Enhanced immunogenicity of *Renibacterium salmoninarum* in chinook salmon after removal of the bacterial cell surface-associated 57 kDa protein

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ABSTRACT: A study was conducted to determine the effect of endogenous serine protease activity on the immunogenicity of *Renibacterium salmoninarum* cells in chinook salmon *Oncorhynchus tshawytscha*. Salmon were immunized with either *R. salmoninarum* cells possessing p57 (p57⁺) or substantially depleted of p57 (p57⁻). The resultant antisera were examined by whole cell ELISA and immunoblot procedures using p57⁺, p57⁻, proteinase-K-treated, and periodate-treated whole cells. These analyses revealed that the removal of p57 by the endogenous serine protease significantly enhanced the immunogenicity of the cell, resulting in a 20-fold increase in detectable antibody titers. The bulk of this antibody activity was directed at sites blocked by the presence of the p57 molecule. Furthermore, proteinase-K and periodate treatment of *R. salmoninarum* cells revealed that the increased antibody activity almost exclusively reacted with carbohydrate moieties on the p57⁻ cell.

KEY WORDS: *Renibacterium salmoninarum* · Bacterial kidney disease · Humoral immune response · Antibodies · Immunogenicity · Whole cell ELISA

INTRODUCTION

Renibacterium salmoninarum, the causative agent of bacterial kidney disease (BKD) of salmonid fish, is a slow-growing, Gram-positive, obligate intracellular diplobacillus (Young & Chapman 1978, Fryer & Sanders 1981, Evenden et al. 1993). Despite advances in the understanding of pathogenicity, immune interactions, and the development of new disease management techniques, BKD continues to plague the culture of salmonids. The ability of the organism to be transmitted both horizontally (Mitchum & Sherman 1981, Bell et al. 1984) and vertically (Evelyn et al. 1984), to survive and possibly replicate within phagocytic cells, along with its fastidious requirements and slow growth, have made it one of the most difficult fish pathogens to study and control (Evenden et al. 1993, Fryer & Lannan 1993).

Currently, there is no vaccine for BKD. Numerous attempts to vaccinate intraperitoneally (IP) with untreated, heat- or formalin-killed whole cells (Baudin-Laurencin et al. 1977, Sakai et al. 1989), soluble extracellular proteins (ECP) or crude cell fractions (Evelyn et al. 1984) have failed to reliably protect salmonids (Paterson et al. 1981, McCarthy et al. 1984, Shieh 1989). While untreated whole cells and soluble ECP antigens do not appear to be protective, it is possible other cell-associated antigens may serve as protective antigens in vaccines against BKD.

Despite previous studies aimed at elucidating immune interactions of *Renibacterium salmoninarum* within the host, only limited information is available concerning the immune response of salmonids to specific antigens of *R. salmoninarum*. Elevated humoral antibody titers have been observed after vaccination with adjuvant and in natural infections (Evelyn 1971, Weber & Zwicker 1979, Paterson et al. 1981, Bruno 1987, Sakai et al. 1989). Turaga et al. (1987a) demonstrated by immunoblotting that coho salmon recognize

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R. salmoninarum ECP, which contains p57. Further, chinook salmon recognize p57 and 3 other proteins of unresolved molecular weight (MW) (Bartholomew et al. 1991)

The p57 molecule is well characterized and a focal point of extensive diagnostic, pathogenic and prophylactic studies. This hydrophobic protein possesses hemagglutinating (Daly & Stevenson 1987) and leukoagglutinating (Wiens & Kaattari 1991) properties. Also, p57 and other extracellular products (ECP) are known to suppress plaque-forming cells of salmonid leukocytes *in vitro* (Turaga et al. 1987a). Rockey et al. (1991) identified an endogenous serine protease that degrades the soluble p57 component of ECP and cell surface-associated p57 at elevated temperatures. In addition, we have demonstrated that heat-induced proteolytic activity causes ECP to lose the ability to suppress the *in vitro* antibody response.

Based on this information we have reasoned that removal of the immunosuppressive putative virulence factor, p57, may expose new antibody binding sites and enhance the antibody response to other cell surface antigens. We have compared the salmonid humoral immune responses to formalin-killed *Renibacterium salmoninarum* [which possesses cell-associated p57 (p57⁺)], and heat-treated formalin-killed *R. salmoninarum* [which lacks most of the cell-associated p57 (p57⁻)]. Analysis of specific serum antibody titers indicated that removal of p57 from whole cells by heat treatment (37°C) was critical for enhancing immunogenicity and antigenicity. Further, analysis of p57⁻ serum reactivity against heat-treated p57⁻ cells digested with periodate provided indirect evidence that carbohydrate antigens may also be important immunogens.

