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Outer Membrane Protein P5 Is Required for Resistance of Nontypeable Haemophilus influenzae to Both the Classical and Alternative Complement Pathways

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The complement system is an important first line of defense against the human pathogen Haemophilus influenzae. To survive and propagate in vivo, H. influenzae has evolved mechanisms for subverting this host defense, most of which have been shown to involve outer surface structures, including lipooligosaccharide glycans and outer surface proteins. Bacterial defense against complement acts at multiple steps in the pathway by mechanisms that are not fully understood. Here we identify outer membrane protein P5 as an essential factor in serum resistance of both H. influenzae strain Rd and nontypeable H. influenzae (NTHi) clinical isolate NT127. P5 was essential for resistance of Rd and NT127 to complement in pooled human serum. Further investigation determined that P5 expression decreased cell surface binding of IgM, a potent activator of the classical pathway of complement, to both Rd and NT127. Additionally, P5 expression was required for NT127 to bind factor H (FH), an important inhibitor of alternative pathway (AP) activation. Collectively, the results obtained in this work highlight the ability of H. influenzae to utilize a single protein to perform multiple protective functions for evading host immunity.

Haemophilus influenzae is a pathogenic Gram-negative bacterium that colonizes the human nasopharynx and can invade the mucosal epithelium or disseminate to other sites, causing otitis media, upper and lower respiratory tract infections, and meningitis. A vaccine targeting the polyribosylribitol phosphate capsule of the most invasive serotype, H. influenzae type b (Hib), was introduced in the early 1990s, effectively reducing the incidence of Hib disease (1), although it remains significant in countries lacking vaccine coverage. Nontypeable Haemophilus influenzae (NTHi) strains lack an outer surface capsule and are therefore unaffected by the Hib vaccine (2). NTHi strains are important causes of sinusitis, conjunctivitis, and pneumonia (3, 4) and are the second most common cause of bacterial otitis media behind Streptococcus pneumoniae (5). NTHi strains are also among the most prevalent organisms found in the lungs of patients with exacerbations of chronic obstructive pulmonary disease (COPD) (5–8) and cystic fibrosis (CF) (9–11). Although NTHi strains are infrequently associated with invasive disease, and most instances of bacteremia occur in children with underlying medical issues (3, 12), emerging evidence suggests that healthy individuals are also at risk of invasive NTHi infection (13–17).

To survive in the host and cause disease, NTHi must defend itself against immune mechanisms. The complement system is an important first line of defense against invading pathogens that mediates lysis of Gram-negative bacteria through terminal complement, targets microbes for phagocytosis by opsonization, and stimulates the inflammatory response (18). Invasive NTHi strains are likely to encounter complement in blood, whereas in noninvasive infections, they are likely to be exposed to complement in the middle ear exudates during otitis media (19, 20), the nasopharyngeal mucosa during inflammation (21, 22), and the lungs during exacerbation of COPD and asthma (23). Moreover, recent evidence indicates that the ability of NTHi strains to resist killing by complement correlates with the severity of pulmonary and invasive disease (24). Thus, bacterial defense against complement appears to be an important feature of both invasive and noninvasive NTHi infections.

Complement activation on a pathogen may proceed through one or more of three pathways: the classical pathway, the mannose-binding lectin (MBL) pathway, or the alternative pathway (AP). All three pathways lead to the deposition of complement protein C3 on the microbial surface and subsequent clearance through phagocytosis of pathogens opsonized with C3 or lytic pathway activation (18). Classical pathway activation is initiated by immunoglobulin (select IgG subclasses or IgM) or C-reactive protein (CRP), bound to the surface of a pathogen (18, 25), whereas the lectin pathway is activated through binding of MBL or ficolins to select surface carbohydrates on microbes. Both pathways lead to the assembly of the classical C3 convertase C4b2a, which cleaves C3 and promotes downstream activation of the lytic pathway. The AP is activated by the cleavage of C3, which can be initiated through the action of the classical and lectin C3 convertases or by spontaneous hydrolysis of C3 (26). The C3b fragment released from the cleavage of C3 associates with a cleavage product of factor B, Bb, generating the AP C3 convertase. C3b generated by the C3 convertases can stimulate the production of more C3 con-
vertases, effectively amplifying the pathway. Much like the classical and MBL pathways, AP activation results in downstream lytic pathway effects and clearance of pathogens (18).

