INFECTION AND IMMUNITY, Feb. 1991, p. 631–637
0019-9567/91/020631-07$02.00/0

Vol. 59, No. 2

Monoclonal Antibody Characterization of a Leukoagglutinin Produced by *Renibacterium salmoninarum*†

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Received 21 August 1990/Accepted 27 November 1990

***Renibacterium salmoninarum* causes a chronic disease of salmonid fish known as bacterial kidney disease. High concentrations of bacterially produced extracellular protein (ECP) are present in plasma, kidney, and spleen tissue of naturally and experimentally infected fish. ECP agglutinated salmonid leukocytes in vitro at concentrations which correspond to levels found in highly infected fish. Association of biological activity with the structure of the major protein constituent of ECP, p57, was accomplished by monoclonal antibody (MAb) analysis. Location of the antigenic binding sites recognized by the MAbs was determined by two-dimensional electrophoresis and Western immunoblotting of the proteolytic breakdown fragments of p57. Eight MAbs have been classified into three groups on the basis of their differential recognition of these proteolytic breakdown products. Group I MAbs bound a region proximal to the amino terminus of the protein. Two of these MAbs were also able to block leukoagglutinating activity. Group III MAbs bound to a region associated with the bacterial cell surface, while group II MAbs bound a region between group I and group III. These analyses have allowed the identification of potential structural and functional regions of p57.**

Renibacterium salmoninarum is the etiologic agent of bacterial kidney disease in salmonid fish (21). This chronic disease causes important economic losses of cultured salmon worldwide (10); however, control of the disease has been limited because of the absence of an effective vaccine (19) and because of vertical (intraovum) transmission (9).

Little is known about the virulence factors of *R. salmoninarum*, which is a gram-positive, slowly growing, facultative intracellular bacterium (10, 26). Isolate virulency has been correlated with cell surface hydrophobicity, autoaggregation, and the production of a 57-kDa protein (2, 3). High concentrations of a 57/58-kDa protein doublet (also referred to as antigen F [11]), hemagglutinin [5,7]), or p57 [this paper]) can be found in experimentally and naturally infected fish tissues and sera (24, 25). Additionally, p57 is the predominant protein antigen found on the bacterial cell surface and is the major component of the extracellular protein (ECP) isolated from bacterial culture supernatants (11, 25). The function of this protein in vivo is unknown. However, a diverse number of in vitro activities have been attributed to p57, including hemagglutination of rabbit and other mammalian erythrocytes (5), agglutination of salmonid spermatozoa (6), restoration of cell surface hydrophobicity (7), and suppression of antibody production (20b, 23).

In order to further characterize this important protein of *R. salmoninarum*, we have produced a panel of monoclonal antibodies (MAbs) which recognize p57. Several of these MAbs inhibit hemagglutinating activity and a novel leukoagglutinating activity of p57. Two-dimensional (2-D) electrophoresis and N-terminal amino acid sequencing have facilitated the correlation of p57 structure with biological function.

(This work was presented in part at the 90th Annual Meeting of the Society for Microbiology, Anaheim, Calif., 13 to 17 May 1990 [25a].)

MATERIALS AND METHODS

Animals. Coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), cutthroat trout (*Oncorhynchus clarkii*), and rainbow trout (*Oncorhynchus mykiss*) were held in 12°C well water at the Oregon State University Fish Disease Laboratory. The fish weighed between 400 and 600 g and were fed Oregon Moist Pellets (Bioproducts, Warrington, Oreg.).

BALB/c mice (Simonsen Laboratory Inc., Gilroy, Calif.) were maintained by the Laboratory Animal Resource Center at Oregon State University.

ECP preparation. *R. salmoninarum* ATCC 33209 was cultured in 2.8-liter flasks containing 1 liter of medium. Kidney disease medium (KDM-II) was prepared according to the method of Evelyn (8), excluding the addition of serum, and was subsequently ultrafiltered with a PTGC-10,000 filter packet (Millipore Corp., Bedford, Mass.) to remove molecules larger than 10 kDa. Cultures were incubated at 17°C with constant rotational shaking for 10 days. ECP was harvested by the method of Turaga et al. (24). Briefly, bacterial cells were removed by centrifugation at 6,000 × *g* for 30 min (4°C), and the supernatant was concentrated 10× by ultrafiltration. ECP was further concentrated by two 50% saturated ammonium sulfate precipitations and dialyzed against three 1-liter changes of 10 mM phosphate-buffered saline (PBS; pH 7.2). Total protein was assessed by the method of Lowry et al. (17) by using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as a standard. ECP for the agglutination assays was prepared by dialysis against one liter of RPMI 1640 (pH 7.4; GIBCO Laboratories, Grand Island, N.Y.). The ECP was filter sterilized (pore size, 0.45 μm) and stored at −70°C until needed or at 4°C for immediate use.

