Pseudomonas aeruginosa antigens as potential vaccines

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Abstract

Pseudomonas aeruginosa is one of the most important opportunistic bacterial pathogens in humans and animals. This organism is ubiquitous and has high intrinsic resistance to antibiotics due to the low permeability of the outer membrane and the presence of numerous multiple drug efflux pumps. Various cell-associated and secreted antigens of P. aeruginosa have been the subject of vaccine development. Among pseudomonas antigens, the mucoid substance, which is an extracellular slime consisting predominantly of alginate, was found to be heterogeneous in terms of size and immunogenicity. High molecular mass alginate components (30–300 kDa) appear to contain conserved epitopes while lower molecular mass alginate components (10–30 kDa) possess conserved epitopes in addition to unique epitopes. Surface-exposed antigens including O-antigens (O-specific polysaccharide of LPS) or H-antigens (flagellar antigens) have been used for serotyping due to their highly immunogenic nature. Chemical structures of repeating units of O-specific polysaccharides have been elucidated and these data allowed the identification of 31 chemotypes of P. aeruginosa. Conserved epitopes among all serotypes of P. aeruginosa are located in the core oligosaccharide and the lipid A region of LPS and immunogens containing these epitopes induce cross-protective immunity in mice against different P. aeruginosa immunotypes. To examine the protective properties of OM proteins, a vaccine containing P. aeruginosa OM proteins of molecular masses ranging from 20 to 100 kDa has been used in pre-clinical and clinical trials. This vaccine was efficacious in animal models against P. aeruginosa challenge and induced high levels of specific antibodies in human volunteers. Plasma from human volunteers containing anti-P. aeruginosa antibodies provided passive protection and helped the recovery of 87% of patients with severe forms of P. aeruginosa infection. Vaccines prepared from P. aeruginosa ribosomes induced protective immunity in mice, but the efficacy of ribosomal vaccines in humans is not yet known. A number of recent studies indicated the potential of some P. aeruginosa antigens that deserve attention as new vaccine candidates. The outer core of LPS was implicated to be a ligand for binding of P. aeruginosa to airway and ocular epithelial cells of animals. However, heterogeneity exists in this outer core region among different serotypes. Epitopes in the inner core are highly conserved and it has been demonstrated to be surface-accessible, and not masked by O-specific polysaccharide. The use of an in vivo selection/expression technology (IVET) by a group of researchers identified a number of P. aeruginosa proteins that are expressed in vivo and essential for virulence. Two of these in vivo-expressed proteins are FptA (ferripochelin receptor protein) and a homologue of an LPS biosynthetic enzyme. Our laboratory has identified a highly conserved protein, WbpM, and P. aeruginosa with a deficiency in this protein produces only rough LPS and became serum sensitive. Results from these studies have provided the foundation for a variety of vaccine formulations.

Keywords: Pseudomonas aeruginosa; Cell wall; Outer membrane; Lipopolysaccharide; A-band; B-band; Core oligosaccharide; Lipid A; Common antigen; Vaccine

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1. Introduction

*Pseudomonas aeruginosa* is a typical species of the genus *Pseudomonas* which is a member of the family *Pseudomonadaceae* [1]. *P. aeruginosa* is one the most important opportunistic pathogens in animals and humans. Results of bacteriological investigations conducted in different hospitals have shown *Pseudomonas* spp., mainly *P. aeruginosa*, to be among the most frequently isolated organisms from pus, sputum, blood and other clinical material of patients with Gram-negative hospital infections. *P. aeruginosa* is also associated with complications in patients with cystic fibrosis (CF), or following surgery, trauma, and thermal burn [2–4]. In some studies, the occurrence of nosocomial infections was the highest in surgery, gynecology and obstetrics but lower in prenatal and pediatric services. Gram-negative rods were isolated in 53.7% of nosocomial cases, with *P. aeruginosa* representing 9.9%. *P. aeruginosa* was ranked the second most frequent pathogen in surgery and third in medicine [5].

*P. aeruginosa* infection is a serious threat to hospitalized patients. *P. aeruginosa* sepsis in burn patients is well known to have high mortality rates (up to 60%) in spite of complex therapy, including the use of broad spectrum antibiotics, normal donor’s plasma, electrolyte solutions, and vitamins [2,6]. As an opportunistic pathogen, the effectiveness of its virulence factors to help this bacterium establish an infection depends on the vulnerability of the host’s state of health. In the case of pulmonary infections in CF patients, the bacteria are apparently capable of reaching the lungs due to the impairment of the clearance mechanism of ciliated epithelial cells. *P. aeruginosa* possesses type IV pilus adhesins and non-pilus adhesins for the initial anchoring to host tissues and specific receptors. The bacteria begin to grow as microcolonies (or biofilms) due to the production of mucoid substance or alginate, which likely protects the bacteria from phagocytic killing [7,8]. As the bacteria continue to survive in the lung, damage to lung tissue occurs, due in part to the secreted toxins and enzymes produced by *P. aeruginosa*. The extensive damage to the lungs of these patients is the main cause of death. Interestingly, pulmonary pseudomonas infections in CF patients rarely spread to blood stream infections. A second common form of pseudomonas infection occurs in patients with severe burn wounds. It is generally believed that a significant role in virulence is played by an array of secreted toxins and proteases. Infection
of the burn wound not only produces local tissue
damage but it can leak into the bloodstream and
cause a systemic infection. LPS (endotoxin) will
likely be released by both live organisms and disinte-
grated bacterial cells. The toxic effect of endotoxin
can cause death due to septic shock.

The majority of *P. aeruginosa* strains produces
characteristic pyocyanin, a water and chloroform
soluble phenazine pigment. During cultivation of
strains at neutral or alkaline pH, the growth medium
assumes blue-green color while at acid pH the me-
dium turns red. Some strains of *P. aeruginosa* pro-
duce a dark, reddish-brown pigment called pyorubin
[9]. Up to 90% of strains produce a diffusible green
fluorescent pigment called pyofluroesin [1]. *P. aeru-
ginosa* is capable of growth at 42°C but not at 4°C.
Biochemical characteristics of *P. aeruginosa* include
its ability to reduce nitrate to nitrogen gas, liquefy
gelatin in broth with 15% gelatin, produce acid from
glucose and mannitol, and in ability to produce acid
from adonitol, dulcitol, inositol, sucrose and sorbi-
tol. The DNA of *P. aeruginosa* has a G+C of 67
mol% [10].

Control against *P. aeruginosa* infection is difficult
due to its high intrinsic resistance to antibiotics. The
low permeability of the major outer membrane (OM)
porins [11] and the presence of multiple drug efflux
pumps [12] are factors that contribute to mechanisms
d of drug resistance in this species. In a recent study,
16 of 132 hospital isolates of *P. aeruginosa* tested for
antibiotic susceptibility were found to be resistant to
eight drugs out of 12 [13]. Thus far, three different
multiple drug efflux pumps encoded by the *mex*-
*mex**-opr* operon of genes have been identified
(the * and ** denote specific letter designations of
the genes in these operons, e.g. *mexA-mexB-oprM*)
[14–16]. Therefore, the development of immunother-
apy and immunoprophylaxis as alternatives for con-
trolling *P. aeruginosa* infections is warranted. It is
essential to have a thorough understanding of the
antigens in this bacterium in order to determine the
ones that can elicit protective response in the host.

Numerous antigens are expressed by *P. aeruginosa*
which are localized in pili, flagellar, extracellular
slime (ES) layer, OM, ribosomes and likely in other
surface-associated structures. Some secreted products
such as exotoxin A, and proteases also possess im-
munogenic properties. Surface-accessible ES and cell
envelope antigens are crucial for stimulation of pro-
tective immunity in human beings or animals, thus
they can be considered as protective antigens. The
protective properties of flagella and ribosomal anti-
gens of *P. aeruginosa* are still topics of debate in the
literature. We believe that the leading role in the
stimulation of specific protective immunity against
*P. aeruginosa* infection belongs to surface-exposed
antigens.

### 2. Antigenic properties of exproducts

*P. aeruginosa* produces a number of secreted viru-
ience factors including exotoxin A, exoenzyme S,
proteases I (neutral protease) and III (alkaline phos-
phatase), elastase, hemolysins (thermolabile phos-
phatase C and thermostable acid glycolipid), enter-

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<td><strong>Probable role of extracellular products of <em>P. aeruginosa</em> in virulence and immunogenicity</strong></td>
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<td><strong>Extracellular antigen</strong></td>
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<td><strong>Proteases:</strong></td>
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<td>Rhamnolipids</td>
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<td>Exotoxin A</td>
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exotoxin, collagenase, lecinthinase, lipase [2,17]. The probable role of these secreted pseudomonas antigens is summarized in Table 1.

The antigenic activities of some of these exoproteins have been studied [2]. In patients with various forms of P. aeruginosa infections, circulating antibodies to elastase, proteases, exoenzyme S, toxin A and other exoproteins of cell metabolism were found whereas in CF patients, elevated levels of anti-exoenzyme S-antibodies were revealed [2]. In a study by Woods and Sokol [27], a direct correlation (r=0.8089) was observed between increased levels of anti-exoenzyme S-antibody measured by ELISA and the severity of clinical symptoms as indicated by Shwartzman scores of pulmonary symptoms [27].

To date, a controversy exists between the naming of exoenzyme S isolated from P. aeruginosa strain 388 [28] and strain DG1 [29]. The exoS gene from P. aeruginosa strain 388 described by Frank and her colleagues encodes a 49 kDa protein that has ADP-ribosyl transferase activity. This group has also identified an enzymatically inactive 53 kDa form that is encoded by a separate gene [28]. In comparison, the exoenzyme S gene described by Sokol et al. [29] yielded a 68 kDa protein that was toxic to HeLa cells, but did not exhibit ADP-ribosyl transferase activity when it was expressed in Escherichia coli [29]. In addition, the reported cyanogen bromide fragment used to isolate the exoenzyme S gene from P. aeruginosa DG1 was not found within the predicted amino acid sequence of exoS from strain 388 [30]. Based on these results, there appears to be a need for the clarification of the nomenclature of the two forms of exoenzyme S before one can assess the contribution of exoenzyme S as a vaccine candidate. In another study by Homma et al. [31], antibodies against P. aeruginosa and in particular against elastase were demonstrated by passive hemagglutination (PHA) in patients. Interestingly, the anti-elastase antibodies were also detected in healthy subjects, likely due to a history of P. aeruginosa infection. Based on these observations, protease and elastase have been included into anti-P. aeruginosa vaccines described by Homma et al. [31,32].

Exotoxin A is an ADP-ribosyl transferase which suppresses protein synthesis of the host cell. It forms a single polypeptide chain with MW of approximately 66–71 kDa [33–35]. The native toxin was shown to be a proenzyme composed of an enzymatically active fragment A (26 kDa) and a binding fragment B (45 kDa) [33,34]. Subsequently, Ogata et al. [36] utilized data based on mutational studies and X-ray crystallographic analysis and showed that exotoxin A has three functional domains. After internalization of the toxin and cleavage by cell-associated proteases, a fragment containing domain III and part of domain II would be generated. This fragment is the active component that acts on EF-2 intracellularly. While many clinical isolates of P. aeruginosa can produce exotoxin A, most strains do not produce large amounts of this toxin since toxin production was down-regulated by the presence of even minute amounts of iron in most growth media. In a study by Bjorn and co-workers [37], they found that the yields of exotoxin A produced by P. aeruginosa strain PA103 (a well-known exotoxin A producer) were influenced by the amount of iron in the culture media. An increase of iron concentration from 0.05 to 1.5 μg/ml reduced the toxin production by 90%.

Toxin A is a lethal agent to a wide variety of animals including primates; it exerts a cytotoxic effect in vitro and a necrotizing activity in vivo [35]. Exotoxin A is a reasonably potent immunogen even when low toxicity doses were used in eliciting antibody response. Toxoid prepared from the exotoxin A induces high titers of specific antibodies. Anti-toxoid serum provided passive protection in mice against the lethal effects of exotoxin A. Cross-protection to heterologous P. aeruginosa strains and high doses of exotoxin A were also observed. It is assumed that different strains of P. aeruginosa produce serologically identical exotoxin [35,38–40]. According to the data of Ogger and Berdal [41], LD50 of toxin A for infant mice was 80 ng. Rabbit anti-toxin A serum when mixed with purified toxin A provided neutralizing effect and showed an increase of LD50 to 2500 ng. An exotoxin A epitope from strain PA-103 cross-reacts with fragment A of diphtheria toxin, but no homology in the DNA sequence was established between the diphtheria toxin gene and toxA of P. aeruginosa [42].