The mechanisms by which NTHi defends itself against host complement are not fully understood; however, current evidence implicates multiple cell surface structures. Lipooligosaccharide (LOS) glycans are critical for mediating this function, as mutations that truncate the LOS lead to severe defects in complement resistance and virulence in animal models (27–30). Surface proteins have also been shown to be involved, including P6, via an unknown mechanism, and proteins E and F, which were shown to bind the host complement regulator vitronectin (31, 32). Importantly, other complement regulators, such as factor H (FH), factor H-L-1, and C4-binding protein (C4BP), are also bound by NTHi as mechanisms of complement defense (31); however, the specific surface structures that mediate these interactions have not been elucidated (31).

We recently identified a role for the periplasmic disulfide oxidoreductase DsbA, an enzyme critical for maturation and stability of proteins exported to the cell surface containing disulfide bonds (33), in the resistance of *H. influenzae* to complement in human serum (34). Bioinformatic identification of putative DsbA substrates revealed a subset with potential roles in complement resistance (35); the outer membrane protein P5 was selected from this list as a candidate because it is ~50% identical to *Escherichia coli* outer membrane protein A (OmpA) (36), a factor previously implicated in adhesion of *H. influenzae* to various mucosal surface structures (42–46). However, a role for P5 in complement resistance has not been previously reported. In this study, we elucidate the mechanism of NTHi complement resistance mediated by P5.

**MATERIALS AND METHODS**

**Strains and culture conditions.** *H. influenzae* RdAW (referred to here as Rd) (GenBank accession no. NZ_ACSM00000000), a capsule-deficient serotype d derivative (47), and nontypeable *H. influenzae* strain NT127 (GenBank accession no. NZ_ACSL01000014.1), originally isolated from the blood of a child with meningitis (27, 48, 49), were grown in brain heart infusion (BHI) broth supplemented with 10 μg/ml hemin and 10 μg/ml NAD (sBHII) or on sBHII agar plates at 35°C. Development of competence for transformation of *H. influenzae* was accomplished as previously described (50). For selection of Rd- and NTHi-derived strains, the following antibiotics were used: 8 μg/ml tetracycline (Tc), 20 μg/ml kanamycin (Km), and 10 μg/ml gentamicin (Gm). For strain generation, plasmids and PCR products were constructed by using standard molecular biology techniques (51). For complementation of mutants, DNA fragments were amplified by PCR, cloned between adjacent SapI restriction sites of the chromosomal delivery vector pXT10, which does not introduce SapI sites in the termini of the fragments. The resulting 2,716-bp PCR product containing the Km resistance (Kmr) gene and homology to *H. influenzae* fragments including 932 bp of the 5′-end of the translational start site using primers 5′-GACCCGGTGTCACAACTCCTGTG-3′ and 3′-ACGTTAGTG-3′ were inserted into strain RP5G (Rd P5-bar) and RP5G (Rd P5-), respectively. Complementation of the mutations in RP5G and NTP5V was performed in the presence or absence of 10 mM Mg2⁺ EGTA to block calcium-dependent bacterial killing.

**Growth analysis.** Strains were cultured in triplicate in sBHI broth at 35°C for 16 h (starting inoculum of an optical density at 600 nm (OD₆₀₀) of 0.01) in a VersaMax, microplate reader (Molecular Devices, Sunnyvale, CA) set to read the absorbance at 600 nm every 10 min. Growth yields were obtained by calculating the averages and standard deviations of the final readings of each triplicate set of wells. Doubling times were determined by using nonlinear regression analysis with an R² value of >0.995 (Prism 5.03; GraphPad Software, La Jolla, CA) and are reported as the averages and standard deviations of each triplicate set of wells.

