1. Thus neither a mutation in mucA nor alginate production were sufficient to promote the altered LPS profile observed in many mucoid CF isolates.

Alginate production has also been implicated in the development of increased resistance to antibiotics in CF isolates. P. aeruginosa isolates from CF patients typically produce high levels of chromosomal \(\beta\)-lactamase (group 1 cephalosporinase, encoded by \textit{ampC}) which has been attributed to the major resistance mechanism seen in chronic infections (Campbell et al., 1997; Giwercman et al., 1991). The amount of inducible-\(\beta\)-lactamase was reduced to \(\approx 72\%\) in all the alginate-producing variants (Table 1), so the resistance phenotype typical of CF strains is apparently not associated with the mucA22 allele. The inability to attain full inducibility in the mucoid variants may be due to the presence of capsular-like polysaccharide that prevents benzylpenicillin from entering the cells.

\textit{P. aeruginosa} secretes a number of proteases which are believed to play a major role in the pathogenesis of this organism, especially during acute infections (Kharazmi, 1989; Morihara & Homma, 1985). These proteases, elastase, LasA (staphyloolytic) protease and alkaline protease, have been inversely correlated with alginate production (Ohman & Chakrabarty, 1982). The mucoid mucA22 variants of PAO1 showed little or no secretion of LasA protease and only \(44\%\) elastase activity compared to parent strain PAO1 (Table 1). However, it is not clear if this phenotype is associated directly with the mutation in mucA or indirectly through another gene activated by \(\sigma^{22}\), the alternative \(\sigma\) factor.

The OMP profiles of alginate-overproducing strains show the presence of a prominent \(\approx 54\) kDa protein which is absent in non-mucoid strains (Goldberg & Ohman, 1987; Grabert \textit{et al.}, 1990). There is some evidence that the \(54\) kDa protein could be the product of \textit{algE} (Rehm \textit{et al.}, 1994). \textit{algE} is transcribed as part of an \(18\) kb operon of biosynthetic genes and its product may be involved in polymer export to the bacterial surface (Chu \textit{et al.}, 1991; Rehm \textit{et al.}, 1994). The \(54\) kDa protein was observed in all Alg\(^{+}\) variants of PAO1 (Fig. 4) and we showed directly that this protein is the \textit{algE} gene product. The OMP profile analysis also showed that the mucA22 defect (which deregulates \(\sigma^{22}\) activity) led to changes in the expression of several OMPs, some of which were up-regulated and others were down-regulated. Experiments are under way to discern the nature of these proteins.

The results described above showed that activated PMNs, or \(\text{H}_2\text{O}_2\) released by these cells, can cause mucoid conversion of \textit{P. aeruginosa} when growing in the biofilm mode as it does in the CF lung. Mucoid conversion following \(\text{H}_2\text{O}_2\) treatment was associated with the generation of the mucA22 allele, a common mutation that occurs when \textit{P. aeruginosa} undergoes mucoid conversion in the CF lung. Mutations in mucA, which encodes an anti-\(\sigma\) factor, lead to the deregulation of an alternative \(\sigma\) factor (\(\sigma^{22}\), AlgT or AlgU) required for expression of the alginate biosynthetic operon. Phenotypic characterization of the mucoid variants, which included a genetically engineered mucA22 mutant, showed that all had similar characteristics and suggested that the only mutation incurred as a result of \(\text{H}_2\text{O}_2\) treatment was in mucA.

We propose that mucoid conversion is a response to exposure to toxic oxygen by-products and that this response is probably a mechanism of defence by the bacteria. It has been shown previously that \(\text{H}_2\text{O}_2\) can induce gene activation in plants and mammalian cells. The activated genes have been proposed to protect the cells against the toxicity of reactive oxygen species (Scandalios, 1997; Shirasu \textit{et al.}, 1996; Sundaresan \textit{et al.}, 1995). Our findings demonstrate a similar phenomenon in bacteria. Furthermore, these findings provide evidence for the first time suggesting that \(\text{H}_2\text{O}_2\) released by PMNs, the predominant cells of inflammation, is an important factor in the conversion of \textit{P. aeruginosa} to the intractable mucoid form in the CF lung. Additional studies would be warranted to elucidate the definitive role of PMNs in the mucoid conversion. Our findings reveal new possibilities in the design of preventive and therapeutic measures using antioxidants and anti-inflammatory agents in CF patients with \textit{P. aeruginosa} infections.

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