METHODS

Animals. Spring chinook salmon *Oncorhynchus tshawytscha* used in this study were derived from spawned parents that tested negative for BKD by ELISA (Pascho et al. 1991). These fish were obtained from the Dworshak National Fish Hatchery in Ahsahka, Idaho, USA. Three tanks each of 35 fish weighing approximately 20 to 50 g upon initial injection were maintained in 12°C, sand-filtered, UV-treated, pathogen-free lake water at the Northwest Biological Science Center of the National Biological Survey in Seattle, WA, USA. Fish were fed Oregon Moist Pellets (OMP) (Bioproducts, Warrington, OR, USA). Coho salmon *Oncorhynchus kisutch*, weighing 200 to 500 g upon initial immunization, were maintained at the Salmon Disease Laboratory (SDL) at Oregon State University (OSU), Corvallis, as a source of standard antisera.

Bacterial strains. *Renibacterium salmoninarum* isolate D-6 (obtained from C. Banner, Oregon Department of Fish and Wildlife, OSU) was cultured on a shaker for 8 to 14 d to an optical density (OD) of 1.0 (525 nm) in kidney disease medium (KDM II) at 17°C (Evelyn 1977), excluding calf serum. After centrifugation (6000 × g, 30 min, 4°C) bacterial pellets were washed (16 000 × g, 2 min) 3 times with 1 ml cold 10 mM phosphate buffered saline (PBS) [0.85% NaCl, 10 mM Na$_2$PO$_4$ (pH 7.2)], resuspended to a wet weight concentration of 200 mg ml⁻¹, and stored at –70°C. Soluble ECP was harvested from the supernatant as previously described (Turaga et al. 1987a).

Preparation of whole cells. The p57⁻ cells were prepared by incubating p57⁺ cells at 37°C for 10 h. Microfuged cell pellets were washed twice in cold PBS. Both cell preparations were then formaldehyde-killed with a 0.3% solution (EM Science, Cherry Hill, NJ, USA) in PBS overnight at 4°C. After washing the cells twice, pellets were resuspended to 200 mg ml⁻¹ in PBS and stored at –70°C.

Production of monoclonal antibodies (mAbs). Monoclonal antibodies (mAbs) 4D3, 3H1, and 1A1 (Wiens & Kaattari 1989, 1991) were used to identify p57 and its breakdown products in immunoblots. Ascitic fluid was purified using the caprylic acid procedure of Russo et al. (1983). After precipitation with 50% ammonium sulfate, the precipitate was resuspended in PBS and extensively dialyzed against PBS, diluted 1:1 in glycerol and stored at –20°C.

Antigen preparation and immunization. Chinook salmon were immunized with either p57⁺ or p57⁻ whole cell antigens (100 μg bacterial wet weight per fish) emulsified 1:1 in Freund's complete adjuvant (FCA, Sigma, St. Louis, MO, USA) and PBS for 4 min at 100 units on a Virtis '23' mixer (Virtis, Gardiner, NY, USA). A preparation of PBS alone in FCA served as a negative control. Each fish was injected with a total volume of 50 ml intraperitoneally using a 1 cc syringe fitted with a 26½ gauge needle (Becton Dickinson, Rutherford, NJ). A boost with Freund's incomplete adjuvant (FIA) followed 15 wk after primary immunization at half the concentration in the same total volume.

Collection of antiserum. Individual serum samples were collected from randomly selected fish in the p57⁺, p57⁻, and control groups at 0 and 12 wk post primary immunization and at 17.5 wk post secondary immunization (i.e. 32.5 wk post primary immunization). Blood was taken from the caudal vessel of animals anesthetized in 10% benzocaine (Sigma). Blood was allowed to clot at room temperature (RT) for several hours. Samples were centrifuged (Beckman model TJ-6) at 500 × g for 5 min at 4°C, the serum removed, aliquoted and stored at –70°C.

Protein and carbohydrate digestion of whole cells. Aliquots of 80 µl of a p57+ or p57- cell stock (10 mg ml⁻¹) were treated with 20 µl of 0.2 M meta-periodate (Sigma) in dd-H$_2$O, or with 20 µl PBS (control). Cells were incubated at 4°C for 24 h as described by Liang et al. (1992). The same cell concentration was used for the proteinase-K treatment (1 mg ml⁻¹) (Sigma) in 0.1 M tris HCl, pH 8.0. Cells were incubated in the dark at RT for 24 h (Mack et al. 1992). Periodate and proteinase were removed by washing the cells twice in PBS, and by resuspending the cells in the original buffer.