**Serum bactericidal assay.** The sensitivity of P5 mutants to serum was determined as previously described (52). Briefly, strains from log-phase cultures were diluted in Hanks’ balanced salt solution (HBSS) with 0.15 mM calcium and 1 mM magnesium (HBSS++) to 1.3 × 10⁶ CFU/ml and incubated at 37°C for 30 min with or without pooled normal human serum (NHS) from healthy anonymous donors aged 18 to 65 years (final concentrations are specified in the figures) (Innovative Research, Novi, MI) and plated onto sBHII agar for CFU enumeration. The reaction was also performed in the presence or absence of 10 mM Mg²⁺ EGTA to block the classical and lectin pathways and selectively activate the alternative pathway. Heat-inactivated serum used in this assay was generated by incubation of NHS at 56°C for 30 min. C1q-depleted sera and purified C1q were obtained from Complement Technologies, Inc. (Tyler, TX). Results of the serum bactericidal assay are reported as percent survival, which was calculated by dividing the CFU recovered from serum-treated samples by the CFU recovered from the sample that lacked serum. Statistical analyses
with the protein-coding region of aacC1, encoding gentamicin resistance, to generate nonpolar deletions in Rd and in NT127, a clinical NTHi strain isolated from the cerebrospinal fluid of a patient with meningitis (48). The amino acid sequence of P5 varies between strains (see Fig. S1 in the supplemental material). Therefore, complementation was achieved by expressing each strain’s respective P5 allele at the xylose locus, as previously described (47). The set of isogenic strains comprised the parent strain (Rd), an Rd P5 mutant (RPSG), a complemented Rd P5 mutant (RPSX), the NT127 parent strain carrying the “empty vector” (NTV), an NT127 P5 mutant carrying the empty vector (NTPSV), and a complemented NT127 P5 mutant (NTPSX) (Table 1).

The strains were evaluated for in vitro growth in rich media. Rd P5 mutant strain RPSG exhibited generation times and growth yields similar to those of parent strain Rd or complemented strain RPSX (Table 2). However, NT127 P5 mutant strain NTPSV exhibited 57% and 50% increases in generation time and 21% and 26% decreases in growth yield compared with parent strain NTV and complemented strain NTPSV, respectively (Table 2). These data suggest that P5 is important for optimal growth of NTHi strains but not for growth of Rd.

In considering P5 as a candidate mediator of complement resistance, it was important to evaluate potential indirect effects on cell surface composition and stability. The outer surface LOS structures are critical mediators of serum resistance of H. influenzae that could potentially be altered by P5 (27, 28, 57, 58). By silver staining of SDS-PAGE gels, the LOS bands were found to have similar mobility between Rd, RPSG, and RPSX or between NTV and NTPSV (see Fig. S2 in the supplemental material), suggesting that P5 does not mediate structural changes in the LOS. To evaluate potential effects of P5 on membrane stability, we examined whether the loss of P5 resulted in enhanced sensitivity to detergents. H. influenzae strains exposed to a range of SDS concentrations exhibited similar sensitivities at all doses (see Table S1 in the supplemental material). Similarly, no differences were detected in sensitivity to polymyxin B between strains Rd and RPSG (data not shown). Thus, P5 mutants resist membrane disruption by both a negatively and a positively charged detergent to the same extent as their wild-type counterparts, suggesting that P5 mutants are not defective for outer membrane stability.

P5 mutants exhibit increased sensitivity to killing by human serum. To investigate a potential role of P5 in complement resistance of H. influenzae, strains were assayed for survival in the presence of normal human serum (NHS). To exclude potential effects of variable growth rates between strains, serum bactericidal assays were performed with bacteria resuspended in HBSS++,
which prevents replication, and viability of all strains was unaffected by incubation in HBSS alone (data not shown). A range of serum concentrations was established for wild-type strains Rd and NTV. The average percent survival of Rd was 92.8% ± 21.0% in 1% NHS across 3 independent experiments, including the biological replicates shown in Fig. 1A as well as data from two other experiments (not shown) (n = 9), with an interquartile range of 72.6% to 114.3%. Rd yielded no colonies after incubation in serum at concentrations of 2% or higher. The average percent survival of NTV was 72.6% to 114.3%. Incubation with 3% NHS reduced the survival of the P5 mutant to less than the LLD of 0.25%, which was an average of 584-fold lower than that of the parent strain and 272-fold lower than that of the complemented strain at this concentration. Again, heat inactivation eliminated the bactericidal activity of NHS on all NTHi strains (data not shown). Together, these results indicate that P5 is required for complement resistance of both Rd and an NTHi clinical isolate.