Control preparations of chicken egg albumin (grade V; Sigma) or KDM-II medium were precipitated by 50% satu-

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† Technical report 9336 of the Oregon Agricultural Experimental Station.

TABLE 1. MAb isotype characterization and activity against *R. salmoninarum* ECP

MAb	Isotype	A_{405}[a] (SD)	Western blot[b]
4H8	IgG1(κ)	1.40 (0.18)	+
4C11	IgG2b(κ)	1.46 (0.04)	+
4D3	IgG1(κ)	1.36 (0.04)	+
2G5	IgG1(κ)	1.16 (0.05)	+
3H1	IgG1(κ)	1.17 (0.04)	+
1A1	IgG1(κ)	1.12 (0.10)	+
4D10	IgG1(κ)	1.33 (0.15)	+
2G9A	IgG1(κ)	1.18 (0.04)	+
MOPC 21	IgG1(κ)	0.06 (0.03)	−

[a] A_{405} activity of 1 μg of protein A-purified MAb per ml tested in triplicate by ELISA. Plates were coated with 1 μg of ECP per ml.

[b] Immunoreactivity with the 57/58-kDa protein (p57). + Reactivity; −, no reactivity.

rated ammonium sulfate fractionation and dialyzed against PBS followed by RPMI 1640 as described above.

Production and purification of MAbs. Female BALB/c mice were injected intraperitoneally with a mixture of 10 mg of PBS-washed *R. salmoninarum* cells mixed 1:1 with Freund complete adjuvant. Two months postimmunization, the mice were injected with 1 mg of wet, packed cells and 10 μg of *Escherichia coli* lipopolysaccharide (LPS) serotype O26:B6 (Difco Laboratories, Detroit, Mich.). Mice were sacrificed 3 days postchallenge, and the spleens were removed aseptically. Splenocytes were then fused with SP2/0 cells by using the polyethylene glycol method (4). Hybridomas were screened by enzyme-linked immunosorbent assay (ELISA) for antibodies against ECP (see below). Five hybridomas of the immunoglobulin G (IgG) isotype were identified and subcloned twice by limiting dilution. Isotyping was performed with an ICN Immunobiologicals kit (Lisle, Ill.). The production of MAbs 2G5, 4D3 (25), and 3H1 (20a) has been previously described.

Cell-free ascites fluid was separately prepared from each hybridoma. Each antibody was purified by protein A chromatography with the MAPS system by following the manufacturer's instruction (Bio-Rad Laboratories, Richmond, Calif.). Protein content was determined by the method of Lowry et al. (17). A commercial hybridoma ascites (MOPC 21; Sigma) was purified as described above and used as a control. Antibodies were dialyzed in RPMI 1640 for use in agglutination assays.

ELISA. ECP at a concentration of 1 μg/ml was diluted in 24 mM carbonate-bicarbonate buffer, pH 9.6, and coated onto enzyme immunoassay flat-bottom plates (Costar, Cambridge, Mass.) overnight at 17°C. Wells were blocked with 1% bovine serum albumin (BSA; fraction V, Sigma) diluted in 0.1% Tween–Tris-buffered saline (50 mM Tris, 1 mM EDTA, 8.7% NaCl [pH 8.0]). Hybridoma tissue culture supernatant or protein A-purified MAb was added for 1 h, and after a washing, a 1:4,000 dilution of peroxidase-labeled goat anti-mouse immunoglobulin (Hyclone, Logan, Utah) secondary antibody was added. Substrate [0.2 mg of 2,2′-azinobis(3-ethylbenzthiazoline sulfonic acid) (diammonium salt) per ml in 9.5 mM citrate buffer (pH 4.0), and 0.05% of 30% H_2O_2] was applied for 30 min, and the A_{405} was