Determination of antibody activity by ELISA. In preparing the coating antigen for the enzyme-linked immunosorbent assay (ELISA), it was anticipated that different treatments might alter the cell's plastic binding capacities. Therefore, it was necessary to determine the plate coating efficiency of each *R. salmoninarum* preparation (i.e. untreated, heat-, proteinase-, and periodate-treated). Upon determination of this property, the appropriate coating antigen concentration was applied to the ELISA plate wells to yield a comparable number of bound bacteria per well. We chose concentrations of cells which would bind 50% of the ELISA well surface (Fig. 1). To accomplish this, the peroxidase saturation technique of Munoz et al. (1986) was applied with minor modifications. Briefly, dilutions of whole cells were incubated in wells
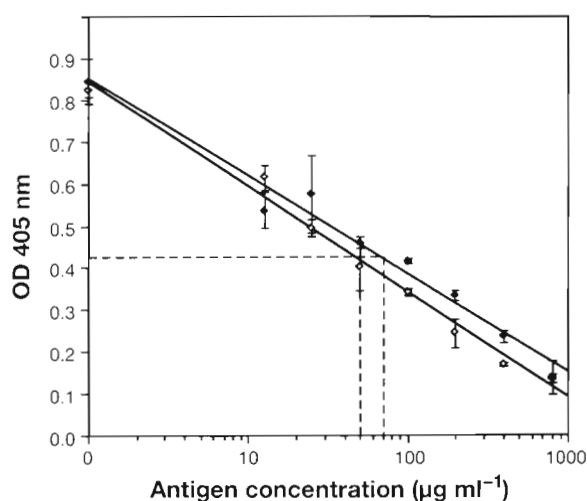

Fig. 1. Determination of whole cell antigen concentration needed to occupy 50% of the surface of an ELISA plate well. Plates were coated with graded concentrations of each antigen, and antigen adsorption in the well was determined using horseradish peroxidase to determine percentage of non-occupied surface sites (Munoz et al. 1986). Logarithmic curve-fit interpolation was calculated, and surface antigen concentration to be employed was calculated as that corresponding to the intersection of the 50% saturation line. Results corresponding to p57+ whole cell antigens (◇) and p57- whole cell antigens (●) are plotted together. Error bars represent 2 SD about the mean
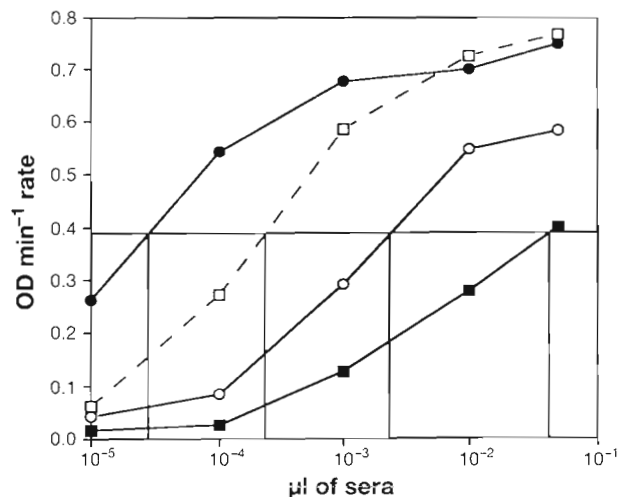
overnight at 17°C. The *R. salmoninarum* suspensions were then removed and replaced with a saturating concentration (1 mg ml⁻¹) of horseradish peroxidase (HRPO). The peroxidase solution was incubated for 1 h at RT. Plates were washed 10 times with a 0.1% Tween 20 in tris-buffered saline (TTBS; 50 mM tris, 1 mM EDTA, 8.7% NaCl, pH 8.0) with a Titertek Automatic Microplate Washer (Flow Laboratories, McLean, VA, USA) and blocked with 200 µl well⁻¹ of 1% bovine serum albumin (BSA; Fraction V, Sigma) in TTBS for 1 h at RT. Substrate (H$_2$O$_2$) and 3',2' azino-bis (ethyl)benzthiaoline sulfonic acid (ABTS) solutions were then added as per the original protocol (Arkoosh & Kaattari 1990).

The concentration of antibody activity was determined by the use of a modification of the ELISA as described by Arkoosh & Kaattari (1990). Formalin-fixed p57+ (50 µg ml⁻¹) or p57- cells (70 µg ml⁻¹) were coated on E.I.A./R.I.A. ELISA plates (Costar, Cambridge, MA, USA) overnight at 17°C. Plates were then blocked with 1% BSA–TTBS for 1 h at RT and washed 5 times in TTBS. Each plate containing 5 dilutions of each test serum and the standard were incubated for 90 min at RT.

A standard antiserum was derived by pooling salmon sera to whole *Renibacterium salmoninarum*. This pool was made sufficiently large so that enough standard serum would be available for the completion of all experiments within this study. The titer of the standard reflected the dilution of the standard required to achieve 50% of the maximum OD. Plates were washed 10 times after each step of the assay. The presence of anti-*R. salmoninarum* antibody in the serum samples was detected using a biotinylated mouse anti-trout immunoglobulin monoclonal antibody, 1-14 (DeLuca et al. 1983), diluted 1:1500 in 1% BSA–TTBS. After washing, streptavidin-conjugated HRPO (Sigma) diluted 1:200 in 1% BSA–TTBS was added and incubated 45 min. After washing, substrate (H$_2$O$_2$) and ABTS were added. Plates were read kinetically for 10 min at 405 nm with a Titertek Multiskan Automatic Reader (Flow Laboratories). The initial reaction rates were then calculated.

Titers were determined by first calculating the volume of each serum required to achieve 50% of the maximum OD rate (Arkoosh & Kaattari 1990). This value was then transformed into a titer (units of antibody activity µl⁻¹) using the following equation:

$$\frac{\text{Volume of standard} - MAX_{50}}{\text{Volume of test} - MAX_{50}} \times 10\,000\text{U µl}^{-1} \text{ (Titer of standard)}$$

Direct comparison of the 50% point with the standard serum allowed normalization of data values from all sera and reduced plate-to-plate variation. Sample titrations are depicted (Fig. 2).

Fig. 2. Determination of serum antibody activity from 3 samples. Data were expressed as initial reaction rate (OD min^{-1}). Amount of antibody activity was calculated as described in the text using the method described by Arkoosh & Kaattari (1990). In this example the standard ($\square$) was assigned a value of 10 000 U μl^{-1}; sample 1 ($\bullet$): 93 000 U μl^{-1}; sample 2 ($\bigcirc$): 1000 U μl^{-1}; sample 3 ($\blacksquare$): 80 U μl^{-1}

Preparation of whole cells for SDS-PAGE and immunoblotting. Whole cells (20 mg ml^{-1}) were mixed 1:1 in sample application buffer (SAB) (120 mM tris, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 3 mM bromophenol blue) or 5 µl (100 mg ml^{-1}) of cells were mixed with 120 µl dd-H$_2$O and 125 µl of SAB and boiled for 3 min. Resulting boiled extracts were then electrophoresed in 10% SDS-polyacrylamide gels (SDS-PAGE) in a Mini-protean II electrophoresis apparatus (Bio-Rad, Richmond, CA, USA) at 200 V for 1 h. Gels were equilibrated in transfer buffer (192 mM glycine, 20 mM tris, pH 8.3, 20% methanol), and the proteins were electrophoretically transferred to immobilon-P (Millipore, Bedford, MA) at 100 V for 1 h at 4°C.

Immunoblotting was performed after blocking the membranes with 1% BSA–TTBS for 1 h at RT or overnight at 4°C. Blots were washed 3 times for 10 min in TTBS, rinsed in dd-H$_2$O, and inserted into a Miniblotter apparatus (Immunetics, Cambridge, MA). Individual salmon serum samples (70 U 70µl^{-1}) and positive control mAbs 4D3, 3H1, and 1A1 (3 µg ml^{-1}) in 1% BSA–TTBS were each added to each well of the apparatus and incubated for 1.5 to 2 h at RT with gentle shaking. After rinsing wells 3 times with TTBS, blots were removed and rinsed with TTBS 3 times for 10 min. After washing, blots were incubated for 1 h in 1-14 (5 µg ml^{-1}) in 1% BSA–TTBS and washed. They were then incubated with peroxidase-conjugated goat anti-mouse-Ig (Hyclone, Logan, UT, USA) diluted 1:5000 in 1% BSA–TTBS for 45 min at RT.

Densitometry. SDS-PAGE and electrotransfer of p57$^-$ or p57$^+$ extracts and molecular weight markers were performed as described above. The membranes were then stained for total protein with colloidal gold solution (Bio-Rad) using a procedure provided by the manufacturer. Blots were washed extensively, dried, and the total protein stained blots were scanned with a helium neon laser beam, Personal Densitometer SI, model PDSI-PC, and Image Quant data recorder (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis. Antibody titers were log-transformed and analyzed using a Student t-test program on the Statgraphics software package to determine significance of each group of mean sera sample values tested in an ELISA against both p57$^+$ and p57$^-$ cells. The reported values in units of antibody activity per µl of serum (U µl^{-1}) were back transformed. P-values exceeding 0.05 were not considered significant.

RESULTS

Quantitative and qualitative analysis of p57$^+$ and p57$^-$ *Renibacterium salmoninarum* cells

To visualize the effects of 10 h of heat treatment on *Renibacterium salmoninarum* surface proteins, p57$^+$ and p57$^-$ cell extracts were electrophoresed and stained for total protein (Fig. 3). There was a substantial reduction in the amount of the p57 molecule present and concomitantly there was an increase in the
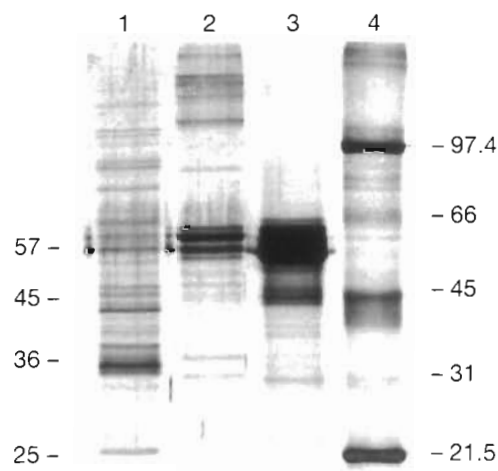

Fig. 3. *Renibacterium salmoninarum*. Analysis of p57 reduction on the cell surface by 10% SDS-PAGE and total protein staining. Total protein stain of 50 µg of *R. salmoninarum* ECP, whole cells (p57$^+$), and whole cells heat-treated at 37°C for 10 h (p57$^-$). Lanes: 1, formalin-killed p57$^-$ cells; 2, formalin-killed p57$^+$ cells; 3, ECP; 4, molecular weight standards in kilodaltons

p36 and other lower molecular weight breakdown products. Fig. 4 illustrates the densitometric profiles of each cell type before and after heat treatment. These profiles indicate that p57 comprised approximately 70% of all protein on p57⁺ cells and that only 14% of the p57 molecule remained after heat treatment.

Determination of antibody activity to p57⁺ and p57⁻ *Renibacterium salmoninarum*

The range of antibody titers present in individual salmon sera at 12 wk post primary injection varied from <45 U μl⁻¹ (undetectable) to 8571 U μl⁻¹ (Table 1). The mean titer against p57⁻ cells from fish immunized with p57⁻ cells was approximately 5-fold higher than the mean titer from fish immunized with p57⁺ cells. Sera from adjuvant injected fish (controls) had non-

detectable titers. At 17.5 wk post secondary injection, chinook immunized with p57⁻ cells had significantly higher antibody titers (p ≤ 0.01; Table 2) than fish immunized with native p57⁺ cells, when reacted with p57⁻ cells (25 704 U μl⁻¹ vs 1288 U μl⁻¹; average values). The mean titers of p57⁺ and p57⁻ immune sera were comparable when assessed against p57⁺ cells. All control fish demonstrated non-detectable titers, as did pre-bleeds from all the immunized fish.

Detection of primary immunogenic surface proteins on p57⁺ and p57⁻ *Renibacterium salmoninarum* whole cells by immunoblotting

Blots of extracts from p57⁺ (Fig. 5A) or p57⁻ cells (Fig. 5B) were probed with 17.5 wk, post secondary sera from 10 individual chinook immunized with p57⁺ cells and 5 chinook immunized with p57⁻ heat-treated cells to attempt identification of any differences in the proteins recognized on untreated, treated, and heat-treated cells. The only cell-associated proteins recognized by either p57⁺ or p57⁻ salmon antibodies were p57 and its breakdown products (ECP). Most of the antibody response to p57⁺ cells was directed against p57, while serum antibodies reacted predominantly with p36 on p57⁻ cells. Recognition patterns of the mAbs are representative of those that react with an amino terminal epitope (group I: mAb 4D3), an internal epitope (group II: mAb 3H1), or a carboxy-terminal epitope (group III: mAb 1A1) of the p57 molecule, as described by Wiens & Kaattari (1991).

Determination of p57⁻ antisera specificity by protein and carbohydrate disruption of p57⁺ and p57⁻ cells

To determine the extent of antibody recognition of protein versus carbohydrate components in the high-titered p57⁻ antisera, the surface protein of p57⁺ and p57⁻ cells was digested by proteinase-K or the surface carbohydrate degraded by periodate prior to reaction with serum from p57⁻ immunized fish (Table 3). Comparable coating concentrations of each digested
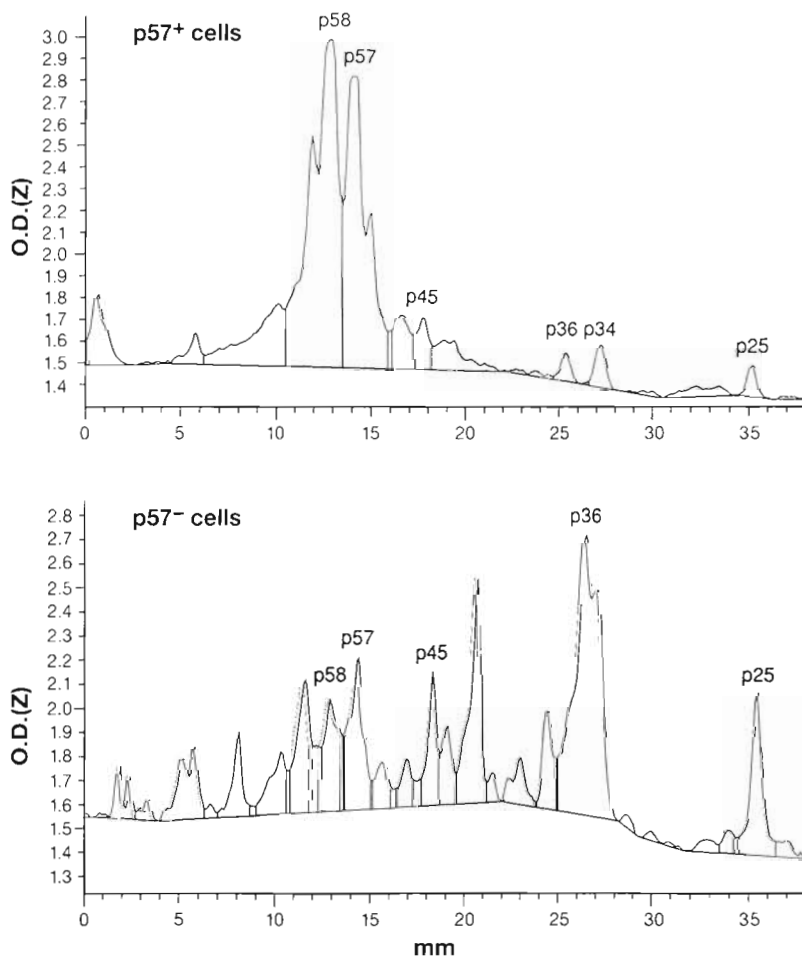

Fig. 4. *Renibacterium salmoninarum*. Densitometer scan of a total protein stained blot of p57⁺ and p57⁻ cells as detected by colloidal gold staining. Peaks are labeled with proteolytic breakdown products corresponding to their molecular weight in kilodaltons. The y-axis is labeled in intensity units signifying signal strength. The x-axis represents the distance scanned in millimeters

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Table 1. Serum antibody titers from chinook salmon immunized with p57⁺ or p57⁻ *Renibacterium salmoninarum* whole cells at 12 wk post primary injection. Each serum was tested on each cell preparation. Control fish were injected with PBS in adjuvant. Values denote non-detectable titers

Antibody activity as measured by ELISA (units μl^{-1})								
p57⁺ sera			p57⁻ sera			Control sera		
Fish	p57⁻ cells	p57⁺ cells	Fish cells	p57⁻ cells	p57⁺ cells	Fish cells	p57⁻	p57⁺
1	90	58	6	857	55	11	<70	<45
2	300	58	7	75	55	12	<70	<45
3	90	58	8	987	55	13	<70	<45
4	300	144	9	750	<45	14	<70	<45
5	225	88	10	8571	<45	15	<70	<45
Mean titer[a]	174	83	832*	51	<70	<45		

[a]Mean of the log-transformed data was back transformed
*Significantly different (p < 0.05) from all other titers

Table 2. Serum antibody titers from chinook salmon immunized with p57⁺ or p57⁻ *Renibacterium salmoninarum* whole cells at 17.5 wk post secondary injection. Fish were boosted 15 wk post primary injection as described in 'Methods' Control fish were injected with PBS in adjuvant. Values denote non-detectable titers. NE: not enough serum available to perform the assay

Antibody activity as measured by ELISA (units μl^{-1})								
p57⁺ sera			p57⁻ sera			Control sera		
Fish	p57⁻ cells	p57⁺ cells	Fish	p57⁻ cells	p57⁺ cells	Fish	p57⁻ cells	p57⁺ cells
20	122580	93000	30	70707	136	40	<70	<45
21	1520	1000	31	1077	95	41	<70	<45
22	95	80	32	77778	12667	42	<70	<45
23	10556	3500	33	8750	475	43	<70	<45
24	1056	1000	34	73684	1357	44	<70	<45
25	1650	987	35	1280	88	45	<70	<45
26	138	113	36	145450	6563	46	<70	<45
27	206	75	37	8889	636	47	<70	<45
28	917	5807	38	NE	NE	48	<70	<45
29	1375	487	39	800000	65625	49	<70	<45
Mean titer[a]	1288	955		25704*	1096		<70	<45

[a]Mean of the log-transformed data was back transformed
*Significantly different (p < 0.05) from all other titers

cell type (using the peroxidase saturation technique) revealed that an average of only 4% (10 000 U μl^{-1}) of p57⁻ reactivity remained after carbohydrate disruption. However, 85% (212 000 U μl^{-1}) of the reactivity remained after protein digestion. Further, periodate treatment of p57⁺ cells slightly enhanced the reactivity of the p57⁻ serum to p57⁺ cells. However, titers to untreated p57⁺ cells were approximately 180-fold lower than to p57⁻ cells (1404 U μl^{-1} vs 250 000 U μl^{-1}), indicating that this latter apparent increase was relatively marginal.

DISCUSSION

The nature of *Renibacterium salmoninarum* antigens and their relative immunogenicity in salmonids remains largely uncharacterized. This study utilized a novel method for removing a majority of the immunosuppressive, putative virulence factor, p57, from the surface of whole cells by heat-induction (37°C) of an endogenous serine protease (Rockey et al. 1991). The removal of p57 was associated with increased antigenicity and immunogenicity. Analysis by ELISA of salmon antiserum to p57⁻ *R. salmoninarum* revealed a significantly elevated (20-fold) specific antibody response.

A similar phenomenon has been observed upon immunization of carp with heat-killed *Aeromonas hydrophila* cells. This preparation induced a higher agglutinating antibody titer over a period of 32 wk than did formalin-killed cells (Lamers & Van Muiswinkel 1986). The authors speculated that heating may release more antigenic material without altering antigen structure or proper antigen processing by macrophages.

In an attempt to identify the nature of the antigens remaining on the p57⁻ cell, immunoblot analysis was performed with p57⁺ and p57⁻ antisera against p57⁺ and p57⁻ cells. Only 3 or 4 proteins were detected on either cell type by the salmon antisera, namely the p57 molecule and its proteolytic breakdown products. The identification of no new antigenic species suggests that the immunoblot method may not be able to capture these putative antigens. Wethered et al. (1988) examined human sera reactions to fungal proteins also by immunoblot procedures and found that they could only visualize a few proteins, even though the antibody titers were quite high. These authors suggested that the antibody response may be directed against carbohydrate antigens which are not detectable on an immunoblot. Such an explanation may apply to our immunoblot results. In fact, it can be seen that some individual sera demonstrated little or no reactivity when immunoblotted, even though the same concentration of antibody activity as determined by the ELISA (1 U μl^{-1} on whole cells) was used for each serum. This indicates that other
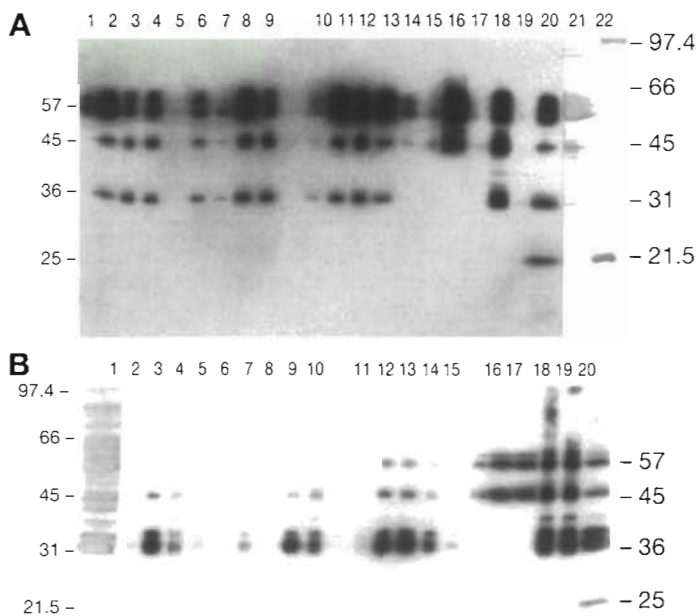
Fig. 5. *Renibacterium salmoninarum*. Immunoblot analysis with anti-p57⁺ and p57⁻ immune chinook serum at 17.5 weeks post secondary immunization. (A) p57⁺ whole cells or (B) p57⁻ whole cells were probed with serum against p57⁺ cells and p57⁻ cells from individual fish, and 3 different anti-p57 mAb controls. Anti p57⁺ sera: lanes A1 to 9 and B2 to 10; anti p57⁻ sera: lanes A10 to 14 and B11 to 15; mAb 4D3: lanes A16 and B16, 17; mAb 3H1 lanes A18 and B18, 19; mAb 1A1. lanes A20 and B20. Total protein stain of p57⁺ whole cells: lane A21; total protein stain of p57⁻ whole cells: lane B1; molecular weight markers: A22

antigens were present on the intact cell that could not be detected on the immunoblots. Also, recognition shifted in p57⁻-immunized fish from p57 to p36. This correlates with the decrease in p57 and the increase in p36 after treatment at 37°C as demonstrated by total protein staining (Fig. 3) and densitometry (Fig. 4).

Table 3. *Renibacterium salmoninarum*. Effects of enzymatic and chemical treatment of cells on anti-p57⁻ reactivity. Antibody titers were determined as described in 'Methods' Values represent the average antibody titers (units µl⁻¹) of antisera derived from 2 salmon immunized with p57⁻ cells and bled 17.5 wk post secondary immunization. Untreated, proteinase-K, and periodate-treated whole cells (both p57⁺ and p57⁻) were coated onto ELISA wells. Concentrations of each treated cell type were adjusted to ensure that there were equivalent amounts of bound cells in each well

	p57⁻ cells	p57⁺ cells
Untreated	250000	1404
Proteinase-K	212000	1123
Periodate	10000	6037

Carbohydrate disruption on p57⁻ cells by periodate resulted in a reduced ability of the cells to react with the p57⁻ sera (Table 3). Although cell-associated proteins did elicit an antibody response, periodate sensitive moieties, probably carbohydrate components, were responsible for most of the elevated serum antibody reactivity to p57⁻ cells. These results suggest the importance of *Renibacterium salmoninarum* carbohydrates (possibly polysaccharides) as immunogens and antigens.

It is not known why the reactivity of the relatively low-titered anti-p57⁺ sera increased after periodate treatment of *Renibacterium salmoninarum*. Perhaps, in situations where the anti-carbohydrate response is blocked, most of the antibodies are directed towards non-periodate or proteinase K-sensitive determinants, such as p57.

One explanation for the observed phenomena would be that p57 acts to occlude the interaction of antibodies or antigen receptors with immunogenic surface molecules. In this case, proteolytic modification by an endogenous serine protease may increase exposure of immunogenic carbohydrate epitopes masked by p57. There are reports of occlusion by different components of bacterial species, which include blocking of antigens by S layer proteins, concealment of cell wall and O antigens by capsular polysaccharide (King & Wilkison 1981, Kadurugamuwa et al. 1985, Williams et al. 1986, Johne et al. 1989), and shielding by the LPS O antigen (Mutharia & Hancock 1983, Jessop & Lambert 1985, Pollack et al. 1989) and outer core (Gigliotti & Shenep 1985, Nelson et al. 1990).

Dooley et al. (1985) report masking of the LPS core oligosaccharide-polysaccharide linkage by the S layer protein in the fish pathogen *Aeromonas hydrophila*. Sulfo-NHS-biotin cell surface labeling has been used to demonstrate occlusion of outer membrane (OM) antigens by the 52 kDa major surface array protein or S layer in *A. hydrophila*. The restricted access of this compound as shown by immunoblotting, demonstrated that the OM proteins from selected strains of *A. hydrophila* are shielded from immune recognition (Dooley & Trust 1988).

There are many ways in which a physical barrier on a bacterial surface may be disrupted, thereby unmasking surface antigens. Chemical degradation is known to partially remove O antigens on rough mutants, unmasking bacterial surface sites (Luderizt et al. 1966). There is also documentation that heat treatment (55°C) of Gram-negative bacterial cells destroys the integrity of the OM allowing better penetration of antibodies for reaction with core epitopes in O-polysaccharide⁻

mutants (Tsuchido et al. 1985). Further, removal of the *Escherichia coli* K antigen by heating or by enzyme treatment unmasks O antigens allowing agglutination by anti-O serum (Orskov et al. 1963, Stirm et al. 1971). In addition, heating is known to increase antibody binding to whole bacteria (Nelson et al. 1991).

Other effective abrogating mechanisms of specific humoral immunity are processes that prevent antibody synthesis. The soluble ECP of *Renibacterium salmoninarum* has been shown to inhibit antibody production in coho salmon B cells *in vitro* (Turaga et al. 1987b). In addition, heating purified extracellular and cell-associated ECP, which resulted in the degradation of p57 and other immunoreactive ECP components, caused a reduction of its immunosuppressive function (Rockey et al. 1991). Perhaps in this study, we are seeing the same effect as indicated by the increase in antibody production after p57 degradation by a proteolytic enzyme. In this study, perhaps p57 was inactivated after cleavage by the heat-activated serine protease.

Bacterial components from a number of mammalian and fish pathogens have been demonstrated to alter immune responsiveness of the animal host. Extracellular products of oral bacteria have been known to inhibit lymphocyte mitogenesis (Higerd et al. 1978). Hastings & Ellis (1988) speculated that the poor immunogenicity of the formalin-activated ECP protease of *Aeromonas salmonicida* in trout may be due to suppression of the antibody-producing system by the protease.

In conclusion, we have shown that there is an enhanced antibody response following modification of the whole bacterial cell using a novel approach. We reasoned that elimination of virulence factors from the bacterial immunogen might enhance immunogenicity. The elimination of the virulence factors was achieved by inducing endogenous proteolytic activity. The removal of the virulence factors significantly increased the magnitude of the antibody response to the injected whole cell antigen and enhanced its antigenicity (ability to react with antibody) as judged by the immunoassay results. Our findings are most consistent with the possibility that p57 may be occluding crucial immunogenic carbohydrate antigens.

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