C3 and C4 deposition on P5 mutants. The strains were next evaluated for complement C3 fragment deposition. Activation of C3 results in covalent binding of C3b to bacterial targets; C3b is then converted to iC3b by the actions of factor H and factor I. The amount of iC3b covalently bound to bacteria was measured by incubating strains in 5% NHS and performing Western blotting with a monoclonal antibody directed against a neoepitope on the α′ chain of iC3b, a cleavage product of C3b (54, 55). Detection of the α′ chain of iC3b covalently binds to bacterial targets (the 68-kDa α′ chain of iC3b migrates as a covalently linked complex with its target) by Western blotting permitted us to determine whether targets for C3b/iC3b deposition were altered by the loss of P5. The amount of C3 deposition on each mutant relative to that on the wild-type strain was evaluated by determining the total amount of iC3b bound by using densitometric analysis of visible bands in each lane. As expected, nonopsonized strain Rd or strain NTV controls did not produce detectable iC3b signals on Western blots (Fig. 2A). Deposition of iC3b on RP5G was increased by 1.5-fold compared to that on parent strain Rd and by 3-fold relative to that on complemented P5 mutant strain RP5X (Fig. 2A). It is not clear why complementation reduced iC3b binding to a level somewhat below that of the wild type; however, insertion of the gene encoding P5 (HI1164) at the xyl locus may lead to increased expression as a result of the change in genomic location. Similar to data obtained for Rd strains, iC3b deposition on NTP5V was increased 2.1-fold compared to that on its parent strain, NTV, and was increased 6.8-fold compared to that on complemented strain NTP5X (Fig. 2A). The targets for C3 fragments on the P5 knockout mutants were similar to those on the wild-type and complemented strains.

The classical pathway is important to initiate killing of NTHi (61). C4b is an essential component of the classical pathway C3 convertase, and increased C4b deposition on the surface of H. influenzae results in greater bactericidal activity (28). To determine if mutation of P5 affects C4b deposition onto H. influenzae, strains were incubated in 5% NHS and evaluated for total C4b deposition by Western blotting with an anti-C4 polyclonal antibody. NHS alone served as a positive control for the ~95-kDa and ~75-kDa α and β chains, respectively (Fig. 2B). Activation of C4 results in cleavage of its α chain to the ~87-kDa α′ chain, which binds covalently (through either ester or amide linkages) and migrates as a complex with its bacterial targets. C4b binding relative to the wild type was determined by using densitometry of visible bands.
bands in each lane. RP5G was found to bind 2.7-fold more C4b than Rd and 6.75-fold more C4 than complemented strain RP5C (Fig. 2B). Strain NTP5V bound 2.4-fold more C4b than NTV and 2.2-fold more C4b than NTP5X (Fig. 2B). As seen with iC3b, targets for C4b on the wild type and the P5 deletion mutants were similar. Taken together with results shown in Fig. 2A, the data indicate that P5 plays a role in the inhibition of classical pathway activation on the surface of H. influenzae strains. The increased C3 fragment deposition that accompanies the loss of P5 may result from increased classical pathway activation and/or independently from increased alternative pathway activation and is examined below.

**Immunoglobulin binding to P5 mutants.** Antibody binding to the surface of a pathogen initiates complement activation via the classical pathway. We evaluated the effects of P5 on antibody binding to the surface of H. influenzae. Strains were incubated with NHS, and binding of IgG and IgM was measured by flow cytometry. Data are represented as the percentage of wild-type levels (strain Rd or NTV). Levels of IgG binding were similar between the parent strains, P5 mutants, and complemented strains.
for both Rd and NT127 (Fig. 3A and C). In contrast, IgM binding to the P5 mutant was increased by ~50% over that of the Rd and NT127 parent and complemented strains (Fig. 3B and D), and these differences were statistically significant. The observed increase in binding of IgM to the surface of P5 mutants could contribute to their enhanced killing by normal human serum.