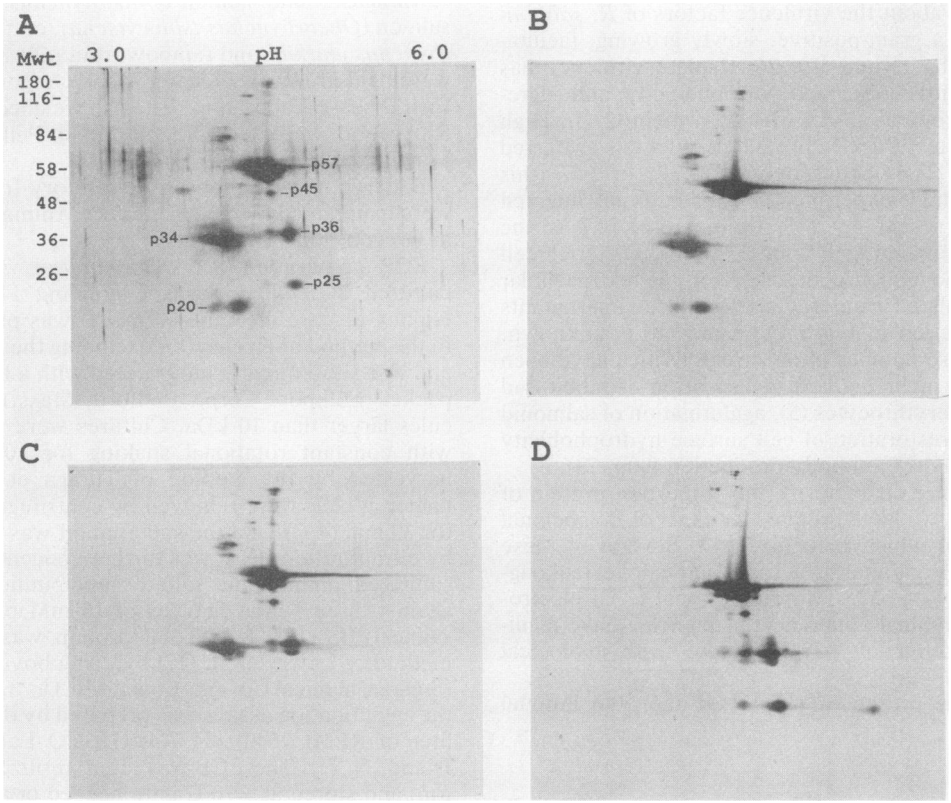

FIG. 1. Total protein stain and Western blots of *R. salmoninarum* ECP separated by 2-D electrophoresis. Proteins were transferred to nitrocellulose and stained with colloidal gold (A) or probed with MAbs 4H8 (B), 3H1 (C), and 1A1 (D), representative of groups I through III, respectively. Mwt, Molecular mass markers (in kilodaltons).

TABLE 2. N-terminal amino acid sequences of various proteins[a]

Protein	Amino acid[b] at residue no.:														
	1				5					10					15
p57	NH_2-	S	Q	G	E	G	N	S	X	T	X	T/G	V	Q	D/G	F
p34	NH_2-	S	Q	G	E	G	N	S	X	T						
p20	NH_2-	S	Q	G	E	G										
p20[c]	NH_2-	S	Q	G/F	E/D	G	N	S/Q	X							

[a] Proteins were sequenced after SDS-PAGE and transferred to PVDF membranes.
[b] X, Unidentified residues.
[c] Sequenced from a 2-D gel.

determined on an enzyme immunoassay autoreader (Biotek Instruments, Burlington, Vt.)

Gel electrophoresis and Western immunoblot analysis of anti-p57 MAbs. ECP prepared as described above consistently contains p57 and a number of lower-molecular-mass proteolytic breakdown fragments due to the activity of an endogenous serine protease (20b). Preliminary localization of antigenic binding sites recognized by each MAb was determined by 2-D electrophoresis (20) of ECP followed by Western blotting. *R. salmoninarum* ECP (3 μg) was mixed 1:1 with 1-D sample buffer (9.5 M urea, 2.0% Triton X-100, 5% 2-mercaptoethanol, 1.0% pH 3-5 ampholyte, and 1.0% pH 4-6 ampholyte [Bio-Rad]). Proteins were focused at 500 V for 10 min and then at 750 V for 3.5 h. 2-D gels (1.0 mm, sodium dodecyl sulfate [SDS]–10% polyacrylamide) were run for 1.5 h at 100 V. Proteins were transferred to nitrocellulose either for 14 h at 30 V or for 1.5 h at 100 V (22). Blots were then stained with a colloidal-gold total protein stain (Bio-Rad) or probed with approximately 5 μg of the appropriate MAb per ml as described previously (25).

Microsequencing. *R. salmoninarum* ECP was separated by 1-D or 2-D electrophoresis and transferred (80 V, 10 to 15 min) to Immobilon-PVDF (Millipore) in transblotting buffer [10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 20% methanol (pH 11.0) (18). Membranes were stained with either Coomassie blue or Ponceau red for 1 min and destained with 50% methanol–10% acetic acid. Bands were excised, rinsed with nanopure water, dried, and frozen at −20°C until sequencing. Amino terminus sequencing was performed at Oregon State University Center for Gene Research with a gas-phase microsequencer (model 475A; Applied Biosystems, Foster City, Calif.) and was followed by high-performance liquid chromatography.

TABLE 3. Minimum concentration of MAbs required for complete inhibition of leukoagglutination or hemagglutination mediated by *R. salmoninarum* ECP

MAb	Group	Minimum concn (μg/ml) required for complete inhibition of:	
		Leukoagglutination	Hemagglutination
4H8	Ia	50	100
4C11	Ia	50	50
4D3	Ib	NI[a]	250
2G5	II	NI	NI
3H1	II	NI	NI
1A1	III	NI	NI
4D10	III	NI	NI
2G9A	III	NI	NI
MOPC 21	Control	NI	NI

[a] NI, No inhibition at a MAb concentration of 1,000 μg/ml, except for MAb 2G9A, which was only tested up to a concentration of 250 μg/ml.

Peripheral blood leukocyte separation and in vitro tissue culture. Peripheral blood (2 to 8 ml) was collected in a sterile heparinized VACUTAINER system from the caudal vein of anesthetized fish (15). Peripheral blood leukocytes were separated by Histopaque 1077 (Sigma) gradient centrifugation, as previously described (14). Leukocytes were resuspended in tissue culture medium (10% fetal calf serum [Hyclone], 50 μg of gentamicin sulfate per ml, and 2% sodium bicarbonate in RPMI 1640 [GIBCO]). Cultures were incubated in 96-well flat-bottom plates (Corning, Corning, N.Y.) at 17°C under a blood-gas mixture.

Agglutination assays. (i) Leukoagglutination. Peripheral blood leukocytes (10^7 cells per ml) were coincubated with dilutions of ECP, control protein (chicken egg albumin), or concentrated KDM-II medium in a total volume of 100 μl. All incubations were done in triplicate. Agglutination was assessed by microscopic examination of wells with a CK Olympus inverted microscope (Boyle Instruments, Gig Harbor, Wash.). Cells were photographed after 24 h.

Determination of MAb inhibition of agglutination was assessed by preincubation of 5, 10, 25, 50, 100, 250, 500, or 1,000 μg of (each) MAb per ml for 1 h with 50 μg of ECP per ml at 17°C before addition of leukocytes.

(ii) Hemagglutination of rabbit erythrocytes. Blood from New Zealand White rabbits was mixed 1:1 with Alsever's solution and centrifuged at 500 × *g* for 10 min. Erythrocytes were resuspended in tissue culture medium (1.5×10^7 cells per ml) and incubated with 50 μg of ECP per ml. Inhibition of agglutination was determined as described above.

Analysis of leukocyte-adherent ECP. Peripheral blood leukocytes (2×10^6) in tissue culture medium were mixed with 200 μg of ECP per ml, a control protein (MAb 2G5, 200 μg/ml), or RPMI 1640 alone in a total volume of 200 μl. This preparation was suspended at 17°C for 3 h with agitation every 15 min. Unbound ECP was removed by five 1-ml washes with RPMI 1640 followed by centrifugation at 500 × *g* for 10 min after each wash. Following the final wash, the cell pellet was lysed by the addition of 10 μl of extraction buffer (10 mM Tris hydrochloride [pH 7.2], 0.15 M NaCl, 0.02% NaN_3, 0.5% [wt/vol] Nonidet P-40 [13]). After 15 min on ice, insoluble cellular constituents were removed by a 10-min centrifugation in a microfuge E (Beckman Instruments Inc., Palo Alto, Calif.). The supernatant was removed and frozen at −70°C until electrophoretic analysis (16).

Analysis of bacterial cell-associated protein. (i) Bacterial cells. *R. salmoninarum* was cultured as described above, and the bacterial cells were washed three times (1 [wet weight]:

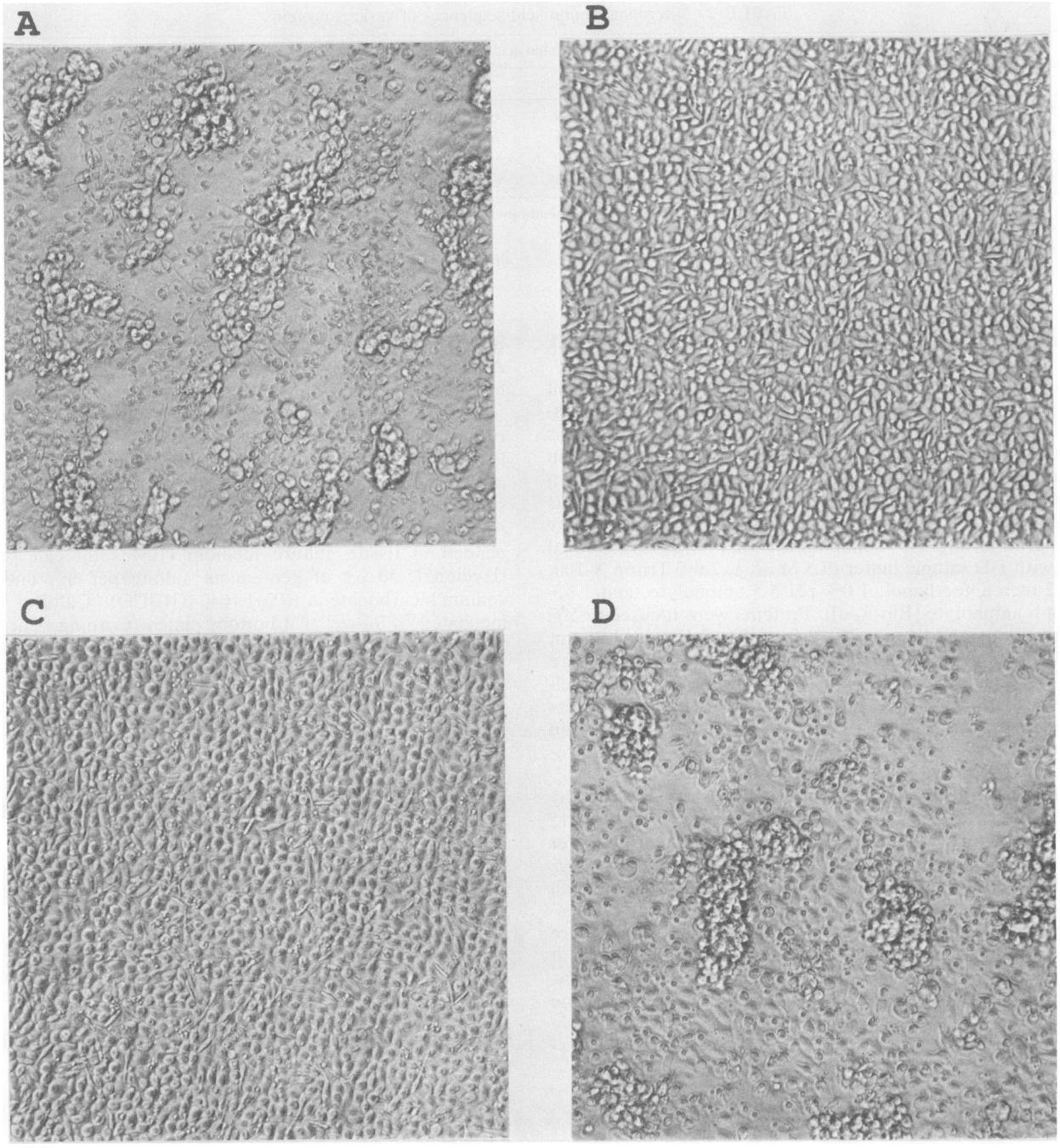

FIG. 2. Light micrographs of cultured peripheral blood leukocytes from coho salmon. Leukocytes (1×10^6) were incubated with 50 μg of ECP per ml (A) or tissue culture medium alone (B). Agglutination was inhibited by preincubation of ECP with 50 μg of MAb 4H8 per ml (C) but not with 1,000 μg of MOPC 21 per ml (D). All incubations were performed in a total volume of 100 μl at 17°C for 24 h. Magnification, ×160.

100 [volume of PBS]) and pelleted by a subsequent 2-min centrifugation in a microfuge. Cells were finally resuspended in an equal (wt/vol) amount of PBS, and 2 μl was mixed with 48 μl of double-distilled water and 50 μl of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. Samples were boiled for 3 min and subjected to electrophoresis.

(ii) Fluorescent antibody analysis. Tissue smears of infected coho salmon kidneys were made on precleaned glass slides (American Scientific Products, McGaw Park, Ill.). After being air dried, slides were blocked by incubation in 0.25% BSA in PBS for 0.5 h. Slides were subsequently probed with 10 μg of protein A-purified MAbs per ml for 0.5 h. Unbound antibody was removed by washing with 0.25% BSA in PBS, and 1:100 goat anti-mouse immunoglobulin fluoroscein isothiocyanate-labeled second antibody (Hyclone) was applied for 0.5 h. Slides were observed with a standard microscope (Zeiss) utilizing an IV F1 epifluorescent condenser and a 12-V, 100-W halogen tungsten light source.

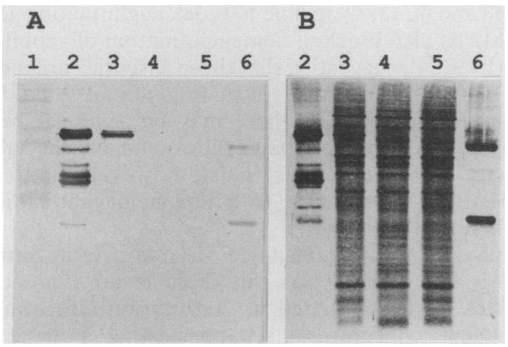

FIG. 3. Western blot (A) and total protein stain (B) of coho salmon leukocyte protein extracts obtained from leukocytes incubated with *R. salmoninarum* ECP or a control protein (protein A-purified MAb 2G5). Lanes: 1, prestained molecular weight markers (Sigma); 2, 2 μg of ECP; 3, leukocytes incubated with 200 μg of ECP per ml; 4, leukocytes incubated in tissue culture medium alone; 5, leukocytes incubated with 200 μg of MAb 2G5 per ml; 6, 2 μg of 2G5. The Western blot was probed with 5 μg (each) of MAbs 4D3 and 3H1 followed by a 1:500 dilution of a second antibody, goat anti-mouse horseradish peroxidase.

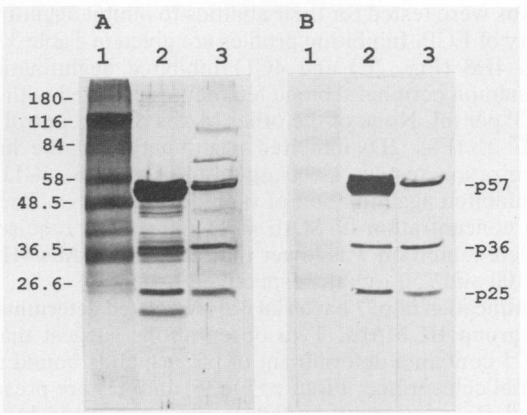

FIG. 4. Total protein stain (A) and Western blot (B) of washed *R. salmoninarum* cells. Lanes: 1, prestained molecular weight markers (Sigma); 2, 3 μg of *R. salmoninarum* ECP; 3, 2.5 μl of *R. salmoninarum* cells washed three times. The Western blot was probed with 5 μg of purified MAb 1A1 per ml.

RESULTS

Characterization of MAbs. All MAbs used in this study were of the IgG isotype. ELISA and Western blots were used to determine the relative activities and specificities of the MAbs. All eight MAbs bound to ECP in the ELISA and recognized the 57/58-kDa protein (p57) on Western blots (Table 1). A control MAb, MOPC 21, had no reactivity against ECP in either of the assays.

Antigenic binding site analysis of p57 with MAbs. The relative locations of antigenic binding sites on p57 were established by determining the pattern of MAb binding to proteolytic fragments of p57 present in ECP. Five major proteolytic fragments with approximate molecular masses of 45, 36, 34, 25, and 20 kDa were resolved from ECP by 2-D electrophoresis (Fig. 1A). The pIs of p57 and proteolytic fragments were acidic. Different isoelectric forms were present as well as minor amounts of higher-molecular-mass aggregates. Replicate blots were probed with each MAb, and the MAbs were divided into three groups on the basis of the patterns of immunoreactivity. Group I MAbs, 4H8, 4C11, and 4D3, bound to p34 and p20, which have a more acidic pI than p57 (Fig. 1B). MAb 4D3 also recognized p45, which was not recognized by MAbs 4H8 or 4C11, indicating a subgroup within this group (not shown). Group III MAbs, 1A1, 4D10, and 2G9A, bound proteolytic fragment p45, and two fragments, p36 and p25, which have a more basic pI than p57 (Fig. 1D). Group II MAbs, 2G5 and 3H1, recognized both p36 and p34 (Fig. 1C). These data suggest that group II MAbs recognize a determinant located between those recognized by group I and III MAbs, since group II MAbs bound proteolytic fragments recognized by both of the other groups.

Identification of the amino terminus of p57. Both p20 and p34 had the same amino-terminal sequence as p57, suggesting that they are proteolytic fragments from the amino terminus of p57 (Table 2). Residue 8 was consistently unidentifiable even after carboxymethylation, suggesting possible posttranslational modification or an artifact of the sequencing methodology.

Inhibition of p57 biological activity with group I MAbs. *R. salmoninarum* ECP agglutinated coho salmon leukocytes in a dose-dependent manner with a lower threshold of 10 μg/ml. Agglutination of coho salmon leukocytes results on the addition of 50 μg of ECP per ml (Fig. 2A). Leukocytes were not agglutinated in tissue culture medium alone (Fig. 2B) or when incubated with similar concentrations of control protein, chicken egg albumin, or concentrated KDM-II medium proteins (not shown). Agglutination was not salmonid species specific, since leukocytes from chinook and coho salmon and rainbow and cutthroat trout were also agglutinated. Fish erythrocytes and mouse splenocytes were not affected.

The component of the ECP responsible for leukocyte agglutination was determined by analysis of leukocyte-adherent ECP. The p57 component of the ECP was detected in the leukocyte extract (Fig. 3A, lane 3), but none of the lower-molecular-mass components were found to be present (Fig. 3A, lane 2). Binding was deemed specific because the goat anti-mouse horseradish peroxidase conjugate did not detect the control protein (MAb 2G5) in the membrane extracts (Fig. 3A, lane 5). Approximately equal amounts of leukocyte extracts were run in each lane, as determined by the protein stained blot (Fig. 3B). These results suggest that the p57 component of ECP was binding to and agglutinating leukocytes. Further, agglutinating activity of the ECP was sensitive to incubation at 37°C, a temperature which enhances the activity of the endogenous serine protease.

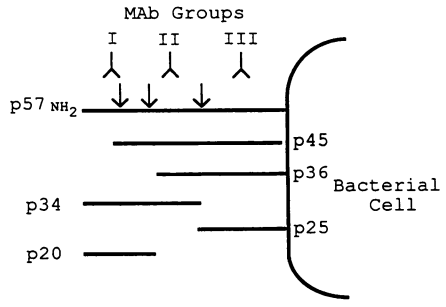

FIG. 5. Proposed structural model of p57. Protein has been depicted in a linear manner. Lower-molecular-mass proteolytic products with a minimum number of proteolytic sites are indicated (↓).

MAbs were tested for their abilities to inhibit agglutinating activity of ECP. Inhibition profiles are given in Table 3. Only MAbs 4H8 (Fig. 2C) and 4C11 inhibited agglutination of coho salmon peripheral blood leukocytes cultured with 50 μg of ECP per ml. None of the other MAbs or the control MAb MOPC 21 (Fig. 2D) inhibited agglutination at the highest concentration tested, 1,000 μg/ml. MAbs 4H8, 4C11, and 4D3 inhibited agglutination of rabbit erythrocytes. The minimum concentration of MAb 4C11 (50 μg/ml) required for complete inhibition was lower than those of MAbs 4H8 and 4D3 (100 and 250 μg/ml, respectively).

Identification of p57 bacterial cell-associated determinant by using group III MAbs. Two observations suggest that p36 and p25 contain a determinant of p57 which is bound to the bacterial cell surface. First, p57, p36, and p25 are present in washed *R. salmoninarum* cell extracts separated by SDS-PAGE (Fig. 4A, lane 3) and were identified by MAb 1A1 (Fig. 4B). Second, group III MAbs, which bind p36 and p25, were unable to immunofluoresce bacterial cells present in infected coho salmon kidney tissue. Since equal concentrations of MAbs had comparable binding activity against ECP (Table 1), the inability of group III MAbs to immunofluoresce bacterial cells suggests that the antigenic binding site is sterically unavailable when p57 is bound to the bacterial cell surface.

DISCUSSION

In this study MAbs were used to investigate the structure and function of p57, which is the predominant extracellular and cell surface protein produced by the fish pathogen *R. salmoninarum*. Analysis was facilitated by an autologous serine protease which cleaves p57 in broth culture, resulting in fragments with molecular masses of 45, 36, 34, 25, and 20 kDa (20b). Resolution of ECP by 2-D electrophoresis followed by Western blotting allowed the MAbs to be divided into three groups on the basis of their differential recognition of these proteolytic fragments. Group I MAbs recognize p34 and p20, which have an identical amino-terminal sequence to p57. Therefore, unless a repetitious sequence exists, group I MAbs bind proximal to the amino terminus of the protein (Fig. 5). Since agglutinating activity was inhibited by group I MAbs, the agglutinating domain may be near the amino terminus. Antigenic binding sites recognized by group I and II MAbs are exposed on the surface of the bacterial cell, as demonstrated by immunofluorescence experiments. However, group III MAbs recognize an antigenic binding site which is sterically unavailable when p57 is attached to the bacterial cell surface. The fragments p36 and p25, recognized by the group III MAbs, also are present in cell extracts from washed bacterial cells. These observations suggest that p36 and p25 may contain a determinant of p57 which is important for the attachment of the protein to the bacterial cell surface. This determinant may be responsible for the in vitro reassembly of the protein onto a *R. salmoninarum* strain lacking p57 previously described by Daly and Stevenson (7).

Previously, p57 was demonstrated to possess agglutinating activity against salmonid spermatozoa and a number of mammalian erythrocytes but not fish erythrocytes (5, 6). We have described a novel activity of p57, the agglutination of salmonid leukocytes. In this study, ECP was used to agglutinate leukocytes, and two experiments demonstrate that p57 is associated with this activity. First, by using leukocytes as an ECP absorbent, p57 was the only component found in membrane extracts. Second, two MAbs which recognize p57, 4H8 and 4C11, were able to block agglutinating activity. These MAbs also blocked hemagglutination of rabbit erythrocytes by ECP, suggesting that these activities are mediated by the same site on p57. Assuming that p57 binds in a receptor-specific manner, there may be a similar receptor determinant on both the rabbit erythrocyte and the salmonid leukocyte. Differences must exist, however, because another MAb, 4D3, was able to inhibit hemagglutination but not leukoagglutination.

The physiological significance of leukocyte agglutination by p57 is still unclear. As yet there is no evidence that leukocytes are agglutinated in vivo during infection, even though high concentrations, approaching 1 mg/ml, are present in moribund fish (24). It is interesting to note that p57 possesses characteristics resembling a number of proteinaceous adhesins (1, 12). These include acidic pI, hemagglutinating activity, and contribution to bacterial cell surface hydrophobicity. To our knowledge no filamentous structures have been described on *R. salmoninarum*, but p57 may function as a nonfimbrial adhesin for the bacterial attachment to cellular receptors allowing intracellular invasion by the bacteria, as proposed by Daly and Stevenson (5, 7). Cloning and sequencing of the p57 gene and the development of isogenic mutants of *R. salmoninarum* will be useful for the further confirmation of the proposed model and the role of this protein in bacterial virulency and adhesion.

ACKNOWLEDGMENTS

Support for this research was received from the Bonneville Power Administration grant DE-79-89BP95906 and from a Sigma Xi grant-in-aid of research.

We thank B. Wiens, D. Rockey, L. Gilkey, J. DeKoning, and J. Piganelli for their critical reviews of the manuscript and R. McPharland for amino acid sequence analysis.

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