Antibodies to toxin A, elastase, alkaline phosphatase, and phospholipase C were found in sera of healthy subjects and patients with P. aeruginosa infections. It was established that five of 39 burn pa-
patients with wounds colonized by \textit{P. aeruginosa} had elevated antibody titers to alkaline phosphatase. Increased antibody levels to exotoxin A or phosphatase C were observed in 15 of 22 patients with \textit{P. aeruginosa} septicemia, otitis, and pneumonia. In an examination of 112 serum samples from healthy subjects of different age groups (0–3, 4–15, 16–75 years old), Granstom et al. [43] showed that antibodies (IgG) to alkaline phosphatase and phospholipase C significantly increased in older children and adults. However, antibody levels to elastase and exotoxin A were not increased. Antibody titers to all four antigens were increased slightly but not significantly in the youngest to the older children.

Murine monoclonal antibodies (mAb) specific for \textit{P. aeruginosa} strain PA-103 toxin A neutralized toxin in vitro in tissue cultures and provided passive immunization in vivo by prolonging the survival of mice in experimental burn infections. Monoclonal antibodies displayed specificity for strain PA-103 toxin but did not react with toxin purified from strains PAO-PR1 or PAO1. These results suggested that structural and epitope differences exist among toxins from different strains [44]. Monoclonal antibodies against \textit{P. aeruginosa} strain PA-103 toxin A reacted with two discrete structural domains of the toxin and have two distinct functional profiles. One group of antibodies reacts with a 46 kDa peptide, likely the binding fragment B of the toxin. These antibodies neutralize cytotoxic and lethal activity of the toxin but have no effect on its ADP-ribosyl transferase activity. The antibodies interfered with binding of toxin to membrane receptors on mouse fibroblast L cells, although the epitope for the antibody appears to be distinct from the actual receptor-binding site. The second group of antibodies is likely specific towards the enzymatically active fragment A. They reacted with intact toxin A and with a cloned enzymatically active carboxy-terminal polypeptide. These antibodies also neutralized ADP-ribosyl transferase activity of activated toxin and of the clone peptide, but did not inhibit binding of toxin to membrane receptors or inhibit cytotoxic and lethal effect [45].

Recent data on studies of extracellular toxic products of \textit{P. aeruginosa} have shown that many of these contribute to virulence, possess immunogenicity and elicited neutralizing antibodies. However, the role of these extracellular antigens in the induction of host protective immunity is not yet known. First, the correlation between the production of toxic substances (e.g. proteases, elastase, collagenase, hemolysins) and virulence of \textit{P. aeruginosa} strains is still not clearly defined. Some strains that do not produce these extracellular substances are virulent in mice (Stanislawsky, unpublished data). Second, ES and/or OM proteins induce protection in mice against \textit{P. aeruginosa} strain PA-103. This strain produces toxin A and does not or only poorly synthesize proteases. We have data to show that ES and OM proteins could elicit protective immunity in mice against \textit{P. aeruginosa} strains known to produce toxin A, proteases or elastase (see below). It is highly unlikely that, toxoid of toxin A, proteases, elastase or any of the secreted toxic proteins can elicit long-term protective immunity in animal hosts.

3. Antigens of extracellular slime polysaccharide (alginate)

Extracellular polysaccharide antigens have been targets for stimulating specific protective immunity against numerous bacterial infections [37,46–51]. Alginate, as described earlier, is the predominant component of the mucoid substance or ES of \textit{P. aeruginosa}. Alginate and ES will be used synonymously throughout this review. Alginate has been used to induce protective immunity in experimental animals [52–54].

Alginate is a linear polymer of β(1→4)-linked β-mannuronic acid and its C-5 epimer L-guluronic acid [55]. The involvement of \textit{algA}, \textit{algC}, and \textit{algD} for the synthesis of mannuronate and \textit{algG}, \textit{algF}, \textit{algI} and \textit{algJ} for the acetylation and epimerization of mannuronate to form the C-5 epimer L-guluronic acid are clearly defined [8]. At present the exact mechanism for the polymerization of alginate polymers is not known. A number of genes, namely \textit{algU} (also named \textit{algT} [56]), \textit{algW}, \textit{mucA}, \textit{mucB} (also named \textit{algN} [57]) \textit{mucD}, \textit{algB}, \textit{algR} and \textit{algZ}, are involved in the regulation, sensing of environmental signals and switching on of the alginate biosynthetic pathway. The intricate regulatory circuitry involving these genes in controlling the expression of alginate causing mucoidy in \textit{P. aeruginosa} has been described
in an excellent review by Govan and Deretic [8]. Interestingly, from our studies, a surface growth association of alginate production by *P. aeruginosa* have been observed in which copious amounts of alginate could be induced when the bacteria are cultured on agar medium covered with sterile cellophane filter disk. A yield of crude alginate in the range of 6–84.8 mg/petri plate could be achieved [53].

*P. aeruginosa* alginate could be separated by ultrafiltration into fractions with components: (1) >300 kDa; (2) 100–300 kDa; (3) 30–100 kDa and (4) 10–30 kDa. The results of immunochemical and serological investigations of the fractions [53] showed that *P. aeruginosa* extracellular slime layer consists of alginate with apparent molecular masses (MWs) of 10–30 kDa which can associate to form larger polymers with a MW of up to 300 kDa and more. Components (1), (2) or (3) have a negative charge and component (4) is positively charged. The studies of alginate components with different MWs in vitro showed that high MW alginate components (30–300 kDa) are similar or identical in their antigenic specificity. The low MW alginate component contains its own specific epitope as well as common epitopes that are also found in the high MW alginate [53]. Results by others [58,59] showed that alginate of various *P. aeruginosa* strains possess conserved and strain-specific epitopes. The protective properties of alginate components with different MW as immunizing antigen and the passive protection potential of anti-alginate sera were examined in mouse protection tests. The results of these experiments showed that alginate caused protective immunity in mice against homologous and partially against heterologous *P. aeruginosa* strains [53].

Cryz et al. [60] showed that native *P. aeruginosa* alginate (MW of >640 kDa) was low in immunogenicity and only elicited weak antibody responses in rabbits and mice. However, depolymerized alginate (MW of <60 kDa) conjugate with toxin A induced in animals a high levels of antibodies to alginate. The protective activity of the conjugate vaccine, however, is not yet known.

A serological cross-reaction was demonstrated between high MW alginate components and LPS [53] but according to an earlier study by others [61], their alginate preparation appears to contain a large proportion of LPS as a co-purified component. The inclusion of a small proportion of LPS in alginate vaccines may have an adjuvant effect and enhance the stimulation of the host protective response against alginate. However, in the experiments where passive protective response alginate is assessed, the anti-alginate sera were pre-absorbed to remove anti-LPS antibodies before being used for the mouse protection studies [53].

Nadaud [62] suggested that alginate derived from ES of *P. aeruginosa* contains specific antigenic determinants different from LPS. The author showed that alginate has ‘a’- or ‘b’-specific antigens. Seventy three percent of the clinical isolates agglutinated with only one of the two a- or b-antisera and 17% with both of them, while 10% remained unagglutinated with either of the antisera. It was established that the protective properties of alginate-a or alginate-b antigens are distinct as compared to the protective properties of LPS antigens of Fisher immunotypes [62].

Antisera to alginate of different O-serogroups (immunotypes) showed cross-reactivity in passive hemagglutination (PHA) and agar immunoprecipitation test [63,64]. Alginate of some strains exhibited cross-reactivity in PHA, independent of the O-serogroup (immunotypes) of the given strains. Comparing these data with those of PHA analysis using LPS antigen and anti-LPS sera [64], it is clear that the alginate antigens being assessed, has unique antigenicity and the reactions in the immunoassays were not due to contaminating LPS antigens. Although there was some evidence of antigenic relationship between alginate and a number of different O-serogroups that they were derived from, certain alginate preparations do not show cross-reactivity with strains of various serogroups. Accordingly, alginate differs in antigenic specificity from LPS [64], this observation is consistent with the data of other authors [62]. Results of the immunoprecipitation test also support our view that this test may be used for classification of alginate antigens [64].

In mouse challenge experiments, antisera to alginate also showed cross-protective activity against intraperitoneal challenge with 24 heterologous *P. aeruginosa* strains from different O-serogroups including the toxigenic strain PA-103, a high producer of exotoxin A [64]. Based on these observations, alginates of certain strains of *P. aeruginosa*
can be selected for the production of a antisera for passive immunotherapy. The capability of alginate from different immunotypes (O-serogroups) to induce cross-protection against all serotypes of *P. aeruginosa* in mouse model studies appeared to be highly varied. Interestingly, alginate from various strains induced immunity to a challenge with the toxigenic PA-103 strain (Table 2) [63]. The best protection against PA-103 was induced by alginate from strains 170001, 170010, 170022, and 170023 (Table 2). These strains belong to serogroups O3, O6, O15, and O12 respectively.

Pier et al. [65,66] reported that purified alginate when used as a vaccine was well tolerated by adult volunteers. In the two preparations of alginate vaccines that they used to immunize human volunteers, designated Lot 1 and Lot 2, they found Lot 1 to be lowly immunogenic. Lot 2 which has a larger average size of alginate polymers, when used at an optimal dose of 100 μg elicited long-lived opsonic antibodies in 80–90% of the vaccinated individuals [67].

### 4. Cell envelope antigens

Due to the accessibility on the bacterial surface, LPS and OM proteins of *P. aeruginosa* are particularly important targets for vaccine studies. Based on SDS-polyacrylamide gel electrophoresis (PAGE) and silver staining analysis, LPS of *P. aeruginosa* generally occurs as ladder-like bands revealing the heterogeneity in size of the LPS molecules [68]. In this heterogeneous mixture, smooth (S) forms of LPSs are those that contain O-antigen polysaccharide attached to core-lipid A and these will be visualized as the multiple bands in the upper part of the gel lanes. Rough (R) forms of LPSs are those that are devoid of O-antigens. In SDS-PAGE, R-LPS will appear as a fast migrating band near the gel front. In addition, there can also be a population of SR form of LPSs which contain one O-antigenic unit attached to core-lipid A. SR-LPS can be identified in SDS-PAGE gels as the second fast migrating bands above the bottom band. *P. aeruginosa* mutants deficiency in the *rfc* gene which encodes an O-polymerase usually exhibits an SR phenotype [69].

#### 4.1. Serological grouping of *P. aeruginosa*

Lanyi and Bergan have earlier provided a comprehensive review on the historical perspectives of the basis for distinguishing among various O-serotypes, and the use of such O-serotype determinations in epidemiological studies of *P. aeruginosa* infections.
[6]. Since the beginning of this century, many classifications based on serogrouping have been elaborated. Owing to the large number of designations for the O-antigens, and debates on whether or not partial O-antigens should be included in the antigenic schemes, the results from different authors were difficult to compare. In an attempt to establish a standardized serotyping scheme, Lanyi and Bergan [6] recommended that antigens be designated according to the most widely recognized Habs scheme [70]. It will further be subdivided into subgroups as described by Lanyi [71] and supplemented by antigens from other serotyping schemes, namely Sandvik [72], Verder and Evans [73], Meiert [74], which are not included in Habs' or Lanyi's systems. Subsequently, Akatova and Smirnova [75] and Homma [76] recommended the use of the serogroup designation system of Lanyi and Bergan and supplemented the scheme with new subgroups from the strain collection of Wokatsch [77]. Liu et al. [78] preferred a scheme named the International Antigenic Typing Scheme (IATS) which designates the O-antigens by continuous numbering, as opposed to the a, b, c system of Lanyi and Bergan [6]. Table 4 shows a detailed comparison of the antigenic constituents of these systems. It is advantageous that in the compiled schemes of Lanyi and Bergan and IATS the numbering of O-serogroups 1, 2, 3, 4, 6, 7, 9, 10, 11 and 12 corresponds to each other and to the original Habs system. The Habs groups O2 and O5 were combined in the Lanyi-Bergan scheme into one group, O2, on the basis of sharing a well-defined partial antigen. Habs’ groups O7 and O8, for the same reason, have also been united into the O7 complex. A study by Lam et al. [79] using monoclonal antibodies raised against O7- and O8-serogroups also demonstrated strong cross-reactivities among these two serogroups and supported the placement of both into the O7-serogroup. Strain Sandvik II, and Verder Evans strain 1M-1 are related in an a, b-a, c manner and were therefore classified into one group (O13) of the Lanyi-Bergan scheme [6]. On the basis of the

<table>
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<tr>
<th>Chemo Reference Strains</th>
<th>O-ANTIGENS</th>
<th>Structure of repeating units of O-specific PS</th>
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<tr>
<td></td>
<td>Lanyi-</td>
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<tr>
<td></td>
<td>Bergan</td>
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<td></td>
<td>Fisher</td>
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<tr>
<td>1</td>
<td>170014</td>
<td>IV (-4)-β-D-GlcN2Ac2U-(1-3)-α-D-FucNAc-(1-3)-α-GalNAc-(1-4)-α-D-GalNAc-(1-6)OAc</td>
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<tr>
<td>2</td>
<td>170003</td>
<td>2a,2b (-4)-β-D-ManNAcAmU-(1-4)-β-D-ManN2Ac2U-(1-3)-β-D-FucNAc-(1-6)OAc</td>
</tr>
<tr>
<td>3</td>
<td>Wokatsch</td>
<td>2a,2b,2e (-4)-β-D-ManNAcAmU-(1-4)-β-D-ManN2Ac2U-(1-3)-β-D-FucNAc-(1-6)OAc</td>
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<tr>
<td>4</td>
<td>170004</td>
<td>(2a),2c (2a) (-4)-β-D-ManNAcAmU-(1-4)-α-L-GulN2Ac2U-(1-3)-β-D-FucNAc-(1-6)OAc</td>
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<td>5</td>
<td>170005</td>
<td>2a,2d (-4)-β-D-ManNAcAmU-(1-4)-β-D-ManN2Ac2U-(1-3)-α-D-FucNAc-(1-6)OAc</td>
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<td>6</td>
<td>170006</td>
<td>2a,2d,2e (-4)-β-D-ManNAcAmU-(1-4)-α-L-GulN2Ac2U-(1-3)-α-D-FucNAc-(1-6)OAc</td>
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<td>7</td>
<td>170007</td>
<td>(2a),2d,2f (-4)-α-L-GulNAcAmU-(1-4)-β-D-ManN2Ac2U-(1-3)-α-D-FucNAc-(1-6)OAc</td>
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<tr>
<td>8</td>
<td>Fisher VII or 170047</td>
<td>VII (-4)-α-L-GulNAcAmU-(1-4)-β-D-ManN2Ac2U-(1-3)-α-D-FucNAc-(1-6)OAc</td>
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results of a number of studies [6,75,76], Lanyi suggested that *P. aeruginosa* be divided into 15 O-serogroups and 31 O-subgroups [71].