Resistance to alternative pathway activation. The P5 mutants were next examined for their sensitivity to the AP alone. Strains were treated with NHS in buffer in the presence or absence of 10 mM Mg\(^{2+}\)/EGTA, which inhibits classical/MBL pathway activation, and assayed for survival. Incubation with Mg\(^{2+}\)/EGTA-containing buffer alone did not decrease the viability of any of the strains (data not shown). When strains were incubated in 20% NHS in the presence of Mg\(^{2+}\)/EGTA, survival of the Rd P5 mutant was similar to that of the parent strain (Fig. 4A). In contrast, the NT127 P5 mutant exhibited a statistically significant 2.2-fold decrease in survival compared to that of the parent strain (Fig. 4A). Strains treated with 20% Mg\(^{2+}\)/EGTA serum were next analyzed for C3 binding by Western blotting. Consistent with the survival data, C3 binding to the Rd P5 mutant and wild-type strain Rd were similar, whereas the NT127 P5 mutant exhibited a 1.6-fold increase in C3 binding compared to parent strain NTV (Fig. 4B). The bactericidal results obtained with Mg\(^{2+}\)/EGTA serum were confirmed by using 20% C1q-depleted serum; C1q is required for classical pathway activation but does not participate in the lectin pathway. C1q-depleted serum (final concentration of 4%) supplemented with C1q at a physiological concentration (70 µg/ml) was used as a control in which all three pathways were intact. Depletion of C1q did not restore survival of the NT127 P5 deletion mutant to wild-type levels (Fig. 4C). Similar survival rates of the NT127 P5 mutant in Mg\(^{2+}\)/EGTA serum (only the AP is functional) and in C1q-depleted serum (Fig. 4C, white bar) (AP and lectin pathways are functional) suggested that the lectin pathway did not contribute to increased killing of the P5 deletion mutant. Supplementation of C1q-depleted serum with purified C1q restored ~99% killing of both the wild type and the P5 deletion mutant even in 4% serum, confirming that an intact classical pathway was required for killing at low serum concentrations (Fig. 4C). At higher (20%) serum concentrations, the AP alone could compromise the survival of the P5 deletion mutant for only NT127 and not Rd. Taken together, these data strongly suggest that P5 is important for interfering with AP activity on select strains of *H. influenzae*.

Inhibition of the AP mediated by NT127 P5 could be the result of binding of AP inhibitor factor H (fH). The NT127 P5 mutant (NTP5V) bound barely detectable amounts of purified fH in a
flow cytometry assay compared with the parent strain (NTV) (Fig. 4D). In contrast, binding of fH to the Rd P5 mutant was equivalent to that of the parental strain (Fig. 4E), consistent with the negligible effect of Rd P5 on survival and iC3b binding in serum possessing only the AP (Fig. 4A and B). These results indicate that P5 is required for fH binding to NT127, which constitutes a probable mechanism by which P5 variants can contribute to NTHi AP evasion.

**DISCUSSION**

Complement is a major effector of the innate immune response and is present in an increased abundance at mucosal surfaces in the context of infection and other inflammatory conditions (31). The association between complement deficiencies and increased susceptibility to infection has long been recognized for invasive *Haemophilus* infections (62, 63). Evidence that colonization by NTHi at the mucosal surface requires evasion of complement was obtained in a chinchilla model of middle ear infection. Specifically, depletion of complement using cobra venom factor restored virulence to a serum-sensitive NTHi mutant (which could not sialylate its LOS as a result of a deletion of the *siaB* gene, encoding cytidinemonophospho-N-acetylmuramic acid synthetase) that was otherwise avirulent in complement-sufficient animals (30). Recently, strains isolated from pulmonary infections were shown to exhibit higher levels of serum resistance than nasopharyngeal isolates (64, 65). While the mechanism of complement-mediated defense against NTHi in the lung is not fully understood, defects in complement-mediated phagocytosis of NTHi have been identified with macrophages isolated from patients with COPD in comparison to those of healthy nonsmokers (66), suggesting the importance of opsonophagocytosis in controlling NTHi in pulmonary infections. For these reasons, in this work, we sought to elucidate new factors involved in complement evasion by NTHi.

We previously found that *H. influenzae* mutants deficient in periplasmic disulfide bond formation as a result of a mutation in the *dsbA*-encoded disulfide oxidoreductase were killed more readily by serum complement (34). By informatics-based approaches, outer membrane protein P5 was identified as a candidate DsbA-dependent protein with a potential role in this phenotype (34, 35). In this report, we show that P5 in both Rd and an NTHi strain are required for full serum resistance. P5 regulated the classical pathway, and loss of P5 was associated with increased IgM binding and C4 deposition, with no apparent change in levels of IgG binding (Fig. 2 and 3). Although the increase in IgM binding to P5 mutants was only 50% over the level of binding to wild-type strains, IgM is very efficient at activating complement compared with IgG, as a single IgM molecule is sufficient to engage the C1 complex and initiate the classical pathway (67). Moreover, surveys of clinical NTHi isolates have revealed a correlation between higher levels of IgM binding and decreased serum resistance (64, 65). The epitope targeted by serum IgM on P5 mutants is currently not known; however, IgM that is bactericidal for NTHi in normal human serum is directed primarily against the LOS (68). Thus, the loss of P5 may increase the exposure of IgM-binding epitopes on the LOS, leading to decreased serum resistance of the strain.

