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Protection of Immunocompromised Mice against Lethal Infection with *Pseudomonas aeruginosa* by Active or Passive Immunization with Recombinant *P. aeruginosa* Outer Membrane Protein F and Outer Membrane Protein I Fusion Proteins

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Recombinant outer membrane proteins (Oprs) of *Pseudomonas aeruginosa* were expressed in *Escherichia coli* as glutathione *S*-transferase (GST)-linked fusion proteins. GST-linked Oprs F and I (GST-OprF$_{190–350}$ [GST linked to OprF spanning amino acids 190 to 350] and GST-OprI$_{21–83}$, respectively) and recombinant hybrid Oprs (GST-OprF$_{190–342}$-OprI$_{21–83}$ and GST-OprI$_{21–83}$-OprF$_{190–350}$) were isolated and tested for their efficacy as vaccines in immunodeficient mice. GST-OprF-OprI protected the mice against a 975-fold 50% lethal dose of *P. aeruginosa*. Expression of GST-unfused OprF-OprI failed in *E. coli*, although this hybrid protein has been expressed without a fusion part in *Saccharomyces cerevisiae* and used for immunizing rabbits. The immune rabbit sera protected severe combined deficient (SCID) mice against a 1,000-fold 50% lethal dose of *P. aeruginosa*. Evidence is provided to show that the most-C-terminal part of OprF (i.e., amino acids 332 to 350) carries an important protective epitope. Opr-based hybrid proteins may have implications for a clinical vaccine against *P. aeruginosa*.

Pseudomonas aeruginosa is an opportunistic gram-negative pathogen. It represents a major cause of hospital-acquired infections, especially in burned and other immunocompromised patients, including transplant or cancer patients (20, 22, 30, 34, 38). Because of the general antibiotic resistance of *P. aeruginosa*, research interest has focused on immunotherapy (23, 24, 46). The two major antigenic cell envelope components of the organism are the lipopolysaccharides (LPSs) and the outer membrane proteins (Oprs). LPS-based vaccines have been used successfully in animal models as well as in patients (9–11, 15, 24, 28). To overcome the problems of toxicity due to the lipid A portion of the LPS molecule, Cryz et al. coupled lipid A-free oligosaccharides isolated from eight different *P. aeruginosa* immunotypes to exotoxin A of *P. aeruginosa* (10).

We have directed our attention towards the cloning and expression of *P. aeruginosa* Opr genes. We have been interested in the potential of Oprs as an immunoprophylactic tool because Oprs are antigenically cross-reactive among all 17 known serogroups of the International Antigenic Typing Scheme (36). We have cloned the genes coding for OprF (14) and OprI (13) and have used recombinant OprI expressed in *Escherichia coli* for successfully vaccinating mice (16, 17).

The use of Oprs as a target for protective immunity may have several advantages. Apart from the fact that Oprs induce a cross-reacting immunity among the different serotypes of *P. aeruginosa*, genes coding for *P. aeruginosa* Oprs can be transfected into apathogenic carrier strains like *aroA Salmonella dublin*, which can be used for oral immunization (43). Several investigations have shown that presentation of antigens to distinct inductive sites of the mucosal immune system like the Peyer's patches can induce the production of secretory immunoglobulin A against the presented antigen (31). Prevention of colonization of the lung by *P. aeruginosa* in patients with cystic fibrosis has not been achieved to date by parenteral immunization of vaccines based on carbohydrate antigens of *P. aeruginosa* (6). Cloned genes coding for Oprs would also be applicable to a recent approach in the development of vaccines that involves the use of immunization with naked DNA (8). This method of immunization seems to have, besides others, the important advantage of eliciting both humoral and cellular immune responses (12). Activated T cells have been considered in recent investigations by Buret et al. (6) to have enhanced the clearing of *P. aeruginosa* from the lungs in a rat model.

The degree of protection which could be achieved by vaccination with OprI is, however, limited compared with the degree of protection of LPS vaccines (16, 33, 45). Apart from the possibility that the access of antibodies to their targets may play a role, this fact could be explained by the observation that the total copy number of a single Opr exposed at the bacterial surface is considerably lower than the number of LPS copies, thus promoting the binding of fewer antibody molecules to the bacterial surface. One might therefore conclude that an outer membrane vaccine consisting of a mixture of different Oprs or fusion components of these could increase the degree of protection.

Whereas recombinant OprI can be expressed in *E. coli* in large quantities and purified to apparent homogeneity by one

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single affinity chromatography purification step (43), the expression of recombinant OprF in *E. coli* has been shown to be toxic for the host (14). Hughes et al. (26) published a map of the B-cell epitopes of the OprF of *P. aeruginosa*, which elicit antibodies reactive with whole cells of *P. aeruginosa*. They identified three surface-exposed epitopes corresponding to amino acid residues (aa) 243 to 256, 285 to 298, and 329 to 342. Using monoclonal antibodies (MAbs) and different recombinant fusion proteins produced in *E. coli* and synthetic peptides, we identified seven B-cell epitopes on the C-terminal part of OprF (aa 190 to 213, aa 212 to 240, aa 239 to 250, aa 284 to 316, aa 332 to 350, aa 240 to 316, and aa 190 to 250), of which aa 212 to 240 and aa 332 to 350 are surface exposed. It therefore seems that the C-terminal part (aa 190 to 350) of the OprF protein is rich in B-cell epitopes, some of which are surface exposed.

In the present study, we show that mice immunized with a recombinant hybrid protein consisting of the entire OprI molecule fused to the carboxy-terminal sequence (aa 190 to 342) of OprF, or with the corresponding immunoglobulins isolated from rabbits which have been vaccinated with the OprF-OprI hybrid protein, are protected against a lethal *P. aeruginosa* infection.

MATERIALS AND METHODS

Microorganisms. *P. aeruginosa* International Antigenic Typing Scheme serogroup 1 (ATCC 33348) was obtained from A. Bauernfeind, Max von Pettenkofer-Institut, University of Munich. Bacteria were grown and adjusted to the required concentration as described previously (16, 17). For the expression of recombinant proteins, *E. coli* K-12 W3110 *lacI*^{q}*L8* was used. For expression of Oprs in a yeast, we used *Saccharomyces cerevisiae* HT393 (*leu2 ura3 pra1 prb1 prc1 pre1 cps1*).

Source of DNAs. Three recombinant plasmids were used as the source of DNAs: pFSauI, a pUC19-derived plasmid that contains a 1.0-kb *Sau*3AI fragment of the *P. aeruginosa* porin protein F gene encoding the C-terminal part of the protein from aa 57 to 350 (14); pITaqI, a pUC19-derived plasmid that contains a 626-bp *Taq*I fragment spanning the complete OprI gene (13); and the expression vector pGEX-2a, originating from the vector pGEX-2T (42), modified by the introduction of the polylinker from vector pTRC (1). The vector pGEX-2a contains the *tac* promoter followed by the coding sequence for 26-kDa *Schistosoma japonicum* glutathione *S*-transferase (GST), a cleavage site for thrombin and the pTRC-specific polylinker region.

Characterization of antisera induced against synthetic peptides. Synthetic peptides representing aa 190 to 213 (peptide D1), 212 to 240 (D2), 239 to 250 (D3), 284 to 316 (D4), and 332 to 350 (D5) from OprF were synthesized as described in the literature (39). Peptides were conjugated to keyhole limpet hemocyanin (KLH) with 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Sigma, Taufkirchen, Germany), as described by the manufacturer. Rabbits were immunized subcutaneously at eight different locations near lymph nodes with 200 μg of KLH-conjugated peptide in complete Freund's adjuvant and reimmunized 2 weeks later with 400 μg of the conjugate in incomplete Freund's adjuvant. The animals received two booster injections intravenously of 150 and 100 μg of conjugate at 6 and 9 weeks after the first immunization. Antibody titers against peptides were measured by an enzyme-linked immunosorbent assay (ELISA) on plates coated with a 5-ng/ml concentration of peptide solution in 50 mM sodium phosphate buffer (pH 7.5; phosphate-buffered saline [PBS]) overnight at room temperature. The plates were washed three times with 0.05 M citric acid–0.05 M Tris (pH 7.4) and dried over silica gel for 3 days. Rabbit sera were diluted 1:160 and saturated with *E. coli* proteins as described recently (37). Western blot (immunoblot) analysis with recombinant GST fusion proteins and immunofluorescence determinations against intact *P. aeruginosa* serogroup 11 (ATCC 33358) organisms were carried out by a method reported in the literature (27, 40).

Expression of OprF and OprI as GST fusion proteins. The oligonucleotides p1 (5′-AAAGAGCTCGCTCCGGCTCCGGAACCGGTTGCCGAC-3′), with a *Sac*I restriction site at the 5′ end corresponding to bases 568 to 594 of the OprF gene (14), and p2 (5′-AAAAAGCTTACTTGGCTTCGGCTTCTACTTCGG-3′), with a *Hin*dIII restriction site at the 5′ end complementary to bases 1028 to 1053 of the OprF gene, and 10 ng of the plasmid pF*Sau*I were employed for a PCR using the Perkin-Elmer Cetus GeneAmp Kit, which yielded a 500-bp fragment. The amplified fragment was digested with *Sac*I and *Hin*dIII and introduced into the vector pGEX-2a to obtain plasmid pGEX-OprF, which encodes the C-terminal part of the porin OprF from aa 190 to 350. The oligonucleotides p3 (5′-CGTACCATGGTGAGCAGCCACTCCAAAGAAACGA AGCT-3′), with an *Nco*I restriction site at the 5′ end corresponding to bases 61 to 87 of the coding region of the OprI gene (13), and p4 (5′-AAAAAGCTTC TATTACTTGCGGCTGGCTTTTTCC-3′), with a *Hin*dIII restriction site at the 5′ end complementary to bases 231 to 255 of the coding region of the OprI gene, and 10 ng of the plasmid DNA pITaqI were used in a PCR to amplify a 215-bp fragment, which was then treated with the restriction enzymes *Nco*I and *Hin*dIII to introduce it into the corresponding sites of the expression vector pGEX-2a, to obtain plasmid pGEX-OprI, which encodes aa 21 to 83 of OprI.

Construction and expression of the GST-OprI-OprF and GST-OprF-OprI hybrid genes. The oligonucleotides p1 (see above) and p5 (5′-TTCAACGCG ACGGTTGATAGCGCG-3′; which is complementary to bases 1003 to 1026 of the OprF gene) and 10 ng of the plasmid pFSauI were used to amplify a 470-bp OprF fragment. A second PCR was carried out with 10 ng of plasmid pITaqI (13) and the oligonucleotides p4 (see above) and p6 (5′-GAAGGCCGCGCTAT CAACCGTCGCGTTGAAAGCAGCCACTCCAAAGAAACCGAAGCT-3′), in which nucleotides 1 through 30 correspond to bases 997 to 1026 of the OprF gene and nucleotides 31 through 57 correspond to bases 61 through 87 of the OprI-coding region. This yielded a 240-bp fragment. One hundred fifty nanograms of both obtained DNA fragments and oligonucleotides p1 and p4 were used for a third PCR as described by Horton et al. (25). The obtained 660-bp fragment was digested with the restriction endonucleases *Sac*I and *Hin*dIII and introduced into the vector pGEX-2a to obtain plasmid pGEX-OprF-OprI, which encodes aa 190 to 342 of OprF and aa 21 to 83 of OprI. The oligonucleotides p3 and p7 (5′-AAAGAGCTCCTTGCGGCTGGCTTTTTCCAGCATGCG-3′), with a *Sac*I restriction site at the 5′ end complementary to bases 223 to 249 of the coding region from the OprI gene, and 10 ng of plasmid pITaqI were used to amplify a 210-bp fragment, which was introduced into the vector pGEX-2a with the help of the restriction enzymes *Nco*I and *Sac*I. The obtained plasmid was digested with the enzymes *Sac*I and *Hin*dIII to introduce a 490-bp fragment obtained by digestion of the plasmid pGEX-OprF with the corresponding enzymes. Plasmid pGEX-OprI-OprF encodes aa 21 to 83 from OprI and aa 190 to 350 from OprF, which are separated by a 2-aa linker introduced at the *Sac*I cloning site.

Expression and purification of the recombinant proteins in *E. coli*. The four plasmids pGEX-OprF, pGEX-OprI, pGEX-OprF-OprI, and pGEX-OprI-OprF were transformed into the *E. coli* K-12 strain W3110 *lacI*^{q}*L8*. Plasmids were sequenced to ensure that no mutation was introduced by PCR into the final construct. For large-scale antigen production, 5-liter bacterial cultures containing the plasmids were left to grow until an optical density at 660 nm of 1 was reached and the *P. aeruginosa*-specific recombinant antigens induced by isopropylthiogalactoside (1) were expressed. After disruption of the cells (29), the four different GST fusion proteins were found to be soluble in aqueous solutions. Therefore, the four fusion proteins could be purified from crude bacterial lysates under nondenaturing conditions by affinity chromatography on immobilized glutathione (42) to a purity of about 80%.

Sodium dodecyl sulfate (SDS)-polyacrylamide (15%) slab gel electrophoresis and Western blotting with MAbs against OprF and OprI as well as with polyclonal rabbit antisera against recombinant OprF and OprI fusion proteins (28a) were carried out as described in the literature (29) to confirm the OprF and OprI identities of the recombinant proteins.

Expression and purification of recombinant OprF-OprI in *S. cerevisiae*. For expression of the *P. aeruginosa* Oprs in *S. cerevisiae*, *S. cerevisiae-E. coli* shuttle vectors based on the 2-μm plasmid (7) were used. To express unfused Oprs, the plasmid pEMBLyex4 (7) served as an expression vector, and Yepsec1 (2) was used to express Opr polypeptides fused to the signal sequence of the *Kluyveromyces lactis* killer toxin. The *Nco*I-*Hin*dIII DNA fragment from pGEX-OprF-OprI, which codes for the OprF-OprI hybrid protein, was isolated and cloned into pEMBLyex4 cut with *Bam*HI and *Hin*dIII (yielding pEMBLyex4-F-I) or into Yepsec1 (yielding Yepsec1-F-I). The *Nco*I and the *Bam*HI sites were turned into blunt ends with Klenow enzyme before ligation, whereas the *Hin*dIII sites were not treated. The growth conditions for recombinant yeast-carrying plasmids with the regulatable UAS_{GAL}/CYC1 hybrid promoter have already been described (3). The soluble OprF-OprI hybrid protein expressed in *S. cerevisiae* was purified by affinity chromatography with the MAb 948/12 (28a) directed against epitope D1. The MAb was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Freiburg, Germany), in accordance with the instructions of the manufacturer. Yeast extracts in PBS were loaded onto the column, and unspecific bound material was eluted with 0.1 M glycine buffer (pH 9.0) containing 0.5 M NaCl. Elution of OprF-OprI hybrid protein was carried out in 0.1 M glycine buffer (pH 11.0). The column was regenerated by washing with 0.1 M glycine (pH 2.5) and then with PBS.

Active immunization and protection experiments. Four groups (A to D) of 68 female BALB/c mice (10 to 12 weeks old) each received 100 μg of antigen (GST [group A], 50 μg of GST-OprF plus 50 μg of GST-OprI [group B], GST-OprF-OprI [group C], or GST-OprI-OprF [group D]) suspended in 100 μl of ABM 2 complete as the adjuvant (Sebak, Aidenbach, Germany) on day 0. Booster injections were given with an equal amount of antigen suspended in 100 μl of $Al(OH)_3$ on days 14, 28, and 42. On day 49, animals were bled from the tail vein for serum collection to determine antibody titers in the pooled sera of 7 to 10 mice from each group. Four days later, all of the animals received immunosuppressive treatment. For immunosuppression, mice received three injections of 150 μg of cyclosphosphamide (Serva, Heidelberg, Germany) per g of body weight in 0.25 ml of PBS on days 53, 55, and 57. On day 58, each antigen group was divided into four subgroups, namely, I, II, III, and IV, each containing 16 to 17

TABLE 1. Characterization of B-cell epitopes of *P. aeruginosa* OprF

Peptide	OprF-specific region (aa)	Reaction to[a]:				
		MAbs[b]	Rabbit anti-OprF by ELISA (against peptide)	Rabbit antisera[c] by ELISA (against peptide)	Rabbit antisera[c] by Western blot (against OprF)	Rabbit antisera[c] by immunofluorescence of intact *P. aeruginosa*[d]
D1	190–213	+	−	+	−	−
D2	212–240	+	+	+	−	+
D3	239–250	−	+	+	+	−
D4	284–316	+	+	+	+	−
D5	332–350	+	+	+	+	+

[a] +, positive; −, negative.
[b] MAbs were induced in mice against a recombinant protein representing aa 58 to 350 of OprF; binding to peptides D1 to D5 was analyzed by ELISA.
[c] Rabbits were immunized with peptides linked to KLH.
[d] Estimated with *P. aeruginosa* serogroup 11 (ATCC 33359).

animals. The mice of groups A to D received intraperitoneally either 5×10^1 (subgroup I), 5×10^2 (subgroup II), 5×10^3 (subgroup III), or 5×10^4 (subgroup IV) CFU of *P. aeruginosa* serogroup 1 organisms. Fifteen additional nonimmunized mice underwent only immunosuppression without bacterial challenge. This control group was used to confirm the state of leukopenia and to exclude nonspecific infections. All surviving animals were monitored for 10 days after infection.

Production of specific immunoglobulins and passive immunization. Rabbits were immunized three times with 100 μg of purified recombinant OprF-OprI isolated from *S. cerevisiae* cell extracts (or with cell extracts from *S. cerevisiae* alone as controls) emulsified in incomplete Freund's adjuvant on days 0, 14, and 28. On day 38, blood samples were obtained and allowed to clot overnight at 4°C. The serum was removed, centrifuged, and stored at −20°C. For groups of 30 female SCID mice (18 to 20 g [body weight]; Bomholtgard, Ry, Denmark), every animal in each group received either 0.5 ml of rabbit anti-OprF-OprI serum and 0.5 ml of rabbit anti-yeast serum, or the corresponding 1:10 dilutions, or 0.5 ml of rabbit anti-D2 and -D5 serum in a 1:10 dilution. As an additional control, the animals in one group received 0.5 ml of normal saline. Those in one additional group were injected with 0.5 ml of rabbit serum against heat-inactivated cells of *P. aeruginosa* serogroup 1. After 3 h, the animals of groups 1 to 6 were subdivided into five subgroups (a to e), receiving 0.5 ml of *P. aeruginosa* serogroup 1 suspension (10^1, 10^2, 10^3, 10^4, and 10^5 CFU/ml suspended in mucin, respectively). The surviving animals were observed for 1 week. Five grams of mucin (Sigma) was suspended in 100 ml of distilled water, treated for 10 min with an Ultra Turrax blender, passed through a sieve, and autoclaved for 15 min at 120°C. Shortly before use, the solution was adjusted to pH 7.2 to 7.4 with sterile 1 N NaOH.

Statistics. The survival rates between the different treatment groups were compared by the probit model (19) under the assumption of log-parallel dose-response curves. Moreover, the relative potency of one treatment to that of another was estimated and tested with the aid of the two-sided χ^2 test (44). Risk ratios were calculated by the proportional hazard model (32) with reference based on the GST group.

RESULTS

Epitope mapping of OprF. To identify amino acid sequence sections of OprF representing B-cell epitopes as a rational basis for the choice of an Opr-based *P. aeruginosa* vaccine, we prepared MAbs against a recombinant protein representing aa 58 to 350 of OprF. Binding of the MAbs was analyzed with a series of recombinant subfragments of OprF expressed in *E. coli*. The MAbs discriminated between five different regions, i.e., aa 190 to 213 (D1), aa 212 to 240 (D2), aa 239 to 250 (D3), aa 284 to 316 (D4), and aa 332 to 350 (D5) (28a). The C-terminal part of OprF between aa 190 and aa 350 seemed, therefore, to cover most of the B-cell epitopes of OprF. To further analyze the epitopes, synthetic peptides related to the amino acid regions defined above were prepared and conjugated to KLH. Polyclonal antisera against these peptides were raised in rabbits in accordance with procedures described in the literature (39). Table 1 shows that peptides D1 to D5 were recognized by the corresponding polyclonal antisera. The peptides D1, D2, D4, and D5 reacted with MAbs, and peptides D2, D3, D4, and D5 were also recognized by polyclonal antibodies raised against recombinant OprF, thus confirming that these five epitopes initiate B-cell responses. Antisera raised against D3, D4, and D5 recognized OprF in Western blot analysis, but viable *P. aeruginosa* cells showed positive fluorescence only after incubation with the antisera raised against D2 and D5. These two epitopes, therefore, seem to be surface exposed. Additional MAbs were identified; these did not react with any of the synthetic peptides but recognized GST-OprF and further recombinant subfragments, leading to two additional epitopes, D6 and D7, which correspond to aa 240 to 316 and 190 to 250, respectively (28a). Therefore, the region from aa 190 to aa 350 of OprF was considered to include important antigenic regions, and we decided to ascertain whether recombinant proteins carrying these epitopes are able to confer protection in animal models.

Epitope mapping of OprI. With the MAbs 2A1, 6A4, and 5B4 raised against native OprI, two different epitopes have been characterized (17). MAb 2A1, which had shown protective ability against *P. aeruginosa* infection, recognizes the N-terminally located epitope (17). Subsequent studies showed that MAb 2A1 binds only if the entire amino acid sequence from aa 21 to aa 83 is expressed (data not shown). For the construction of recombinant OprI antigens to be used as a subunit vaccine, the complete region of aa 21 to 83 was therefore considered to be the most adequate antigen.

Expression of Oprs in *E. coli*. The efficacy of a single Opr of *P. aeruginosa* in a vaccine against *P. aeruginosa* infection might be improved by coexpression of the fused epitopes of two different Oprs. Four different GST fusion proteins were expressed in *E. coli* in large amounts, namely, GST-$OprF_{190–350}$ (GST fused to OprF spanning aa 190 to 350), GST-$OprI_{21–83}$, GST-$OprF_{190–342}$-$OprI_{21–83}$, and GST-$OprI_{21–83}$-$OprF_{190–350}$ (Fig. 1). The recombinant proteins could be about 80% purified by affinity chromatography on immobilized glutathione. Western blot analysis of the four recombinant products with the OprI-specific MAbs 6A4 and 2A1 and different OprF-specific MAbs directed against the epitopes D1, D2, D4, D5, D6, and D7 showed that the MAb-specific epitopes were expressed by the recombinant fusion proteins (data not shown).

We also tried to express the two hybrid proteins without the non-*Pseudomonas* component GST with vector pTRC (1). Whereas a high expression could be achieved with the $OprI_{(aa\ 21–83)}$-$OprF_{(aa\ 190–350)}$ hybrid protein, the $OprF_{(aa\ 190–242)}$-$OprI_{(aa\ 21–82)}$ hybrid protein was not expressed in detectable amounts.

Expression of Oprs in *S. cerevisiae*. Since the hybrid protein OprF-OprI was not expressed in *E. coli* in an unfused form, we tried to produce it in an alternative host cell, namely, *S. cerevisiae*. For this purpose, we chose the plasmid pEMBLyex4 as the expression vector; this plasmid carries the regulatable

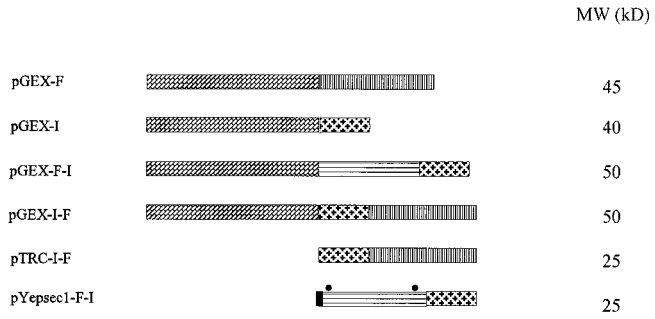

FIG. 1. Schematic overview of the constructed recombinant fusion proteins of Oprs of *P. aeruginosa*. For expression in *E. coli* K-12, the vector pGEX-2a, which codes for GST, was used. Symbols: ▮, signal sequence of *K. lactis* killer toxin; ●, potential glycosylation site; ▨, GST (aa 1 to 225); ▥, $OprF_{190–350}$; ▤, $OprF_{190–342}$; ⊠, $OprI_{21–83}$. MW, molecular mass; F, OprF; I, OprI.

UAS_{GAL}/CYC1 hybrid promoter and is the one with which we had obtained production yields of recombinant proteins of 0.1 g/liter in shaker flasks and 0.9 g/liter in fed batch fermentors (4, 5). Unfortunately, OprF-OprI encoded by pEMBLyex4-F-I (Fig. 1) was expressed in only minute amounts in *S. cerevisiae* (data not shown). It seems as if the OprF-OprI hybrid protein is unstable in the cytoplasm of *E. coli* as well as in *S. cerevisiae* when expressed without fusion to a host cell protein. Since OprF and OprI are exported in members of the family *Pseudomonadaceae* through the periplasmic space, we tried to copy the export in *S. cerevisiae*. To this end, the OprF-OprI hybrid protein was fused to the secretion signal sequence of the killer toxin of the yeast *K. lactis*. This leader peptide has already been shown to support efficiently the secretion of foreign proteins into the culture broth (2) or into the periplasmic space of *S. cerevisiae* (4, 5). The tripartite hybrid protein killer toxin-OprF-OprI encoded by Yepsec1-F-I (Fig. 1) now consists of the following polypeptide stretches: first there are the 16 aa of the *S. cerevisiae* secretion signal sequence; these are followed by 9 aa encoded by a DNA linker and then by the OprF-specific polypeptide stretch from aa 190 to 342 and an OprI peptide including aa 21 to 83. The OprF-specific polypeptide carries the potential glycosylation site asparagine-X-threonine twice (Fig. 1). These glycosylation sites should be recognizable if the fusion protein enters the secretionary pathway. Upon fusion to the killer toxin leader sequence, OprF-OprI was detected in *S. cerevisiae* cell extracts by Western blot analysis when expressed under induced condition of the UAS_{GAL}/CYC1 promoter, but no secreted antigen was detected in the culture broth (data not shown).

The OprF-OprI fusion protein expressed in *S. cerevisiae* did not migrate as a sharp band in SDS-polyacrylamide gels but showed a heterogeneous distribution, appearing in several smearing bands. This indicates posttranslational modification by N-glycosylation. Incubation of the recombinant *P. aeruginosa* antigen with endoglycosidase F resulted in the appearance of a sharp band of lower molecular weight, indicating the entering of OprF-OprI into the secretionary pathway when fused to the killer toxin leader sequence and the glycosylation of at least one of the two potential glycosylation sites (data not shown).

Active immunization with *E. coli*-derived fusion proteins. Mice were immunized four times at 2-week intervals with 100 μg of recombinant GST-linked fusion protein, or GST only, suspended in the adjuvant ABM complete. The antibody titers, each from the pooled sera of 8 to 10 mice, were analyzed by ELISA as well as by Western blotting for binding activity against *P. aeruginosa* and by ELISA against peptides D1 to D5.

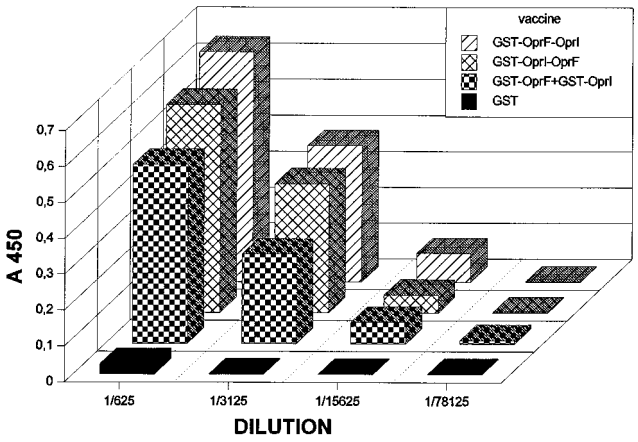

FIG. 2. Determination of antibody titers against *P. aeruginosa* in sera of mice immunized with the indicated GST-linked recombinant outer membrane vaccine or with GST alone. ELISA measurements were carried out as described previously on plates coated with sonicated *P. aeruginosa* serogroup 12 (16). Titers are indicated as the serial serum dilution resulting in an A_{450} above the twofold background value.

Figure 2 shows that specific antibody titers against *P. aeruginosa* were obtained in all immunized groups with serum dilutions up to 1:15,625. Western blot analysis of the sera with *P. aeruginosa* polypeptides showed specific staining of OprI as well as of OprF by sera from all immunized groups. No staining of OprI or OprF was observed in the GST-immunized control group (data not shown). Further analysis of the sera against peptides D1 to D5 (Fig. 3) by ELISA showed that in GST-OprF-OprI- as well as GST-OprI-OprF-immunized animals, the predominant antibody response was directed against peptides D5 and D4. To test whether the induced antibodies against outer membrane fusion proteins protect mice against *P. aeruginosa* infection, mice received three doses of cyclophosphamide for immunosuppression. Leukocyte counts determined in peripheral blood samples from 15 nonimmunized control animals dropped to mean levels below 400/μl. One day later, the animals were challenged with either 5×10^1, 5×10^2,

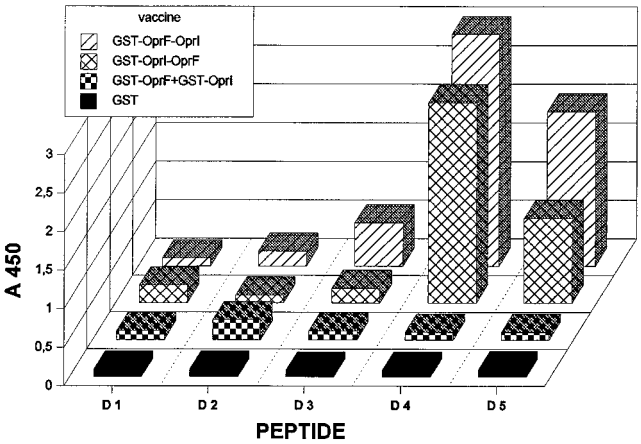

FIG. 3. Antibody determination by ELISA against synthetic peptides D1 to D5 listed in Table 1, which represent B-cell epitopes of OprF. Mice were immunized four times with the indicated recombinant fusion proteins or GST alone. Details are described in Materials and Methods.

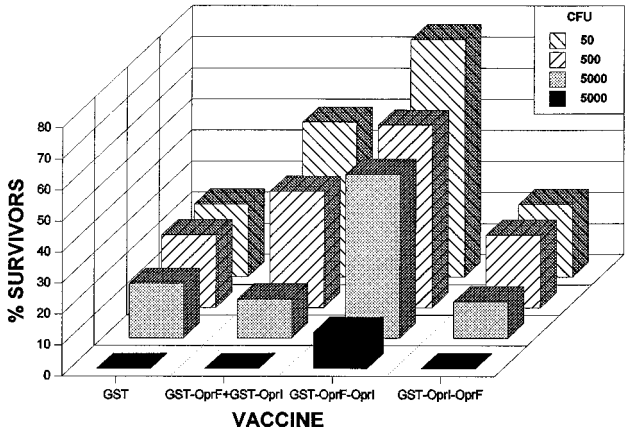

FIG. 4. Survival of BALB/c mice after immunization with the indicated vaccine or GST alone, followed by immunosuppression and intraperitoneal challenge with 5, 50, 500, or 5,000 CFU of *P. aeruginosa* serogroup 1. Bars represent percentages of survivors ($n = 16$ or 17) per challenge dose.

5×10^3, or 5×10^4 CFU of *P. aeruginosa* serogroup 1. Survival of the animals was registered for 1 week. Figure 4 and Table 2 show the survival rates of the animals after four different challenge doses and the 50% lethal dose (LD_{50}) values for each of the vaccines, calculated by probit regression analysis. For groups immunized with GST only or with GST-OprI-OprF, LD_{50} values as low as 1.58 and 2.65 CFU were calculated. Simultaneous vaccination with a mixture of GST-OprI and GST-OprF induced an increase of the LD_{50} value to 83.3 CFU. This difference, however, was found to be not statistically significant. In contrast, after vaccination with the hybrid GST-OprF-OprI, a highly significant shift of the LD_{50} value to 1,540 CFU was calculated ($P \leq 0.001$). Compared with the GST-immunized controls, a protection value of a 975-fold-increased

TABLE 2. Analysis of survival of mice[a]

Vaccine	LD_{50}[b]	95% CI[c] for LD_{50} value	LD_{50} factor[b]	Risk ratio[d]	95% CI for risk ratio
GST	1.58	0.002, 1,015	1	1	
GST-OprF+GST-OprI	83.34	16, 419	52	0.732	0.497, 1.076
GST-OprF-OprI	1,540[e]	414, 5,727	975	0.344[e]	0.223, 0.528
GST-OprI-OprF	2.65	0.013, 560	1.7	0.889	0.616, 1.285

[a] Mice were vaccinated with the indicated GST-linked recombinant Oprs or GST alone as a control.
[b] LD_{50} values were calculated by probit analysis (19).
[c] CI, confidence interval.
[d] Risk ratios were calculated by the proportional hazard model (32), with reference based on the GST group.
[e] $P < 0.0001$ versus GST group.

LD_{50} was calculated for the GST-OprF-OprI group. These results could be confirmed in an identically designed second experiment (data not shown). Analysis of the data by the proportional hazard model and calculation of the risk ratios between the groups defined by the different vaccine preparations are shown in Table 2. Vaccination with GST-OprF-OprI reduced the risk highly significantly ($P \leq 0.0001$) to 0.34 (95% confidence interval, 0.22 and 0.53), compared with the GST-immunized controls.

Passive immunization with antibodies against yeast-derived OprF-OprI. The recombinant *Pseudomonas* antigen was enriched from the supernatants of yeast cell extracts by ammonium salt precipitation and immunoaffinity chromatography, with an anti-OprF mouse MAb directed against epitope D1. Rabbits were then immunized three times with the antigen, and sera were collected from the animals. Whereas the preimmune sera did not show any reactivity with either *P. aeruginosa* OprF or OprI, the sera from the immunized rabbits reacted

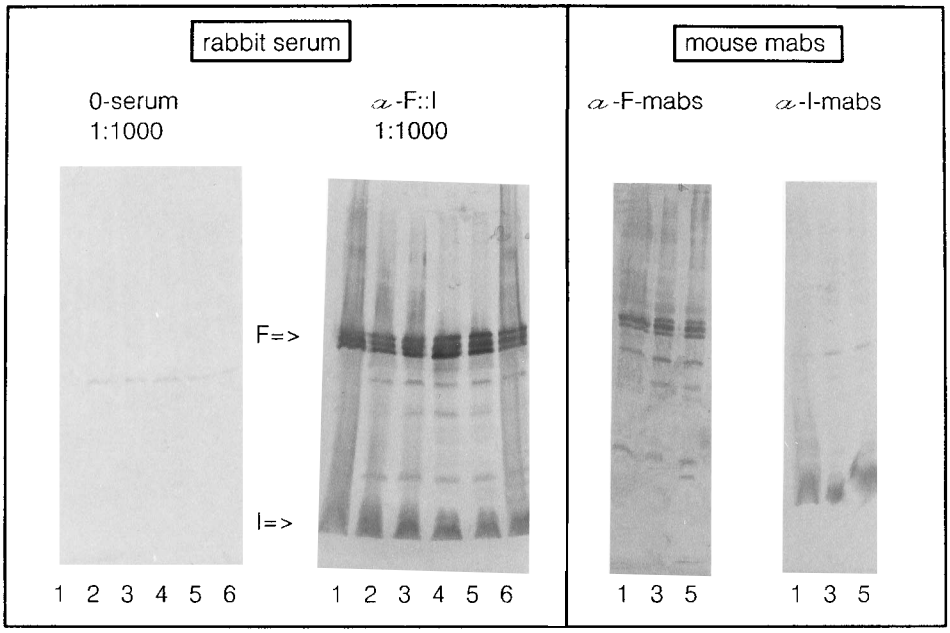

FIG. 5. Western blot analysis of rabbit antibodies induced against OprF-OprI isolated from *S. cerevisiae* with *P. aeruginosa*. (Left) Polyclonal rabbit serum raised against OprF-OprI (preserum [0-serum] and serum after three immunizations [α-F::I]); (right) controls with OprF- or OprI-specific MAbs (α-F-mabs and α-I-mabs, respectively). Cell extracts were prepared from *P. aeruginosa* ATCC 33348 (lane 1), ATCC 33361 (lane 2), clinical isolate 232 (lane 3), clinical isolate 235 (lane 4), ATCC 1227 (lane 5), and clinical isolate 14/9/82 (lane 6). F, OprF; I, OprI.

TABLE 3. Protection against *P. aeruginosa* infection in SCID mice by rabbit anti-OprF-OprI sera

Challenge dose (CFU)[b] or control	No. of surviving animals in group[a]:						
	1 (yeast control)[c]	2 (yeast control diluted 1:10)[c]	3 (OprF-OprI)[c]	4 (OprF-OprI diluted 1:10)[c]	5 (*P. aeruginosa*)[d]	6 (Challenge control)	7 (Mucin control)
5×10^0	5	5	5	5	5	1	
5×10^1	1	1	5	4	5	0	
5×10^2	1	0	5	2	5	0	
5×10^3	0	1	2	0	5	0	
5×10^4	0	0	0	0	0	0	
Mucin (control)							5

[a] Values are numbers of surviving animals after transfer of specific rabbit serum before challenge in each group ($n = 5$). Statistical analysis (probit analysis for parallel line model): group 1 versus group 3, 85-fold increase in potency (significance [by chi-square test], $P = 0.002$); group 1 versus group 5, 325-fold increase in potency (significance, $P = 0.001$).
[b] Female C.B-17 *scid/scid* mice (SCID) were challenged intraperitoneally with the indicated CFU of *P. aeruginosa* serogroup 1 suspended in 0.5 ml of mucin.
[c] Rabbit serum from animals immunized with the indicated antigen.
[d] Rabbit serum from animals immunized with *P. aeruginosa* serogroup 1.

specifically with OprF and OprI from three different ATCC strains of *P. aeruginosa* as well with the three different clinical isolates of *P. aeruginosa* tested (Fig. 5). The protective efficacy of these sera was tested in SCID mice for defense against a lethal challenge with *P. aeruginosa*. As shown in Table 3, mice injected with the control anti-yeast serum were not protected against infection even at a challenge dose of 5×10^1 (Table 3, group 1). On the other hand, mice which received the OprF-OprI-specific rabbit serum were fully protected against a 5×10^2 CFU challenge dose of *P. aeruginosa* (Table 3, group 3), and 40% survival was observed after challenge with 5×10^3 CFU. As an additional control, protection by rabbit serum induced against LPS of the challenge strain, *P. aeruginosa* serogroup 1, was tested. With a challenge dose of up to 5×10^3 CFU, 100% of the animals protected with LPS-specific serum survived (Table 3, group 5). No survival could be observed in this group after a 10-fold-higher challenge dose of 5×10^4 CFU. Statistical analysis was used to compare the protective doses of OprF-OprI-specific serum, of LPS-specific serum, and the anti-yeast control group for protection against *P. aeruginosa* infection. The results showed an 85-fold increase in potency of the OprF-OprI serum in comparison with that of the anti-yeast serum ($P < 0.002$) (Table 3, group 3). In contrast, a 325-fold-higher potency was calculated for the LPS-specific serum than for the anti-yeast serum ($P < 0.001$).

In addition, mouse MAbs and polyclonal rabbit sera against KLH-linked synthetic peptides, representing aa 212 to 240 (epitope D2) and aa 332 to 350 (epitope D5), were prepared. MAbs and rabbit sera were tested in the SCID mouse model and compared with the polyclonal LPS-specific antiserum and a saline challenge control for protection against *P. aeruginosa* infection. As depicted in Table 4, no statistically significant protection was provided by MAbs against D2 (group 1) and D5 (group 2). Calculation of the protective dose of rabbit antisera against epitopes D2 and D5, and of anti-LPS serum in comparison with the saline challenge control group, revealed a 29-fold increase in potency for the anti-D2 serum. However, this value was of only limited significance ($P = 0.03$). In contrast, for the D5-specific serum, a highly significant ($P < 0.001$) increased protectivity (>1,000-fold) compared with that of saline treatment was calculated.

In an identically designed experiment, these results could be confirmed with three different rabbit sera against epitope D5. With each of the sera, a highly significant protection ($P < 0.001$) was obtained (data not shown).

DISCUSSION

The use of *P. aeruginosa* Oprs as a vaccine had already been suggested by Mutharia et al. in 1982 (36). They were able to demonstrate that Oprs cross-react immunologically between the different serogroups of this organism. Protection against *P. aeruginosa* infection by immunization with purified native Oprs could be demonstrated in several animal models (16, 21, 45).

TABLE 4. Protective effect of MAbs and polyclonal antibodies directed against epitopes D2 or D5 of *P. aeruginosa* OprF

Challenge dose (CFU)[b]	No. of surviving animals in group[a]:					
	1 (MAb 943/96 [anti-D2])[c]	2 (MAb 939/736 [anti-D5])[c]	3 (Rabbit serum no. 23 [anti-D2])	4 (Rabbit serum no. 33 [anti-D5])	5 (Rabbit serum diluted 1:10 [*P. aeruginosa* immunized])[d]	6 (Challenge control)
5×10^0	4	5	5	5	5	5
5×10^1	0	2	4	4	5	0
5×10^2	1	0	2	5	5	0
5×10^3	1	0	0	5	5	0
5×10^4	1	0	0	4	5	0

[a] Values are numbers of surviving animals after transfer of specific serum before challenge in each group ($n = 5$). Statistical analysis (probit analysis for parallel line model): group 1 versus group 6 and group 2 versus group 6, not significant; group 4 versus group 6, >1000-fold increase in potency (significance, $P < 0.001$ by chi-square test); group 3 versus group 6, 29-fold increase in potency (significance, $P = 0.03$).
[b] Female C.B-17 *scid/scid* mice (SCID) were challenged intraperitoneally with the indicated CFU of *P. aeruginosa* serogroup 1 suspended in 0.5 ml of mucin.
[c] Volume, 0.5 ml (1 mg/ml).
[d] Rabbit serum from animals immunized with *P. aeruginosa* serogroup 1.

In these preparations, however, contamination by *P. aeruginosa* LPS was still demonstrable. The molecular cloning of the OprI and OprF genes (13, 14) enabled protection studies with recombinant OprI and OprF to be carried out and made it possible to investigate the localization of protective epitopes on these antigens.

These studies indicate that surface-exposed B-cell epitopes are located at the C-terminal part of OprF. This is in accordance with studies published previously by Hughes et al. (26), Siehnel et al. (41), and Finnen et al. (18). However, the exact location of surface-located epitopes is still a matter of debate. Our finding that peptide D5 (aa 332 to 350) elicits antibodies which react with intact whole cells of *P. aeruginosa* (Table 1) is in agreement with the same observation reported by Hughes et al. (26) regarding antibodies raised against a peptide (P10) covering the sequence section between aa 328 and 342. The assumption that this sequence may represent a protective epitope is further supported by work described in the same article in which elicited antibodies possessed opsonic activity against wild-type cells of *P. aeruginosa* and by our own findings which show that antibodies induced in rabbits against peptide D5 are protective against *P. aeruginosa* infection in SCID mice (Table 4). A second surface-located epitope could be located by antibodies elicited against peptide D2 (aa 212 to 240) (Table 1). In contrast, antibodies raised against P6 (aa 213 to 227) synthesized by Hughes et al. did not show any reaction with whole *P. aeruginosa* cells, which suggests the presence of aa 228 to 240 forming a surface-located epitope. It may be considered that a disulfide bridge between Cys-215 and Cys-229 is necessary for forming an epitope, as suggested by the model of Siehnel et al. (41). However, this epitope seems not to be relevant for protection, as shown by the lack of protection observed in SCID mice when D2-specific rabbit antisera were injected before challenge with *P. aeruginosa*. This result is further supported by the finding of only low antibody titers against peptide D2 in *P. aeruginosa*-protected mice immunized with recombinant GST-linked Oprs (Fig. 3). The sequence covered by peptide P9 synthesized by Hughes (aa 285 to 298) is part of the peptide D4 (aa 284 to 316) for the first 14 aa. But, in contrast to the results obtained with the antibodies against P9 evoked in mice, we did not observe a positive fluorescence with intact *P. aeruginosa* cells after incubation with our antibodies against D4. One may speculate that the peptide D4 covers two epitopes of OprF, one of which may be recognized predominantly in rabbits.

From these results, we conclude that at least the most C-terminal part of OprF seems to be a suitable candidate antigen for conferring protection. Our decision to include the entire section of OprF from aa 190 to aa 350 for cloning hybrid antigens as a tool for a subunit vaccine resulted from the consideration that the folding of a large peptide stretch might resemble more the relevant and perhaps protective epitopes of OprF in its native form. This precaution is supported by the identification of MAbs which do not react with any of the synthetic peptides but recognize recombinant OprF fusion proteins carrying aa 240 to 316 (D6) and aa 190 to 250 (D7). When GST-linked fusion proteins of OprF, OprI, OprF-OprI, and OprI-OprF were used to test whether the antigens would protect the animals against *P. aeruginosa* infection, only animals immunized with GST-OprF-OprI were shown to be protected, although antibody titers measured by ELISA against *P. aeruginosa* did not differ significantly between the different immunized groups. Analysis of immune sera by ELISA against the peptides D1 to D5 demonstrated that animals immunized with the hybrid antigens had developed significantly higher titers against D4 and D5 (Fig. 3). Only one of the two groups was protected against *P. aeruginosa* infection. We do not have a simple explanation for this finding. The differences between the amino acid sequences of recombinant GST-$OprF_{190–342}$-OprI antigen, which provided protection in the immunosuppressed mouse model, and those of nonprotective GST-OprI-$OprF_{190–350}$ may lead to the speculation that the C-terminal last 8 aa may have a negative effect on protection. However, this is not supported by the experiments with SCID mice, in which protection was achieved with D5-specific antisera induced by immunization of rabbits with peptide D5 (aa 332 to 350) linked to KLH. On the other hand, the different positions of OprI and OprF in both recombinant hybrid antigens may have an effect on the formation and presentation of different epitopes, thus leading to protection for only one of the hybrid antigens. The observation that, after immunization with all three different antigens, comparable antibody titers against sonicated *P. aeruginosa* could be measured by ELISA, whereas considerably different titers were measured against peptides D1 to D5, suggests that antibodies directed against additional epitopes besides D1 to D5 have also been produced in different amounts.

The finding that GST-linked OprF-OprI possesses considerable efficacy as a vaccine against *P. aeruginosa* infection in cyclophosphamide-treated mice encouraged us to express OprF-OprI without any further fusion peptide. However, OprF-OprI could not be expressed in *E. coli* nor in *S. cerevisiae*. The in-frame fusion of OprF-OprI to the signal sequence of the secretory *K. lactis* killer toxin protein resulted in detectable expression of the recombinant *Pseudomonas* hybrid antigen in *S. cerevisiae*. Because of the heterologous yeast leader sequence, the recombinant antigen entered the secretory pathway but was not secreted into the culture fluid. At least one of the two potential glycosylation sites within the expressed OprF section was modified by an N-linked glycosylation, as shown by endoglycosidase F digestion (data not shown).

Two different models demonstrated the ability of OprF-OprI fusion antigens to serve as a protective vaccine in immunocompromised individuals. The cyclophosphamide model first described by Cryz et al. (9) mimics the clinical situation of patients receiving cytostatic therapy. In previous studies, we tested the protective efficacy of OprI in this model (17). Vaccination with OprI protected the animals against a 36-fold LD_{50}. Vaccination with recombinant GST-OprF-OprI increased protection to a 975-fold LD_{50}. Even at a challenge dose of 5×10^3 CFU, highly significant protection could be obtained. LPS-based vaccines are considered to provide the most powerful degree of protection against *P. aeruginosa*. In a second *P. aeruginosa* protection model in SCID mice, therefore, we included an additional control in which protection by antibodies against LPS of the identical serogroup used for challenge was determined. As shown in Table 3, protectivity as calculated for dose requirement of anti-OprF-OprI serum is in a range comparable to that of the LPS-specific antibodies. However, because the sera had not been adjusted to contain comparable amounts of antibodies against *P. aeruginosa* antigens, a true comparison concerning the protective efficacy of LPS antibodies with that of the OprF-OprI-induced antibodies cannot be made. As already mentioned in the introduction, the Oprs of *P. aeruginosa* are highly conserved and antigenically related among all known serotypes of *P. aeruginosa*. However, their potential as a clinically relevant vaccine has been questioned because of the rather lower protection values observed compared with those of LPS vaccines obtained by immunization with single Oprs. Our results with recombinant OprF-OprI hybrid antigens are the first proof that Oprs are candidates for a clinically applicable vaccine. Work is in progress to improve

the expression of OprF-OprI and to test its safety and immunogenicity in human volunteers.

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