For determination of *P. aeruginosa* O-antigens, slide agglutination is the method of choice [6]. Some isolates, especially those forming abundant slime, agglutinate less readily by the slide method. Such cultures become readily agglutinable after being autoclaved at 120°C for 1 h, or kept at 100°C for 2.5 h followed by centrifugation and homogenization in glycerol and heating to 130°C for 1 h [6,71]. Due to the major group antigen (e.g. O6a), strains belonging to different subgroups of the corresponding O-serogroup usually gave positive agglutination reactions with a single group serum that can easily be scored as 2+ to 4+ by visual assessment. A more accurate determination of partial antigens is performed with absorbed O-sera (e.g. O6b, O6c) (Table 3). O-antigen of *P. aeruginosa* and several other pseudomonads differ from most Gram-negative bacteria in that their living cells are readily agglutinable in the homologous O-serum, but after exposure to 55–75°C the suspension turns to slime, and the cells lose their agglutinability. Heating at 100°C for 60 min renders the cells agglutinable at low titers. After boiling the cells for 2.5 h the titer obtained in agglutination tests increased markedly. The highest O-antibody titers were obtained with bacteria heated to 120–130°C. Treatment with formalin, ethanol and low concentrations of hydrochloric acid reduces agglutinability. The above procedures, however, do not influence the immunogenicity and agglutinin-binding capacity of the O-antigen. As live *P. aeruginosa* cultures agglutinate readily in O-serum, there is no reason to believe that they contain antigen which would mask O-agglutination, like in the case of *Enterobacteriaceae*
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<th></th>
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</thead>
<tbody>
<tr>
<td>19</td>
<td>170011</td>
<td>7a,7b,7c</td>
<td>-3)-β-D-FucNAc-(1-4)-α-PseNAcN-((2-4)-β-D-Xyl-(1-4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-90% OAc</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>170012</td>
<td>7a,7b,7d</td>
<td>-3)-β-D-FucNAc-(1-4)-α-PseNAcN-((2-4)-β-D-Xyl-(1-4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-90% OAc</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>170013</td>
<td>7a,7d</td>
<td>-3)-β-D-FucNAc-(1-4)-α-PseNAcN-((2-4)-β-D-Xyl-(1-4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-90% OAc</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>170020</td>
<td>9a,9b,9d</td>
<td>-3)-β-D-QuinAc-(1-4)-α-δ-FucNAc-(1-4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R)-CH₃</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-90% OAc</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>Wokatsch 16</td>
<td>9a,9c</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>170019</td>
<td>9a,9d</td>
<td>-3)-β-D-QuinAc-(1-4)-α-δ-FucNAc-(1-4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(R)-CH₃</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>170034</td>
<td>10a,10b</td>
<td>-3)-α-L-GalNAcU-(1-3)-α-δ-QuinAc-(1-3)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>-80% OAc</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>170002</td>
<td>10a,10c</td>
<td>-3)-α-L-GalNAcU-(1-3)-α-δ-QuinAc-(1-3)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>-80% OAc</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>170015</td>
<td>11a,11b</td>
<td>-3)-α-L-FucNAc-(1-3)-β-D-FucNAc-(1-2)</td>
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</tr>
<tr>
<td>170016</td>
<td>11a,11c</td>
<td></td>
<td>-80% OAc</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>170023</td>
<td>12</td>
<td>-3)-α-δ-QuinAc-(1-8)-β-Non2Ac2-(2-3)-α-δ-FucAm-(1-2)</td>
<td></td>
</tr>
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<td></td>
<td>-80% OAc</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Sandvik II</td>
<td>13a,13b</td>
<td>-4)-α-D-GalNAcU-(1-3)-β-D-QuinAc-(1-2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-80% OAc</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Verder-Evans</td>
<td>13a,13c</td>
<td>-4)-α-D-GalNAcU-(1-3)-β-D-QuinAc-(1-2)</td>
<td></td>
</tr>
<tr>
<td>IM-I</td>
<td></td>
<td></td>
<td>-80% OAc</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Meitert X</td>
<td>14</td>
<td>-3)-β-D-ManNAc-(1-4)-α-L-Rha-(1-2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-80% OAc</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>170022</td>
<td>15</td>
<td>-4)-α-D-GalNAc-(1-2)-β-D-Ribf-(1-2)</td>
<td></td>
</tr>
</tbody>
</table>

*Data from [105]. ND designates not determined.

Abbreviations: QuiN, 2-amino-2,6-dideoxyglucose (quinoosamine); FucN, 2-amino-2,6-dideoxygalactose (fucosamine); Qin4N, 4-amino-2,6-dideoxyglucose; Bac(2N4N), Bac(NAc)2, 2,4-diamino-, 2,4-diacetamido-2,4,6-trideoxyglucose (bacillosamine, di-N-acetyl-bacillosamine), respectively; GalNAc, GalNAcN, 2-acetamido-, 2-formamido-2-deoxygalacturonic acid (N-acetyl-, N-formylgalactosaminuronic acid), respectively; Glc(2N3NA), Glc(NAc)2A, Man(2N3NA), Man(NAc)2A, Glc(NAc)2A, 2,3-diamino-, 2,3-diacetamido-2,3,4,6-tetrahydrolactose, mann-, guluronic acid, respectively; Pse(5N7N), Pse(5Nac7NFM), 5,7-diamino-, 5-acetamido-7-formamido-5,7,9-tetrahydro-1-glycer-1-manno-nonulosonic acid (psedumonic acid, 5, N-acetyl-2-formylpsedumonic acid), respectively; Non(5N7N), Non(NAc)2, 5,7-diamino-, 5,7-diacetamido-5,7,9-tetrahydro-1-glycer-1-galacto-nonulosonic acid, respectively; Rib, ribose; Xyl, xylose; Glc, glucose; and Rha, rhamnose.

with K-antigen. In *P. aeruginosa* and many other pseudomonads, evidently the slime antigen(s) could be partly responsible for agglutination of live and boiled cells. Exposure to mild heat and chemicals probably results in a change of structure of *P. aeruginosa* rendering the cell nonagglutinable, whereas
heating at higher temperatures restores agglutinability, likely by destroying the agglutination-inhibiting product. Another serotyping scheme was established by Fisher et al. [80] who identified seven immunotypes based on comparison of protective response in mice by homologous and heterologous strains of *P. aeruginosa*. This scheme does not correspond to serogrouping due to O-antigen structures (Table 3).

*P. aeruginosa* isolates frequently show polyagglutinability, i.e. agglutinated with a number of different O-antisera. In contrast, spontaneous agglutinability, i.e. clumping of cells by all O-antisera as well as in physiological saline, is rarely encountered [6,71]. Pitt and Erdman [81] presented evidence that a heat-stable cell constituent distinct from the O-antigen is apparently responsible for polyagglutinability. Studies by Rivera et al. [82] and Lam et al. [83] described the identification of a common LPS antigen known as A-band. This common A-band LPS likely account for the polyagglutinability of many of these *P. aeruginosa* clinical isolates. In contrast, spontaneous agglutination is associated with mutation to serological R-form [6]. We also have data to show that some strains with R-form LPS exhibited polyagglutinability in serotyping reactions [84]. The use of monoclonal antibodies raised against the *P. aeruginosa* IATS serotypes was effective in minimizing the occurrence of polyagglutinability among clinical isolates examined [85–87].

Lanyi et al. [88] showed that *P. aeruginosa* O-antigens could be classified into five immunoelectrophoretic (IE) groups; groups I and III were divided into three and two subgroups respectively. O-antigens identified by agglutination corresponded closely to immunoelectrophoretic patterns. A strain with identical O-antigens or sharing major somatic components fell into the same IE group with one exception, that of IE group V.

Immunoelectrophoresis has supported the assumption of Adam et al. [89] that O-antigens designated by Lanyi in his original system [71] as O8 and O9 correspond to R-antigens. This was further proven by Adam’s (unpublished) results obtained with different LPS-preparations including phenol-chloroform-petroleum ether extracts of the reference strains (170017 and 170018). In light of these studies, these antigens were omitted from the Lanyi-Bergan scheme [6].

### 4.2. Structure and immunochemical specificity of LPS

LPS is a complex molecule consisting of three regions: namely O-specific PS (O-antigen), core oligosaccharide and lipid A. Since the late 1960s many attempts have been undertaken to correlate immuno-specificity of *P. aeruginosa* strains with structures of O-antigens in S-LPS. They have been found to possess the same general architecture as the most thor-

---

**Table 4** O-antigen reference strains of *P. aeruginosa* awaiting further study

<table>
<thead>
<tr>
<th>Reference strain</th>
<th>Serological classification based on slide agglutinations</th>
<th>Chemotype (see Table 3)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fisher VII</td>
<td>2a,2d</td>
<td>8</td>
<td>O-PS structure differs from other subgroups; may have a new subgroup antigen</td>
</tr>
<tr>
<td>Wokatsch 14</td>
<td>3a,3b</td>
<td>9</td>
<td>Discrepancy between serological classification and O-PS structure</td>
</tr>
<tr>
<td>170001</td>
<td>3a,3b,3c</td>
<td>9</td>
<td>Both strains may have partial antigens different from each other and from those of other serogroups</td>
</tr>
<tr>
<td>Habs 3</td>
<td>3a,3b,3c</td>
<td>10</td>
<td>Absence of O-PS</td>
</tr>
<tr>
<td>Habs 6</td>
<td>6a</td>
<td>14</td>
<td>Absence of O-PS in Wokatsch, nature of O9c- and O9d-antigens not clarified</td>
</tr>
<tr>
<td>Fisher I</td>
<td>6a</td>
<td>18</td>
<td>Structure of chemotype 24 is specific for O10a,10b subgroup</td>
</tr>
<tr>
<td>Habs 7</td>
<td>7a,7b,7c</td>
<td>–</td>
<td>O-PS structures identical in O11a11b and O11a11c subgroups; nature of O11b and O11c not clarified</td>
</tr>
<tr>
<td>170020</td>
<td>9a,9b,9d</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Wokatsch 16</td>
<td>9a,9c</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>170019</td>
<td>9a,9d</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Fisher V</td>
<td>10a,10c</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>170016</td>
<td>11a,11c</td>
<td>26</td>
<td></td>
</tr>
</tbody>
</table>

*Data of this table are adapted from Knirel et al. [103]. O-PS designates O-specific polysaccharide.*

*In the Fisher immunotype V strain, the classification based on chemical structure does not correlate with slide agglutination results.*
Table 5
Protective activity of *P. aeruginosa* PAC1R and its mutants with defective LPS

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Percentage of mouse survival at <em>P. aeruginosa</em> challenge with Fisher immunotype I–VII</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAC1R (S-LPS)</td>
<td>60</td>
<td>40</td>
<td>80</td>
<td>20</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>PAC557 (R-LPS)</td>
<td>60</td>
<td>20</td>
<td>80</td>
<td>60</td>
<td>0</td>
<td>20</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>PAC608 (SR-LPS)</td>
<td>60</td>
<td>0</td>
<td>NS</td>
<td>40</td>
<td>0</td>
<td>60</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>PAC609 (SR-LPS)</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>PAC610 (R-LPS)</td>
<td>60</td>
<td>40</td>
<td>NS</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>PAC612 (R-LPS)</td>
<td>40</td>
<td>60</td>
<td>NS</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>LPS of homologous strain</td>
<td>80</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aData adapted from [131]. Animals used in the experiments were 50 mice/group, 10 mice/challenge dose and 5 challenge doses were chosen. One challenge dose = 3X–7X LD50 values.

bInterpretation of results: 80–100% survival was regarded as high protection, 60–70% survival meant medium range protection, and < 50% survival was regarded as no protection. NS designates not studied.

... studied LPS from *Enterobacteriaceae*. The *P. aeruginosa* LPS contains also a hydrophobic lipid part (lipid A), to which an O-specific polysaccharide chain is attached via a core oligosaccharide [90]. The structurally most conservative part of the molecule, lipid A, shares many features that are also seen in lipid A of *Enterobacteriaceae*. Composition analysis of LPS from rough mutants derived from serotype O5 and O6 revealed that the core oligosaccharide is composed of D-glucose, L-rhamnose, 2-amino-2-deoxy-D-galactose, L-glycerol-D-manno-heptose (Hep), 3-deoxy-D-manno-octulosonic acid (Kdo), L-alanine and phosphate. An unusual 7-O-carbamoyl substitution was observed on the second heptose under mild hydrolysis conditions [91,92]. To date, accurate structural determinations of core oligosaccharides from serotypes O3 [93], O5 [94] and O6 [91,92] have been accomplished using high resolution nuclear magnetic resonance and fast atom bombardment mass spectrometry.

4.2.1. O-specific polysaccharide (O-antigens)

The most variable part of the LPS molecule is an O-specific PS chain, which determines the serogroup or subgroup-specific antigens. Analysis of the monosaccharide composition of the O-specific polysaccharides, that was carried out in the 1970s [95–102], revealed the presence of xylose, glucose, rhamnose, glucosamine, 2,4-diamino-2,6,4-trideoxy-D-glucose, and L-galactosaminuronic acid. However, many other amino components failed to be identified, and this has delayed the progress in structural determination of PS until the 1980s. The composition of *P. aeruginosa* O-specific PS [94] differs significantly from that of PS of many other Gram-negative bacteria studied [104]. In enteric organisms, neutral sugars are usually present in abundance. In contrast, neutral sugars occurred infrequently in *P. aeruginosa* PS. Of four neutral sugars found in *P. aeruginosa* O-specific PSs (D-glucose, L-rhamnose, D-xylose, D-ribose) only rhamnose is encountered frequently (in six out of the 13 O-serogroups [103]). Also, the PSs of four O-serogroups, O1, O2, O9 and O12, do not contain any neutral sugars.

*P. aeruginosa* PSs are rich in monoamino and diamino sugars, some of them carrying simultaneously a carboxyl substituent. Among amino sugars widely distributed in nature, hexosamines (D-glucosamine, D-galactosamine) are encountered rather infrequently, whereas 6-deoxy hexosamine derivatives (D-quinovosamine, D- and L-fucosamine) are present in the majority of the serotypes. Acid monosaccharides are represented by three classes: 2-amino-2-deoxyuronic acids, 2,3-diamino-2,3-dideoxyuronic acids, and 5,7-diamino-3,5,7-tetraacetyloxynulosonic acids. Most amino groups of the amino sugars are acetylated, but in some cases they carry acyl substituents that occur rarely in natural carbohydrates. N-Acetyl derivatives of D-galactosaminuronic acids are found both as free acids and as primary amides. Finally, many constituent monosaccharides (L-rhamnose, N-acetyl-D-fucosamine, derivatives of D- and L-galactosaminuronic acid and of pseudaminic acid) carry O-acetyl groups, which have been found in 7 O-serogroups [103]. The O-specific PS chains of LPSs of 15 O-serogroups differ in their composition,
and hence, O-specificity of the respective strains correlates with the monosaccharide composition of O-antigens [103].

The results of chemical investigations show the differences among O-PS primary structures of (or constituent sugar residues) 15 O-serogroups of P. aeruginosa [103,105]. O-PS primary structure of subgroups also differ, but within every subgroup, there are similar compositions. O-PS chains consist of disaccharides. Taking all differences into consideration, 31 chemotypes of P. aeruginosa were determined based on O-PS primary structure: O1-serogroup includes one chemotype; O2-serogroup (6 subgroups) - 7 chemotypes; O3-serogroup (3 subgroups) - 3 chemotypes; O4-serogroup (2 subgroups) - 2 chemotypes; O6-serogroup (4 subgroups) - 5 chemotypes; O7-serogroup (3 subgroups) - 3 chemotypes; O9-serogroup (3 subgroups) - 2 chemotypes; O10-serogroup (2 subgroups) - 2 chemotypes; O11-serogroup (2 subgroups) - one chemotype; O12-serogroup - one chemotype; O13-serogroup (2 subgroups) - 2 chemotypes; and O14- and O15-serogroups - one chemotype, respectively.

The data from structural elucidation studies are important and provide evidence verifying the accuracy of serological typing schemes of P. aeruginosa (Table 3) [6,17,103,105]. As well, these chemical data facilitate the interpretations of cross-reactions in serological tests. For instance, O2-serogroup is the most heterogeneous in terms of the presence of partial O-antigens (serological and immunochemical data). All O-PSs of this serogroup contain a common monosaccharide component, 2-acetamido-3-acetamido-2,3-dideoxy-d-mannuronic acid, which seems to determine the partial O2a-antigen. Among other partial antigens, common to several subgroups, the partial O2b-antigen, characteristic of the O2a,2b and O2a,2b,2e subgroup, is probably related to a fragment of the carbohydrate skeleton, which is the same for both polysaccharides (Table 3). O2d-antigen, common to O2a,2d, O2a,2d,2e and O(2a),2d,2f subgroups, may be due to the presence of the trisaccharide units; for the O2a2d subgroup these are the only type of units, for the O2a,2d,2a subgroup they represent the minor type of units, and for the O(2a),2d,2e subgroup they account for approximately 35% of the total trisaccharide units. The presence in the O-specific PS of two types of trisaccharide units may also account for the fact that some strains, belonging to the O2-serogroup, react in both anti-O2b and anti-O2c sera [106], although no subgroup has been established which contains simultaneously O2b- and O2c-antigens. In fact, the O(2a),2c-PS contains both the units (major type), which seems to be related to the O2c-antigen and the units (minor type), determining the O2b-antigen.

As for the O2c-antigen typical of O2a,2b,2e and O2a,2d,2e subgroups, it is difficult to relate it unambiguously to any common fragment in the corresponding polysaccharides. Despite the common sugar residues shared by these O2-serogroup strains, there is the specificity of O-antigens owing to perhaps the structural organization in every subgroup. This interpretation is supported by evidence provided by results of inhibition of passive hemagglutination or immunoprecipitation tests. Thus, O2a,2d,2f-antigen is the most serologically detectable antigen in O2-serogroup. This subgroup consists of two types of O-PS differing by the structure (Table 3).

Structural analysis and inhibition of passive hemagglutination showed that immunotype VII O-antigen and O2a2d-antigen are not identical [103,105], although using bacterial agglutination and passive hemagglutination gave identical results in the Lanyi-Bergan scheme [6]. The cross-reactions between these antigens in the immunoassays are likely caused by the common fragment of O-PS chain: 4)-β-D-ManN₃Ac₂U-(1→3)-α-D-FucNAc-(1-, and by the presence of O2a,2d-antigen which constitutes approximately 10% of O-antigens found in Immunotype VII. Most probably, immunotype VII O-antigen besides O2d factor can contain other O-factors common with O2a,2d,2f-antigen and related to monosaccharide: -α-1-GulNAcAmU. Immunotype VII (Fisher 7 reference strain) may have a new subgroup antigen. According to O-PS chain structure, the partial O2a,2b,2e-antigen (Wokatsch 25 reference strain) is somewhat different from O2a2b-antigen (170003 reference strain) (Table 3).

The identity of O-PS structure of 170001 and Wokatsch 14 reference strains is established, both strains are included in the same O3a,3b subgroup or chemotype 9 [103,105].

The structure of immunotype I O-antigen is not identical to the structure of O6a-antigen although,
serological cross-reaction is observed between both antigens. Probably, immunotype 1 O-PS contains common O-factor with one or several other subgroups or has an additional O-factor. Thus, O-antigen of immunotype I is isolated into a separate chemotype [103].

O6a- and O6abc-antigens are similar by structure, with both antigens having a common carbohydrate skeleton, however, but O6a-antigen contains the additional O-acetyl group bound to α-D-GalNAcUN.

The structure of O-antigen of Habs 8 strain is identical to that of O7a, 7b, 7d-antigen but not to O7a, 7d-antigen as it could be precluded by the Lanyi-Bergan scheme [6].

The structure of O-antigen of immunotype V is identical to that of O10a, 10b-antigen but not to O10a, 10c-antigen as is shown in the Lanyi-Bergan scheme. Bacterial cells of immunotype V serologically react with anti-O10 serum and could absorb all agglutinins from anti-O10a, 10c serum (anti-170002 serum).

Some discrepancies between serological analysis and structural analysis could be observed. In the case of O11a, 11b and O11a, 11c serotypes, chemical elucidation of LPS structures from both showed identical sugar repeat structures, 3αL-1-FucNAC-(1→3)-β-D-FucNAC-(1→2)-β-D-Glc-(1. The predominant O11a-antigen is designated based on serological results and this antigen likely has the aforementioned structure obtained from chemical analysis (Table 3). The differences between O11a, 11b and O11a, 11c could not be resolved based on the chemical analysis. Therefore, the differences that were contributed by the O11b- and O11c-antigens respectively appeared to be related to other heat-stable antigens that are not part of the PS of the O-antigen of O11. It should be noted that a number of strains were found which reacted in both anti-O11b and anti-O11c sera (Lanyi, personal communications). Whether or not such cultures represent a third subgroup (O11a, 11b, 11c), is not elucidated at this time; for epidemiological purposes, the establishment of O11a, 11b, 11c subgroup does not appear justified. The nature of O11b- and O11c-antigens is not yet clear [103, 105].

Thus far, 31 chemotypes of *P. aeruginosa* were determined on the basis of structural analysis and immunochemical investigations of *P. aeruginosa* O-antigens (Table 3) [103, 105]. It could be concluded that the knowledge of the primary structure of the O-PSs is useful but not sufficient for postulating the existence of some common epitopes. To alleviate this problem, data for macromolecular structure of LPS are also needed.

The data of the structures of the O-specific PSs represent the chemical basis for the classification of *P. aeruginosa* (as an example, chemotype classification). They are valuable for the improvement of the known serological classification and for the identification of different strains of this microorganism. These data are also necessary for the preparation of synthetic or semisynthetic antigens and artificial vaccines, which may be useful for the immun prophylaxis, immunotherapy or diagnosis of *P. aeruginosa* infection. Comparison of data for the structures of O-PSs and for the specificity of the corresponding O-antigens shows good agreement in most cases. Some contradictions revealed in the course of the comparative study on reference strains from various classification schemes may lead to a further extension or alteration of the compiled schemes. Table 4 summarizes some finding [103] for the 'problematic' strains. In particular, it would be advisable to examine Fisher immunotypes I and VII to identify the partial antigens. This type of results may allow the classification of these two immunotypes into separate O-subgroups of O2- and O6-serogroups, respectively. Investigation of the nature of O9c-, O9d- and O11c-antigens may also be of value.

O-specific PSs have been used for the preparation of O-PS-toxin A conjugate vaccine containing 37% of mixed O-PSs of eight serogroups and 63% toxin A. The vaccine induced in mice IgG antibodies to all eight O-serogroup-specific antigens and toxin A. However, it was observed that the immunogenicity of O-PSs varies, with OPs from certain serogroups being highly immunogenic while others only stimulated modest antibody response. Immunization protects mice against challenge by *P. aeruginosa* strains of homologous serogroups [107]. In human volunteers, a significant IgG antibody response to a booster dose of an O-PS-toxin A vaccine was also observed [108].

Antigenic relationships between certain O-antigens of *P. aeruginosa* and *Enterobacteriaceae* were established on the basis of the minor unilateral and a few
bilateral agglutination reactions, for example: *P. aeruginosa* O2-serogroup with *Proteus rettgeri* O1,17; *Salmonella* O21 and O63; *Citrobacter* O32 and O35; *Proteus hauseri* O20; *P. aeruginosa* O4-serogroup with *Salmonella* O64; *Citrobacter* O16 and O22; *Shigella flexneri* O1a; O1b; O4aB; O5 and Y; *P. aeruginosa* O6-serogroup with *E. coli* O26; *P. aeruginosa* O11-serogroup and *Salmonella* O59; and *Proteus hauseri* O60 and O2 etc. [6]. A serological relationship has been revealed between O-antigens of *P. aeruginosa*, *Salmonella arizona* and *Citrobacter* sp. [109]. Specifically, a unilateral serological relationship by immunoblotting between LPSs of *C. freundii* O35, *S. arizona* O59 and *P. aeruginosa* O11 was revealed. In Western immunoblots, O-antiserum to all three bacteria recognized both slow moving and faster migrating bands of the *P. aeruginosa* O11 LPS, while antibodies to O-PS of *P. aeruginosa* did not bind the LPS of either *C. freundii* or *S. arizona*. The cross-reactivity is apparently based on chemical structural similarity between the PSs. Apart from one common monosaccharide (α-FucNAc) the sugar components of LPS among these three bacterial species are different. However, the PSs of all three LPSs have the repeating units of the same size with the corresponding monosaccharide residues having the same absolute configurations and being connected to each other by the same a or b linkages. Conformational analysis [110] confirmed the presence of structurally similar sites in the helix surfaces of each of the two PSs, which may serve as a common epitope. However, the serological relationship of these bacteria could not be discerned by less sensitive methods such as rocket immunoelectrophoresis, and gel immunoprecipitation. When inhibition of ELISA was performed, less than 50% inhibition was achieved even at the use of high amounts (10 mg) of the inhibiting antigen tested [109].

In conclusion, the definitive chemical nature of cross-reactive epitope(s) of *P. aeruginosa* O-antigens is not yet known.

4.2.2. Core oligosaccharide

The core portion of LPS consists of two regions: an inner core containing L-glycero-d-manno-heptose and 3-deoxy-d-manno-octulosonic acid (KDO) and an outer core containing d-glucose, L-rhamnose, d-galactosamine and L-alanine. In addition, phosphate and probably ethanolamine are also components of the core [90–95,99,111,112]. The immunological study of different *P. aeruginosa* rough mutants permits the determination of antigenic properties of the core oligosaccharide.

Ziegler [113] apparently had some difficulty in demonstrating cross-reactivity of polyclonal antisera to rough mutants (core-lipid A region) with LPS of wild-type (smooth strains). The immunogenicity of the core-lipid A epitopes became apparent when de Kievit and Lam [114] were successful in producing monoclonal antibodies (mAb) with specificity against various epitopes of the core-lipid A region. Among those antibodies, mAb 18–19 recognizes core-plus-one epitope of O2/O5/O16 IATS serotypes. Monoclonal antibody 5c101 is specific for the outer core regions of *P. aeruginosa* LPS, and it is cross-reactive only to O2/O5/O16 serotypes. These serotypes are chemically related and share similar backbone sugar residues composed of two uronic acid and one FucNAc residues (Table 3). Another mAb 7–4 recognizes the inner core region and is cross-reactive with all *P. aeruginosa* strains, it is therefore a genus-specific antibody. Monoclonal antibody 177 is specific for a lipid A epitope of *P. aeruginosa* LPS and is broadly cross-reactive to all Gram-negative bacteria examined [114]. Others have reported the production of human monoclonal antibodies to the outer core region of serotype A and G respectively (Homma serogroups) also reacted in vitro with *P. aeruginosa* of several O-serogroups and protected mice against *P. aeruginosa* clinical isolates of different O-serogroups [115–117]. The protective activity of the human monoclonal antibody was apparently related to opsonophagocytic activity [118].

Thus, *P. aeruginosa* LPS core region contains common epitopes which may induce a protective effect in animals. Fig. 1 depicts the core oligosaccharide structure of serotype O5 and O6 and the probable epitopes identified based on immunochemical analysis with mAbs.

4.2.3. Lipid A

Lipid A of Gram-negative bacteria causes endotoxic effect. When conjugated with BSA, this OM constituent was shown to elicit the formation of anti-lipid A antibodies [119]. Composition of fatty acids found in *P. aeruginosa* resembles those for
some other bacteria (e.g. *Acrononas agilis, Chromobacterium violaceum*, and *Rhodobacterium vannieli*) but it differ from those of enterobacterial LPS in several respects [120]. Kulshin et al. showed [121] that the backbone of *P. aeruginosa* lipid A consists of a pyranosidic 1,6-linked β-glucosamine disaccharide (β-D-GlcP-(1→6)-β-D-GlcP), phosphorylated in positions 4’ and 1 and there is a lack of fatty acyl substituent on the O-3 of the lipid A backbone. A report by Bhat et al. [122] depicted the lipid A of strain PAO1 as having a 1→6-linked glucosamine with an unsubstituted phosphate group linked to the C-1 position of the reducing glucosamine and an ester-bound phosphate at the C4’ position of the non-reducing glucosamine. The latter phosphate group is found to be non-stoichiometrically substituted with 4-amino-4-deoxyarabinose (4-AraN). The presence of 4-AraN appeared to be due to an inductive process because it was only detected when LPS was extracted in small batches of cultures and not in large batches grown in fermenters. No pyrophosphate groups were detected and ester-linked fatty acids are located at the amino groups at the C3 and C3’ positions. Another study performed by Karunaratne et al. [123] also showed the same structure of *P. aeruginosa* lipid A plus an observation of tetraacyl fatty acids substituents in addition to the hexaacyl and pentaacyl substituents described by the other authors earlier [121,122].

The structure of lipid A is apparently common to all *P. aeruginosa* strains and may share cross-reactive epitopes to lipid A of other Gram-negative bacteria. It was demonstrated that human monoclonal antibodies against *E. coli* lipid A protected neutropenic rabbits from lethal pseudomonas infection [124]. In one of our studies, an anti-lipid A murine mAb 177 raised against *P. aeruginosa* strain PAO1 cross-reacted with lipid A of all *P. aeruginosa* serotypes and other Gram-negative bacteria [114]. The immunogenicity of lipid A was confirmed by the observation that antibodies to lipid A were detected in patients with Gram-negative infections [125].

### 4.3. Cross-protective immunity induced by *P. aeruginosa* LPS

*P. aeruginosa* LPS (0.1–1 μg) stimulated a chemotype-specific protective immunity in mice on day 5–14 after single or double injections, and O-specific antibodies were determined during this period. While immunization with low doses (0.1–1 μg) elicited an increase in antibody level the use of high doses (100 μg) inhibits antibody production. However, there was no correlation between the level of antibody against O-antigen and chemotype-specific protection level. The primary structure of O-PS determines immunochemical specificity and specific protection of animals [126] and probably, humans.

By serological analysis, Habs 6 (chemotype 14, Table 3) and Immunotype I (chemotype 18) are considered homologous to one another. Using low doses of LPS-Habs 6 and LPS-immunotype I, cross-protections against challenge by either strain of bacteria were observed [126]. Chemical study of O-PS of immunotype I and Habs 6 showed that the structure of both tetrasaccharides was similar but not identical. The differences were determined by the linkage between N-acetylglucosamine (d-QuiNAc) and rhamnose (Rha) residues [103]:

Habs 6 \(-\alpha-\text{d-Qu}i\text{NAc)-(1→3)-}\alpha-\text{l-Rha-}\) (Structure 1)

Immunotype I \(-\alpha-\text{d-Qu}i\text{NAc)-(1→2)-}\alpha-\text{l-Rha-}\) (Structure 2)

In addition, O-PS of immunotype I contains one additional NH₂ group linked to N-formyl-galactosaminuronic acid residue [103]. It appears that such a small difference in the tetrasaccharide of O-PS structure is not recognized by the immune system, neither on the formation of O-specific antibodies nor of protective immunity. The low dose (1 mg) of some other LPS chemotypes stimulated cross-protection in mice although the primary structure of O-PS is different [103,105]. What would be the conditions that favor
cross-immunological responsiveness? It is possible that O-specific PS chains cover the epitopes of the core oligosaccharide in the majority of P. aeruginosa smooth strains. Hence, typical smooth LPS (0.1–1 µg) stimulated chemotype-specific protective immunity. However, some smooth P. aeruginosa strains have O-specific PS chains that do not cover all antigenic determinants of the core oligosaccharide. Previous studies had indicated that up to only approximately 14% of P. aeruginosa LPS are fully capped with long chain O-specific polysaccharide side chain [90]. It has been described earlier that LPS prepared from P. aeruginosa are heterogeneous in size and they exhibited a ladder-like banding pattern in silver-stained SDS-polyacrylamide gel [68]. In an electron microscopy study, using a cryoprotective technique known as freeze-substitution, the B-band LPS of P. aeruginosa serotype O6 strain appeared as patches of fibrous structures on the bacterial cell surface. This indicated that the O-polysaccharide of this serotype do not provide a uniform coverage to the entire cell surface [127]. Therefore, it is highly possible that cross-reactive LPS epitopes such as those in the core oligosaccharides are not completely covered by the hydrophilic long chain O-polysaccharides. It is therefore plausible that low doses of this LPS could stimulate some anti-core protective response. It is known that there are conserved epitopes among core oligosaccharide of LPS in Gram-negative bacteria [128].

In one of our studies, immunization by low doses of LPS (0.1–1 µg) caused specific protection in mice but when high doses (10–100 µg) were used, cross-protection against the challenge with the P. aeruginosa heterologous strains was induced [126]. Using lipid A prepared from various strains, cross-protection in mice was observed [129]. Since the lipid A structure is highly conserved in P. aeruginosa, this cell envelope antigen should theoretically stimulate cross-protection. The only concern is whether lipid A would be sufficiently accessible on the bacterial cell surface for antibodies to interact with.

Data from a study by Cryz et al. [130] suggested that P. aeruginosa LPS core oligosaccharide (R mutants) can be a serotype-specific protective antigen. However, our experiments established that P. aeruginosa R mutants induce cross-protection against some P. aeruginosa immunotypes [131] (Table 5). Thus, LPS core-lipid A region is potentially a good candidate as a common protective antigenic complex.

The conformation of the complex LPS molecule likely plays a role in the induction of protective and, especially, cross-protective immunity. It is our assumption that P. aeruginosa LPS of some chemotypes share similar or identical conformational protective determinants. Intensive immunization with a subtoxic dose of 100 µg LPS per mouse caused cross-protection in mice against P. aeruginosa although the primary structure between the O-antigens was different. In this case we may assume that there exist conformational protective determinants of P. aerugi-

### Table 6

<table>
<thead>
<tr>
<th>Route of immunization</th>
<th>Relationship between chemotypes&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>Hypothesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Intraperitoneal and subcutaneous</td>
<td>1 ↔ 18</td>
<td>1 = 18 = 26, these chemotypes have identical (common) conformational protective determinant(s)</td>
</tr>
<tr>
<td>subcutaneous</td>
<td>8 ↔ 26</td>
<td></td>
</tr>
<tr>
<td>2. Either subcutaneous or intraperitoneal</td>
<td>1 ↔ 8</td>
<td>1 ~ 8 ~ 18 and 4 ~ 24 ~ 26 chemotypes have considerably similar conformational protective determinant(s)</td>
</tr>
<tr>
<td>intraperitoneal</td>
<td>8 ↔ 18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 ↔ 24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 ↔ 26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26 ↔ 4</td>
<td></td>
</tr>
<tr>
<td>3. Subcutaneous alone</td>
<td>1 ↔ 4 or 26</td>
<td>‘Partially’ similar conformational protective determinant(s)</td>
</tr>
<tr>
<td></td>
<td>4 ↔ 8, 24 or 26 etc.</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Data in this table are adapted from Stanislavsky et al. [126]. Conformational protective determinants are revealed in a course of single or double immunization with high (10–100 g) LPS doses.

<sup>b</sup>(↔) designates the chemotype of LPS protects against P. aeruginosa strains with heterologous LPS and vice versa (i.e. a bidirectional relationship).
nosa LPS. The following hypothesis could be made: if the chemotype of LPS protects against P. aeruginosa strains with heterologous LPS and vice versa, it is possible to admit absolute identity of the conformational protective determinants of different LPS chemotypes. Bi-directional protective activities are observed only in the case of subcutaneous or intraperitoneal antigen injections. These protection patterns between different chemotypes probably show that considerable similarity exists between them [126]. If the LPS chemotype protects mice against a strain with heterologous LPS but not vice versa (unidirectionally), it indicates that there could be some similarities of conformational protective determinants between the different LPS chemotypes (Table 6) [126].

The view of the existence of a conformational epitope of O-antigen was supported by the immunochemical data whereby LPS-specific monoclonal antibodies were produced and some of these antibodies showed cross-reactivity among different P. aeruginosa serotypes [116]. The structure of core oligosaccharide and/or lipid A plays an important role in the stimulation of cross-protection and probably also in conformation of LPS molecule. Evidently, within different LPS chemotypes there are identical, and similar or partly similar determinants which cause cross-protection against P. aeruginosa infection.

4.4. Common polysaccharide antigen

It is known that the immunospecificity of P. aeruginosa LPS is determined by a fine structure of O-PS (O-antigen) [103,105]. It has been found that, together with O-antigen which is usually an acid PS, many P. aeruginosa strains synthesize neutral polysaccharide [117,132–134]. One of them is a common polysaccharide antigen (CPA) which was first described by Sawada et al. [132]. A monoclonal antibody to P. aeruginosa strain IFO3080 binds in ELISA to approximately 80% of the tested strains of various serovars of P. aeruginosa and some other pseudomonads (P. maltophilia, P. taetrolens, P. aerofaciens). CPA was isolated from LPS of several P. aeruginosa strains and shown to have the structure of β-rhamnan [132,133]:

\[ -3)\alpha-D-Rhap(1 \rightarrow 3)\alpha-D-Rhap(1 \rightarrow 2)\alpha-D-Rhap(1 - \text{(Structure 3)} \]

It was shown that the monoclonal antibody against CPA also binds to another, structurally distinct neutral polysaccharide antigen (extracellular neutral polysaccharide) of P. aeruginosa [134].

The study of phytopathogenic pseudomonad LPSs shows that P. cerasi produces O-antigen which has the same structure as CPA (Structure 3) [135]. Serological cross-reaction between purified LPS’s of several strains of P. aeruginosa and O-antiserum to P. cerasi indicated that P. aeruginosa strains produce CPA immunologically different from their O-antigens. Since O-antiserum can be completely absorbed by purified LPS of P. cerasi, CPA which is recognized by this serum has the same structure as O-antigen of P. cerasi, namely the structure of β-rhamnan (Structure 3) [135]. In immunoblotting and ELISA experiments, LPSs of Immunotype V and VII CPA did not react with the anti-P. cerasi serum in immunoblotting and showed the lowest reactivity in ELISA as compared to LPS from other P. aeruginosa immunotypes. CPAs that cross-reacted avidly with anti-P. cerasi serum were observed in LPS prepared from immunotype II, IV and VI using both immunoassay methods [135].

These results correlate well with the chemical data. Thus, by mild acetic acid degradation of P. aeruginosa immunotype VI LPS which resulted in the splitting off of lipid A and depolymerization of O-antigen [136], a neutral PS was isolated and characterized as a rhamnan [133]. The same PS was prepared from the acid-degraded P. aeruginosa immunotype IV LPS after removal of O-PS by anion-exchange chromatography. On the contrary, in the acid-degraded P. aeruginosa immunotype III LPS the analogous analysis revealed only a negligible amount of neutral material containing CPA.

It is noteworthy that two series of ladder-like bands (A and B) were observed when LPS was prepared from P. aeruginosa PAO1 and subjected to SDS-PAGE [82,83,137]. Only one series (B-bands) reacted in immunoblotting with mAb MF15-4 specific to the O-antigen of serotype O5 (IATS). Another mAb, N1C9, specific to A-bands revealed the presence of A-series in LPS from 11 out of 17 standard serovar strains of the IATS of P. aeruginosa and 170 out of 250 clinical isolates from patients with cystic fibrosis [82]. Currie et al. [138] also demonstrated by Western immunoblotting using an A-
band LPS-specific mAb that A-band-like antigens are also found in other species of pseudomonads including *P. acidovorans* and *P. putida*. These results are supported by Southern hybridization results in which a DNA probe made an A-band biosynthetic gene, *gae* (GDP-β-mannose conversion protein for A-band common antigen polysaccharide), hybridized with DNA prepared from *P. acidovorans*, *P. putida* and *P. syringae*. The PS prepared from A-band LPS has a structure of α1-2,α1-3,α1-3-linked β-Rha [139] which is essentially the same structure as CPA (Structure 3) described by other researchers [132,133].

The recently discovered *P. aeruginosa* CPA with a rhamnan structure (3) is important for identification of bacteria in this species. A synthetic antigen of CPA has been prepared. As an immunogen, BSA has been conjugated to the synthetic rhamnan through the 6-aminohexyl group (rhamnan-BSA) and has the following structure:

\[-\text{3-}\alpha-\text{d-Rha-(1} \rightarrow 2\text{-}\alpha-\text{d-Rha-(1} \rightarrow 3\text{-}\alpha-\text{d-Rha}
\]
\[/5 \rightarrow 3\text{-}\alpha-\text{d-Rha-(CH}_{2}\text{)\text{,NH}_{2}}\]

(Structure 4)

PS chains in the rhamnan-BSA complex act as the CPA immunodominant carrier (epitope) and this complex was investigated as an antigen [140]. Rhamnan-BSA stimulated the production of specific antibody in rabbits. Results of inhibition of ELISA with synthetic d-rhamnan showed the presence of antibodies against CPA [140].

4.5. Outer membrane protein antigens

OM proteins participate in the formation of pores and other OM structures, and possess various functions [141–143]. The bacterial OM interacts with corresponding membrane receptors of the host immunocompetent cell, stimulating the formation of humoral and cell immunity. A number of proteins with different MWs and functions have been located in the OM of *P. aeruginosa*. According to the electrophoretic classification of OMPs by Hancock [143], the following OM proteins have been identified Table 7): iron-repressible OM proteins (IROMPs) (78–87 kDa), OprC (70 kDa), esterase (55 kDa), OprP (48 kDa), OprD1 (46 kDa) and D2 (45 kDa), OprE (45 kDa), OprF (38 kDa), OprG (25 kDa), OprH1 (21 kDa) and H2 (20 kDa), and OprI (9 kDa). IR-OMPs are synthesized when bacteria are cultivated at low iron nutrient medium. Three to nine types of different IROMPs have been detected under these conditions [144–146]. Such proteins have been called siderophores [144]. Pseudomonads produce two classes of siderophores, pyochelin and pyoverdin [147]. Two ferricyochelin receptor OM proteins were identified, a 75 kDa protein named FptA [148] and a low MW, 14 kDa protein called FBP [149]. FptA fulfills the properties of an IROMP while the low MW FBP does not. OprC and OprE appear to participate in forming specific channels through which the different carbohydrates (specifically three saccharides) permeate [150]. Esterase is a minor OM protein with enzymatic activity [151]. OprP has been identified in *P. aeruginosa* cultivated in medium with low phosphate content (0.15 M or lower). The function of OprP is apparently specific for phosphate transport [152]. Benz and Hancock [146] established that isolated protein P trimers could be reconstituted in planar lipid bilayer membranes from diphytanoyl phosphatidylcholine. OprP forms highly anion-selective pores in lipid bilayer membrane [152].

OprD1 is co-regulated by a glucose-transport system [153]. This protein participates in the formation of porins through which glucose and xylose permeate. Similar proteins are detected in both *P. fluorescens* and *P. putida*. The OprD1-homologues in these bacteria immunologically cross-react with OprD1 of *P. aeruginosa* [154,155]. *P. aeruginosa* mutants resistant to carbenem - β-lactam imipenem have been isolated from clinical material and experimental animals. This class of mutants lost the OM protein with a MW of 45 kDa which is similar to OprD2 [156–158]. OprD2 was implicated to play a physiologically significant role in the uptake of basic amino acids and peptides [158]. This protein has been synthesized by cultivating strains in a medium supplemented with glucose or other saccharides [156]. OprF is a porin [159,160] and constitutes a non-specific channel of the OM which adds stability and determines shape [154]. *P. aeruginosa* protein F and *E. coli* OM protein A share amino acid sequence homologies and are immunologically cross-reactive with each other [161].

[...]

The synthesis of OprG depends on the conditions of bacterial cultivation. OprG content in OM varies...
Table 7
The characterization of OM proteins of *P. aeruginosa*

<table>
<thead>
<tr>
<th>Protein*</th>
<th>MW (kDa)</th>
<th>Conditions favoring expression</th>
<th>Function</th>
<th>Immunological activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IROMPs</td>
<td>78-87</td>
<td>Low iron</td>
<td>Binding of siderophores, iron acquisition</td>
<td>Not studied</td>
<td>[144-146]</td>
</tr>
<tr>
<td>OprC</td>
<td>70</td>
<td>Not determined</td>
<td>Porin</td>
<td>Not studied</td>
<td>[150]</td>
</tr>
<tr>
<td>Esterase</td>
<td>55</td>
<td>Constitutive</td>
<td>Enzyme</td>
<td>Ab response</td>
<td>[151,169]</td>
</tr>
<tr>
<td>OprP</td>
<td>48</td>
<td>Low phosphate</td>
<td>Transport of phosphate</td>
<td>Conserved and induces cross-reactive Ab response</td>
<td>[125,145,152]</td>
</tr>
<tr>
<td>OprD1</td>
<td>46</td>
<td>Glucose as carbon source</td>
<td>Porin, transport of glucose and other substrates</td>
<td>Good immunogen, Ab cross-reactive with OM proteins of <em>P. fluorescens</em> and <em>P. putida</em></td>
<td>[153-155]</td>
</tr>
<tr>
<td>OprD2</td>
<td>45.5</td>
<td>Certain carbon source</td>
<td>Porin, selective uptake of basic amino acid and peptides</td>
<td>Not determined</td>
<td>[156-158]</td>
</tr>
<tr>
<td>OprE</td>
<td>44</td>
<td>Not determined</td>
<td>Porin</td>
<td>Not determined</td>
<td>[150]</td>
</tr>
<tr>
<td>OprF</td>
<td>38</td>
<td>Constitutive</td>
<td>Porin, structural</td>
<td>Protective immunogen, Ab cross-reactive with OmpA of <em>E. coli</em></td>
<td>[159-161,177-179]</td>
</tr>
<tr>
<td>OprG</td>
<td>25</td>
<td>High iron, high Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Unknown</td>
<td>An immunogen, Ab response</td>
<td>[159,162,169]</td>
</tr>
<tr>
<td>OprH1</td>
<td>21</td>
<td>Low Ca&lt;sup&gt;2+&lt;/sup&gt;, Mg&lt;sup&gt;2+&lt;/sup&gt;, Mn&lt;sup&gt;2+&lt;/sup&gt;, Sr&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Stabilizing Mg&lt;sup&gt;2+&lt;/sup&gt;-deprived cells</td>
<td>Ab cross-reactive with <em>P. chlororaphis</em></td>
<td>[163]</td>
</tr>
<tr>
<td>OprI</td>
<td>9</td>
<td>Constitutive</td>
<td>Lipoprotein</td>
<td>Immunogen, Ab response detected</td>
<td>[165,166,169]</td>
</tr>
</tbody>
</table>

*The nomenclature and the electrophoretic properties of OM proteins of *P. aeruginosa* are adapted from Hancock et al. [143].

depending on the carbon source in the medium [159,162]. The function of OprG is not yet known. Probably, this protein participates in penetration of fluoroquinolone or low-affinity iron into bacterial cell. OprH1 is synthesized when *P. aeruginosa* is grown in medium with low concentration of Mg<sup>2+</sup>, Ca<sup>2+</sup>, Mn<sup>2+</sup> and Sr<sup>2+</sup> ions. This protein apparently has a role in blocking the penetration of antibiotics [163]. OprH2 is a lipoprotein which was shown to be bound covalently with cell peptidoglycan [159,164]. Finally, OprI is a low molecular mass lipoprotein which is present in large amounts in *P. aeruginosa* OM. It has high homology to a lipoprotein of *E. coli* OM [165,166].

Some authors also indicate the presence of other OM proteins. For instance, a protein with a MW of 54 kDa was found in *P. aeruginosa* mutants resistant to norfloxacin. This protein likely has a role in the blocking and penetration of norfloxacin into bacterial cells [167]. Proteins with a MW of 26–30 kDa were also observed in the same *P. aeruginosa* mutants. These proteins were similar to extracellular proteins of corresponding *E. coli* mutants [168]. OM proteins with MWs of 9, 14, 29, 21, 25, 26, 30, 40, 44 and 55 kDa have been isolated by saline or Tris-EDTA extraction from *P. aeruginosa* cultivated on meat-peptone agar [169]. Protein A (110–120 kDa) and protein B (11 kDa for 11 O-serogroup strain and 37 kDa for O2-serogroup strain) were isolated by the aqueous extraction [170]. Thus, various cell proteins localize in the *P. aeruginosa* OM. Many of these proteins are involved in the formation of specific and non-specific channels through which nutrients, substrates or antibiotics, and other antibacterial drugs penetrate into the cell. The OM functions as a selective barrier. Some *P. aeruginosa* OM proteins have similar chemical structures with OM proteins of other pseudomonads or *E. coli* and probably with other Gram-negative bacteria. Cross-immunological reactions were observed between these proteins, i.e. these proteins have common epitope(s) that are possibly important for the development of vaccines against *P. aeruginosa* and Gram-negative infections.

Homma et al. [171–173] were the first to study immunological properties of OM proteins of *P. aeruginosa*, particularly protein components that are co-extracted with endotoxins from the filtrate of autolysed culture. Using electrophoresis analysis, two components were shown: (1) LPS-protein and (2)
Table 8
Localization of specific antigens (epitopes) in *P. aeruginosa* and current status of results from characterization and protective property studies

<table>
<thead>
<tr>
<th>Cell structure or localization-Specific antigen (epitope)</th>
<th>Characterization</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extracellular products:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elastase</td>
<td>Species-specific antigens</td>
<td>Protective protein antigen</td>
</tr>
<tr>
<td>Proteases I and III</td>
<td>Species-specific antigens</td>
<td>Protective protein antigen</td>
</tr>
<tr>
<td>Exotoxin A</td>
<td>Species-specific antigen, common epitope with diphtheria toxin</td>
<td>Protective protein antigen, induces antitoxic immunity</td>
</tr>
<tr>
<td>Extracellular slime layer</td>
<td>Species-specific antigens</td>
<td>Protective alginate antigens, specific epitopes of 30–&gt;300 kDa and 10–30 kDa alginate components.</td>
</tr>
<tr>
<td><strong>Cell wall:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OM proteins, specific and non-specific channels</td>
<td>Group and species antigens, common epitope with other Gram-negative bacteria</td>
<td>Protective (vaccine) protein or lipoprotein antigens with MW ranging from 9 to 130 kDa</td>
</tr>
<tr>
<td><strong>LPS:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific PS (B-band repeated units)</td>
<td>O-specific PS antigens</td>
<td>31 chemotypes of O-antigen, 15 O-serogroups (31 O-subgroups), chemotype- and serogroup-specific protective antigens; conjugates of PS with toxoid and other protein carriers are effective vaccines</td>
</tr>
<tr>
<td>A-band</td>
<td>Common antigen</td>
<td>Species-specific neutral PS (rhamnan) antigen</td>
</tr>
<tr>
<td><strong>Core oligosaccharide:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer core</td>
<td>Common antigen</td>
<td>Species-specific epitope, protective antigen</td>
</tr>
<tr>
<td>Inner core</td>
<td>Common antigen</td>
<td>Species-specific epitope, protective antigen</td>
</tr>
<tr>
<td>Lipid A</td>
<td>Common antigen</td>
<td>Common epitope for <em>P. aeruginosa</em> and other Gram-negative bacteria, protective antigen</td>
</tr>
<tr>
<td>Inner/cyttoplasmic membrane</td>
<td>Specific target antigen(s) not defined</td>
<td>Phospholipids, proteins, lipoproteins</td>
</tr>
<tr>
<td>Flagella</td>
<td>H-antigens</td>
<td>Protein (flagellin) serovar-specific antigens, H1- and H2-epitopes (partial antigens), protective antigens</td>
</tr>
<tr>
<td>Pili</td>
<td>Antigens</td>
<td>Protein- (pilin, one polypeptide chain) specific antigens</td>
</tr>
<tr>
<td>Cytoplasmic:</td>
<td>Antigen(s)</td>
<td>RNA-protein complex as protective antigen, specific ribosomal protein vaccine developed, contamination by LPS could be a concern</td>
</tr>
</tbody>
</table>

**nuclein polyribose (nucleic acid and ribosome containing) complex. A protein (‘original endotoxin protein’, OEP) was isolated from the LPS-protein complex, and from the bacterial cell envelope. OEP was shown to exert pyocin and anti-tumor activities as well as protect mice [172] and minks [173] against *P. aeruginosa* challenge.**

Immunochromatography data on two other proteins isolated from the cell envelope of *P. aeruginosa* were also obtained. One of these designated protein A had a pronounced capacity for protection of mice from infection with both homologous and heterologous *P. aeruginosa* strains, whereas the other designated protein B protected animals mainly from infection with homologous strain. Protein A protected mice against infection with toxigenous PA-103 strain. Immunological analysis showed that protein A is probably species-specific while protein B is probably serogroup-specific [170,174]. Thus, two proteins possessing common epitopes and each its own specific epitope were demonstrated by gel immunoprecipitation and crossed-immunelectrophoresis [170]. In a study by Sompolinsky, who prepared sonicated lysates of *P. aeruginosa* antigens for crossed-immunelectrophoresis analysis, a common OM protein antigen was also identified [175].

Using liquid chromatography, we were able to isolate nine proteins from the OM and these were sub-
jected to immunological assessment [176]. Among these proteins, only proteins SF-4 (approx. MW of 40 kDa) and SF-7 (approx. MW of 30 kDa) were practically free of LPS, and gave one precipitation line with antiserum for homologous strain. By immunoelectrophoresis, protein SF-4 contained two anionic components and one cationic, whereas protein SF-7 contained mainly one cationic component (epitopes). Anti-SF-4 and anti-SF-7 sera were found to protect mice passively against *P. aeruginosa*. Thus proteins SF-4 and SF-7 are protective antigens, which are localized in the OM of *P. aeruginosa* [176].

Tris-EDTA extracts of *P. aeruginosa* containing proteins with MWs of 9, 14, 20, 25, 30, 38, 44 and 55 kDa (according to electrophoresis in polyacrylamide gel) protect mice against different immunotypes (serogroups) [169]. Sompolinsky et al. [175] was able to resolve 64 antigens in crude water-soluble extracts of *P. aeruginosa* by crossed immunoelectrophoresis in agarose with polyvalent anti-*Pseudomonas* immunoglobulin. Two common protein antigens were isolated from water-soluble extracts: one with a MW of 59–62 kDa and the other of 62–65 kDa. Gilletland et al. [177,178] showed that isolated porin OprF (38 kDa) protected mice against *P. aeruginosa* infection and established this protein to be a vaccine candidate. Park et al. [179] reported that OprF purified from different *P. aeruginosa* immunotypes provided variable levels of protective effect in mice, i.e. 67% or 100% protection was observed.

There are probably other cell envelope-associated substances which have antigenic properties. As an example, leucocidin (cytotoxin), a toxin bound to bacterial cell. Thus, different OM protein antigens having protective activity may be used as vaccine candidates.

On the basis of OM protein antigens, experimental studies using different versions of *P. aeruginosa* protein vaccines were performed [180]. One of these versions contained proteins with MWs of 20–100 kDa and it protected mice against *P. aeruginosa* burn sepsis [181]. This vaccine elicited a high level of specific antibodies in volunteers. Plasma collected from volunteers with specific anti-*Pseudomonas* response, protected mice against *P. aeruginosa* infection. These plasma preparations provided passive protection and helped the recovery of 87% of the patients with severe forms of *P. aeruginosa* infections [182]. The protective effect of *P. aeruginosa* OM protein vaccine in mice was also demonstrated by other authors [183].

5. Flagellar H-antigens, serotyping of *P. aeruginosa*

Verder and Evans [73] were the first to subdivide *P. aeruginosa* serogroups into serotypes (serovars) based on thermolabile antigens. In an earlier study, Lanyi provided evidence that thermolabile antigens detectable by agglutination and immobilization tests in sera raised against formalinized culture are associated with flagella [184]. He elaborated a scheme, distinguishing two serologically unrelated H-antigen complexes, H1 and H2. Strains with H1-antigen were in their majority characterized by formula H1a while some of them had antigens H1a1b, in which H1b represented a minor antigen. The H2 complex could be divided into different combinations of well-defined partial antigens: 2a2b, 2a2c, 2a2b2f, 2a2c2f, and 2a2d. Antigen combination 2a2d2e2f was shown only in strains which later proved to carry R-like somatic antigens. On the basis of a combination of O- and H-antigens, Lanyi divided his strains into 53 serotypes [184].

Using indirect immunofluorescence technique, Ansgor et al. [185] elaborated a new scheme for H-antigens of *P. aeruginosa*. They were able to distinguish between a complex flagellar antigen ‘a’ and a uniform H-antigen ‘b’. Strains with H-antigen ‘a’ shared a common factor (a0) and fell into 15 different formulae by combination of partial H-antigens a1, a2, a3 and a4. Using classification of H-antigens, Ansgor et al. divided *P. aeruginosa* isolates into 99 serotypes (serovars). By immunofluorescence technique, Ansgor confirmed Lanyi’s H classification with respect to the differentiation of H1-antigens and H2. Some discrepancies between Lanyi’s subdivision of the H2-antigen complex and Ansgor’s subdivision of the H’a’ complex were observed. These differences might be attributed to the use of sera absorbed in a different manner and with different strains, as well as obvious technical differences between agglutination and the immunofluorescence method.

Instead of designating each partial H-antigen with a symbol expressing relationship to the complex H-antigens, Pitt [186] devised a continuous numbering.
of six factors (H1–H6). The corresponding H-specific sera were prepared by suitable absorption. These sera in some occasions agglutinated field strains without immobilizing them, because they likely contained antibodies to other heat-labile antigens. To make the sera more flagella-specific, flagella antigens were prepared from strains lacking pili. Pitt’s antigen H3 was distinct and probably corresponded to the ‘uniform’ antigen of Lanyi H1 and Ansorg Hb. By cross-absorption, Pitt distinguished complex H1, H2, H5 and H4, H6; these antigens may be complex antigens of Lanyi H2 or Ansorg H’a’.

Live and formalized cultures of motile \textit{P. aeruginosa} are effective antigens for producing H-agglutinins in high titers. Immunogenicity and antibody-binding capacity of these antigens remains unaffected after heating at 60°C for 1 h, but the activity is lost after treatment with ethanol and 1 N HCl or after heating at \(\geq 75°C\). Agglutinability of H-antigens remains intact after formalin treatment. However, conditions of heating at \(\geq 70°C\) and exposure to ethanol or 1N HCl cause the loss of H-agglutinability in \textit{P. aeruginosa} cells [184].

In electrophoretic analysis of flagellar antigens isolated by differential centrifugation, a major protein band of 53 kDa was observed. This protein is likely the flagellin since this band is absent in a non-flagellated strain. The highly purified flagellin contained 16 amino acids. It has been observed that motility and chemotaxis play an important part in the virulence of \textit{P. aeruginosa} in the burned mouse model study. It was demonstrated that mice immunized with \textit{P. aeruginosa} flagellar antigen survived when they are subsequently burnt and infected at the burn site. The protection is flagellar antigen-specific and appeared to be associated with the immobilization of bacteria in the burnt skin [187].

Drake and Montie [188] showed that anti-flagella serum passively transferred to mice protects animals against \textit{P. aeruginosa} burn wound sepsis. The specific flagellar antibody was determined as IgG. Results of cross-immunological investigations showed that protection was not only H-antigen-dependent but it was specific for the flagellar antigen type. Antiserum to Hb-antigen protects only against homologous strain but not against Ha-antigen of strain. Antiserum to Ha-antigen was also effective against the homologous strain. However, protective capacity was selectively removed from antiserum by absorbing with \textit{P. aeruginosa} Fla\(^+\) cells. Anti-flagella antibody is hypothesized as exerting its protective capacity in two ways: first by inhibiting the motility or invading bacteria by binding to the flagellum and immobilizing the bacteria, and second by acting as an opsonin, targeting either immobilized or mobile cells for phagocytosis [188].

Rosok et al. [189] produced two murine monoclonal antibodies, belonging to IgG3 and IgG2a isotypes, that bind to \textit{P. aeruginosa} type a flagella and type b flagella respectively. Specificity of each monoclonal antibody for type a or type b flagella was demonstrated using ELISA, indirect immunofluorescence, and immunoblotting. Among a panel of 257 flagellated \textit{P. aeruginosa} clinical isolates, the IgG3 antibody bound to 67.7% of the isolates and the IgG2a antibody bound to the other 30.7%, for a combined coverage of 98.4%. Anti-flagella sera caused an inhibition of the motility of organisms and protected mice against \textit{P. aeruginosa} burn wound sepsis. The anti-flagella antibodies provided specific and significant prophylactic and therapeutic protection against lethal challenge with \textit{P. aeruginosa} strains [189]. However, the role H-antigen(s) plays in the induction of host protective immunity is disputable. In a \textit{Salmonella typhimurium} model, it was demonstrated that very purified flagellin could not elicit protective immunity in mice whereas the flagella preparations with some admixture of LPS-protein complex or isolated cell envelope stimulated specific protective response in mice. These observations are consistent with our result which showed that anti-flagella sera did not have a protective effect but possessed immobilization activity [51]. However, one cannot rule out the possibility that the mechanisms of protective immunity against \textit{Salmonella} and \textit{P. aeruginosa} infection could be different.

In a study by Spangenberg et al. [190], the genetic diversity of flagellins of \textit{P. aeruginosa} was examined by comparing the nucleotide and predicted amino sequences of \textit{fltC} from 20 \textit{P. aeruginosa} strains. Twelve of these strains harbor the a-type \textit{fltC} and eight of them have the b-type. The a-type \textit{fltC} were less conserved and exhibited 57 nucleotide substitutions, among which 39 occurred within a variable central region of the gene. The amino acid sequence was identical among \textit{fltC} of the eight b-type. Between
the a-type and b-type, a difference of 35% in their primary structures was observed. However, both types share strong homology in their predicted protein features, implying that the polymorphic proteins could fold into similar structures during the polymerization of the flagella. These data shed some light towards a better understanding at the molecular level of the antigenic variability among the two flagella types. More importantly, the finding of the homology in protein features between the a-type flagellin and b-type flagellin supports the intention of identifying common flagella epitopes as vaccine candidates against pseudomonas infections.

6. Pilus antigens

Pili (fimbria) are composed of polymerized pilin subunits. In *P. aeruginosa* two types of pili have been shown: (1) thin, polar pili composed of heat-labile antigens [191,192] and (2) thicker, non-polar pili associated with drug resistance plasmids [193]. The polar pili which are flexible filaments, 5.2 nm in diameter and 2.5 μm in average length, are involved in adherence. The pilin subunit is a 15 kDa protein encoded by a single copy of gene found in the chromosome [194]. Data from Paranchych’s laboratory indicated that pilins are assembled in a helical array of 5 subunits per turn, with a pitch of 4.1 nm [195,196]. The presence of an N-terminal residue, *N*-methylphenylalanine (NMePhe), and a highly conserved hydrophobic stretch of 29 amino acids at the N-terminus placed *P. aeruginosa* pili into the class of type IV pili. This class of pili is also produce by an array of Gram-negative bacteria including *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Bacteroides nodosus*, *Moraxella nonliquefaciens*, and *Moraxella bovis*. [197]. Although this N-terminus is conserved, it is likely not a region to be targetted as a vaccine candidate due to at least two reasons. First, immunogenic regions are usually hydrophilic portions of proteins. Therefore the hydrophobic nature of the N-terminus will not qualify this region of a pilin to be a good antigen. Second, this terminus may not be sufficiently accessible for antibodies to interact with it. Interestingly, the C-terminus of *P. aeruginosa* pilin was found to be responsible for adherence to epithelial cell-surface receptors. The receptor for *P. aeruginosa* PAK pilin was identified to be gangliotetraosyl ceramide (asialo-GM1) [198]. In a study by Farinha et al. [199], it was observed that alterations of the pilin by insertional mutagenesis at the C terminus of the pilin gene did not affect normal pilus biogenesis. But the bacteria lost their ability to adhere to human pneumocyte cells and showed a decrease in virulence in mice [199]. More recently, the disulfide bridge at Cys129 and Cys142 residues of *P. aeruginosa* PAK pilin was found to be essential for the appropriate conformational folding and adherence properties of pili [200]. Recent advancements in peptide technology brought forth the designing of heterodimeric coiled-coil synthetic peptides [201]. These coiled-coil peptides could enhance the presentation of pilin epitopes and could bring about a new generation of anti-pili vaccines against *P. aeruginosa*.

In serological studies, antigenic activity of pili was demonstrated by agglutination of live bacteria in anti-pilus serum prepared from live cultures. Pilus antigens are heat-labile and under formaldehyde treatment or bacteriophage action, the pili apparently withdrew into the cell and the pilin is depolymerized [202]. Pilus antigens are serologically heterologous. To obtain a pilus-specific reaction, antisera have to be absorbed with homologous O- and H-antigens. O-agglutinins are removable with boiled cultures, H-agglutinins are absorbed with formalized cells of flagellated strains, or preferably, with non-fimbriated flagellated mutants. The latter can be selected by their resistance to fimbriae-specific phage [203].

Adherence of some bacteria to cell involves pili, thus it is anticipated that antibodies against pili may be protective to the host [204]. Woods et al. [205] suggested that adherence of *P. aeruginosa* to the upper respiratory epithelium of seriously ill patients is correlated with its subsequent colonization of the respiratory tract. The use of either purified pili or homologous anti-pilus antiserum to preincubate buccal epithelial cells decreased the in vitro adherence of the homologous strain, whereas the use of a heterologous antiserum or pili prepared from heterologous strains did not decrease adherence. These findings demonstrated the potential for preventing adherence of *P. aeruginosa* by active or passive immunization against pilus antigens of the microorganism. The role of *P. aeruginosa* pili (pilin) as a pro-
tective (vaccine) antigen is not yet known. Ivanova et al. [206] did not observe any significant correlation between the presence of pili in *P. aeruginosa* strains and virulence. No significant differences could be detected between strains that have pili and strains that are pilus-deficient. Therefore, pili may only have a limited role in the adherence properties of *P. aeruginosa* to host tissues. The factors that could be involved in *P. aeruginosa* adherence are complex and each of the many cell surface structures could have a role. For instance, extracellular slime (alginate) was implicated to be involved in microcolony (biofilm) formation in *P. aeruginosa* found in the lungs of cystic fibrosis patients with chronic pulmonary infection [7]. More recently, the outer core polysaccharides of *P. aeruginosa* LPS were shown to be a ligand for binding and ingestion of *P. aeruginosa* cells by epithelial cells of the airway [207] and the cornea [208]. Nonetheless, as described earlier, a lot of advancements have been achieved in the studies of *P. aeruginosa* pili, the current focus appeared to be the targeting of the C-terminus of the pilin towards vaccine development.

7. Conclusion and future prospects

*P. aeruginosa* is an opportunistic microbe and an etiologic agent responsible for severe complications in individuals with thermal burn, mechanical extensive trauma, cancer, cystic fibrosis and following surgical operations. *P. aeruginosa* infection is a serious problem for public health. Extracellular products and cell structures of *P. aeruginosa* contain numerous antigens with different specificity and the assessment of the potential of these antigens as vaccine candidates is summarized in Table 8.

These antigens have different roles for the formation of host protective immunity against *P. aeruginosa*. Extracellular products, e.g. toxin A, exoenzyme S, proteases or elastase, are protein species-specific antigens. *P. aeruginosa* toxin A and diphtheria toxin have a common epitope. However, the role of these antigens for the stimulation of anti-infection or post-vaccinal immunity is not yet known. Alginate (100–300 kDa or more) consists of the subunits with molecular mass 10–30 kDa. Both specific and common epitopes are likely present and could induce host protective immunity against *P. aeruginosa* infection. Serotype-specific antigens (epitopes) were localized in flagella (protein H-antigens) and the role of flagella in host protective immunity was discussed. The protective role of *P. aeruginosa* pili (fimbriae) antigens has not been confirmed at present.

OM proteins with MWs of 9–130 kDa are protective and species-specific antigens which have an important role for the production of host protective immunity against infection by *P. aeruginosa*, regardless of whether or not the bacteria are synthesizing exotoxin A. LPS of *P. aeruginosa* is a complex molecule containing serogroup- (subgroup-) or chemo-type-specific O-antigens (O-specific PS), core-oligosaccharide and lipid A common antigens (epitopes) localized in B-band LPS. However, A-band LPS, which is composed of α-rhamnan, is a common antigen among *P. aeruginosa* and other pseudomonads. O-specific PS stimulates in host serogroup- (cheto-type-) specific protective immunity and core or lipid A stimulates cross-protection. α-Rhamnan conjugated with BSA did not elicit a protective response (Stanislasky, unpublished results). One possible new vaccine target in LPS antigens is the epitope in the inner core region. Using mAb 7-4, de Kievit and Lam [114] was able to demonstrate the conserved nature of the inner core among all the serotypes. More importantly, this region of the LPS was readily exposed on the cell surface regardless of the chain length of the O-antigen [114]. *P. aeruginosa* ribosomes were capable of inducing a protective response in mouse experiments but the antigenic specificity of ribosomal proteins or RNA is not yet known [209–211]. The copurification of LPS as a contaminant in pseudomonal ribosomal vaccines that caused a serotype-specific protective response has raised some concerns [212]. In the study of the biogenesis of *P. aeruginosa* pili, a number of important proteins namely, PilB, PilC, and PilC of approximate MWs of 62 kDa, 37 kDa, and 27 kDa respectively were identified. The genes encoding these proteins were located adjacent to the pilin structural gene, pilA [213]. PilD was implicated to function as a prepilin peptidase as well as a component of a protein-excretion apparatus in *P. aeruginosa* [214]. Therefore, this class of proteins may also be assessed for their potential as vaccine candidates.

The wealth of information in the literature on re-
cent development of anti-pseudomonal interventions clearly addresses the significance of *P. aeruginosa* as a public health problem. The following are examples of current progress in this area and may provide valuable clues to future directions in this area of research. A toxoid of exotoxin A was formulated in conjugate vaccines with octavalent O-polysaccharide antigens [215] and alginate respectively [60]. These conjugates are in various phases of clinical trials and safety tests. Anti-idiotypic (anti-Id) antibodies [216] which could be classified as Ab2β, that represent the internal image of the original bacterial antigen, were produced against an anti-LPS antibody specific for O-antigen of Fisher immunotype I. These antibodies afforded protection only to animals challenged with *P. aeruginosa* of the homologous serotype. However, the methodology can be exploited to generate anti-Id antibodies with broad cross-reactivity. Taking advantage of surface accessibility of OM proteins, von Specht et al. [217] used recombinant DNA techniques to produce a hybrid protein of OprF-OprI as a candidate vaccine and demonstrated protective properties of this new antigen in immunosuppressed mice. Also, oral immunization of mice with a recombinant OprI expressed in *Salmonella dublin* as a vaccine delivery organism induced IgA in the gut against OprI [217]. In a study by Gilleland and Gilleyland [218], synthetic peptides representing surface-exposed epitopes of OprF were generated and conjugated to keyhole limpet hemocyanin and the conjugate vaccine afforded protection in rats against pulmonary *P. aeruginosa* infection. New technology for antibody engineering were used in the construction of ScFv (single chain peptides of the variable region of antibody) specific for OprF [219] as well as phage display recombinant Fab specific for O-antigens [220] and inner core region [221] respectively of *P. aeruginosa* LPS. However, while these antibodies are useful reagents for engineering humanized antibodies with good affinity to *P. aeruginosa* antigens, they will have limited use as vaccines at present. One outer membrane protein that deserves more attention as a potential new vaccine target is the ferricyochelin receptor, FptA, which is a 75 kDa protein that was shown to be an IROMP [148]. This FptA protein and an LPS biosynthetic enzyme were shown by Wang et al. [222,223], using the in vivo selection/expression technology (IVET), to be essential virulence-related proteins expressed by *P. aeruginosa* in vivo in an animals model infection study. The IVET system was adopted from methods developed by Mahan et al. [224] who studied virulence-related genes of *Salmonella* sp. expressed in vivo. Since the *fptA* gene has already been cloned [148], it should be attainable to manipulate *fptA* by recombinant DNA techniques into a high expression vector system to generate sufficiently large amounts FptA protein which will facilitate the testing of immunogenicity and protective properties of FptA. The identification of a homologue of an LPS biosynthetic enzyme as another important in vivo expressed virulence factor by the IVET study by Wang is not surprising, since LPS of *P. aeruginosa* was demonstrated by Cryz et al. [225] as a major virulence factor of this organism. A recent study by Tang et al. [226] using both a wild-type strain and an LPS-deficient mutant in a neonatal mouse challenge model further confirmed the role of LPS in the pathogenic process *P. aeruginosa* infections. In addition, other studies have demonstrated that mutants with R-LPS are usually sensitive to human serum [227,228]. Therefore, strategies to develop interventions targeting enzymes involved in LPS biosynthesis or chain polymerization could render the bacteria harmless to the host. Our laboratory has accomplished the cloning and sequencing of the entire LPS biosynthetic gene cluster [229], as well as the characterization of the wzz gene (previously known as rol) involved in the modulation of B-band O-antigen chain length [230]. Although many of these genes in the B-band LPS cluster exhibited serogroup specificity based on Southern hybridization analysis, one gene, *wbpM* (new nomenclature to replace the ‘rfb’ designation of genes involved with O-antigen synthesis [231]) which resides outside the serospecific region was found to be of particular interest. Using *wbpM* as a DNA probe in Southern blot analysis, this gene was found to be conserved among all serotypes of *P. aeruginosa*. In addition, a chromosomal mutant of *wbpM* constructed by a gene replacement strategy was shown to produce only R-LPS. Work is under way to determine the localization and immunogenicity of the WbpM protein. One class of proteins that has received a lot of attention are homologues of the LuxR-LuxI proteins which are involved with quorum sensing and control of expression of virulence
determinants as well as secondary metabolites [231–234]. The vaccine potential of this type of protein is unknown at present; however, the importance of these proteins in the pathogenic mechanisms of *P. aeruginosa* warrants further investigations. Naturally, the localization and immunogenicity of this class of protein have to be determined before one can assess their potential as target antigens for further development as anti-*Pseudomonas* reagents.

In conclusion, this review provides a comprehensive account of studies on possible vaccine candidates against *P. aeruginosa*. To date, there is not a single candidate vaccine antigen that can be identified as having an overwhelming advantage over other potential protective antigens. However, the information provided here is useful in ruling out a number of less effective candidate *P. aeruginosa* antigens while pointing to a few, in particular, surface-associated or in vivo-expressed antigens, that have good potential for further development.

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