It is unclear why P5 mutants bind increased amounts of IgM while IgG levels remain equal between the mutant and wild-type strains. However, a similar observation has been made with *Haemophilus ducreyi* mutants deficient in an outer surface protein, DsrA (69). *dsrA* mutants exhibit increased binding of IgM, which was found to be responsible for the increased activation of classical pathway components on the surface of this bacterium, but levels of bound IgG were equivalent between the mutant and wild-type strains (69). It was suggested that DsrA may physically exclude IgM from the surface of this bacterium or that the loss of *dsrA* results in the upregulation of a novel IgM epitope-containing factor on the surface of the bacterium (69). These scenarios are also plausible in the case of P5 mutants; however, further investigation will be necessary to determine exactly how P5 participates in limiting surface IgM deposition on *H. influenzae* strains. Potentially, these examples are indicative of a general strategy by which Gram-negative pathogens utilize outer surface proteins to exclude IgM from their surfaces and avoid complement activation.

A protein similar to P5 in *E. coli*, OmpA, was implicated previously in serum resistance. OmpA has been suggested to bind complement regulatory factor C4BP (38, 39), a host protein that normally functions to limit inappropriate classical pathway activation. Preliminarily studies showed that P5 mutants and wild-type strains bound similar levels of C4BP (S. Ram and C. V. Rosadini, unpublished data). Thus, P5 is unlikely to play a role in the binding of C4BP to our strains. However, we did find that P5 of NT127 is required for defense against alternative pathway activation via its ability to promote the binding of another complement-inhibitory molecule, fH (Fig. 4D). *H. influenzae* strains that bind fH were shown previously to be more sensitive to NHS when fH was depleted (70). Thus, the significant decrease in fH binding to NTHi P5 mutants strongly suggests a mechanism for their loss of resistance to the AP. Interestingly, P5 was not required for Rd strains to bind fH (Fig. 4E), suggesting that this strain binds fH via an alternative mechanism. Furthermore, outer surface loops of P5, which are likely to be involved in fH binding, are different between Rd and NT127 (see Fig. S1 in the supplemental material), which likely accounts for the difference in function. Of note, in a survey of the serum resistance of 18 clinical NTHi isolates, Martí-Lliteras et al. identified a strain with a predicted truncation of P5 that exhibited a moderate level of serum resistance albeit a level lower than that of 16 of the 17 other strains tested (71). Therefore, it is possible that some clinical isolates may possess an alternative fH-binding mechanism contributing resistance analogous to that of Rd, and it will be of interest to evaluate this possibility with isogenic mutants.

Evidence for alternative fH-binding mechanisms within the species raises the possibility that *H. influenzae* strains capable of high levels of fH binding may possess more than one binding mechanism, a strategy common to many other organisms, including *Neisseria meningitidis* (72, 73), *Streptococcus pneumoniae* (74), *Borrelia burgdorferi* (75), and *Candida albicans* (76). Importantly, Hallstrom et al. found that among clinical isolates of NTHi from cases of sepsis, disease severity was correlated with increased serum resistance and binding of complement-inhibitory proteins, including fH (24). An understanding of how pathogenic NTHi strains bind fH and whether differences in this ability involve the acquisition of multiple binding mechanisms is therefore of potential clinical relevance to the severity of invasive infection.

Previously, P5 was implicated in the pathogenesis of *H. influenzae* as an adherence factor for attachment of *H. influenzae* to host mucosal structures (42–46). The work presented here describes new functional roles for this abundant outer membrane protein, including limiting the binding of IgM to the bacterial surface and participating in the binding of fH. Overall, P5’s role in
Role of H. influenzae P5 in Complement Resistance

References:


