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Elevated temperature treatment as a novel method for decreasing p57 on the cell surface of *Renibacterium salmoninarum*

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ABSTRACT: *Renibacterium salmoninarum* is a Gram-positive diplo-bacillus and the causative agent of bacterial kidney disease, a prevalent disease of salmonid fish. Virulent isolates of *R. salmoninarum* have a hydrophobic cell surface and express the 57–58 kDa protein (p57). Here we have investigated parameters which effect cell hydrophobicity and p57 degradation. Incubation of *R. salmoninarum* cells at 37°C for >4 h decreased cell surface hydrophobicity as measured by the salt aggregation assay, and decreased the amount of cell associated p57. Incubation of cells at lower temperatures (22, 17, 4 or −20°C) for up to 16 h did not reduce hydrophobicity or the amount of cell associated p57. Both the loss of cell surface hydrophobicity and the degradation of p57 were inhibited by pre-incubation with the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Cell surface hydrophobicity was specifically reconstituted by incubation with extracellular protein (ECP) concentrated from culture supernatant and was correlated with the reassociation of p57 onto the bacterial cell surface as determined by western blot and total protein stain analyses. The ability of p57 to reassociate suggests that the bacterial cell surface is not irreversibly modified by the 37°C treatment and that p57 contributes to the hydrophobic nature of *R. salmoninarum*. In summary, we describe parameters effecting the removal of the p57 virulence factor and suggest the utility of this modification for generating a whole cell vaccine against bacterial kidney disease.

KEY WORDS: *Renibacterium salmoninarum* · Bacterial kidney disease · p57

INTRODUCTION

Bacterial kidney disease (BKD) is a chronic and systemic disease that is often fatal for salmonids (Fryer & Sanders 1981). The causative agent, *Renibacterium salmoninarum* is a Gram-positive, non-motile, slow growing, fastidious, pathogen (Fryer & Sanders 1981, Bruno 1986, Evenden et al. 1993). BKD is considered one of the most difficult salmonid bacterial diseases to control (Elliott et al. 1989) and many aspects of the pathogenesis and causative agent are still poorly understood. *R. salmoninarum* is a facultative intracellular parasite that has the ability to survive and possibly multiply within phagocytic cells (Bruno 1986, Austin & Austin 1987, Bandin 1993, Gutenberger et al. 1997). The chronic course of disease and intracellular residence suggests that the organism may exploit a variety of pathogenic mechanisms causing the physiological and pathological changes associated with BKD. Research on the pathogenic mechanisms and the development of a vaccine have been hampered by the lengthy incubation times required for primary culture and experimental challenge of fish (1 to 4 mo) (Fryer & Sanders 1981, Toranzo & Barja 1993).

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A characteristic of virulent *Renibacterium salmoninarum* isolates is the tendency of bacterial cells to autoaggregate due to a hydrophobic cell surface (Daly & Stevenson 1987, Bruno 1988). Subsequent work suggested that less virulent isolates lack the saline extractable 57 kDa protein (Bruno 1990) also referred to as p57 (Wiens & Kaattari 1991). Previously we have found that *R. salmoninarum* produces large amounts of p57 during *in vitro* culture and *in vivo* infection (Turaga et al. 1987a, Wiens & Kaattari 1989, Rockey et al. 1991a). P57, in addition to being a secreted protein, is also a predominant cell surface component present on most isolates of *R. salmoninarum* (Getchell et al. 1985, Wiens & Kaattari 1989, Daly & Stevenson 1990, Bandin et al. 1992). A number of *in vitro* functions have been attributed to p57 and to the extracellular proteins (ECP) harvested from bacterial culture supernatant. We and others have previously demonstrated that incubation of ECP or p57 with salmonid lymphocytes causes *in vitro* immunosuppression of antibody production to non-related antigens TNP-LPS or human gammaglobulin (Turaga et al. 1987b, Rockey et al. 1991b, Fredriksen et al. 1997). In addition, ECP agglutinates salmonid leukocytes, and this activity can be blocked by preincubation with monoclonal antibodies that recognize the amino-terminal portion of p57 (Wiens & Kaattari 1991). P57 can also agglutinate rabbit and other mammalian erythrocytes (Daly & Stevenson 1987, 1990). These data support the hypothesis that p57 in ECP has biological activity that may be linked to virulence.

In addition to p57, ECP contains proteolytic activity that is especially active at elevated temperatures (Rockey et al. 1991b), contributing to the instability and degradation of p57 (Griffiths & Lynch 1991). Incubation of ECP at 37°C removes the majority of proteins present in ECP, including p57, as determined by SDS-PAGE and western blotting (Rockey et al. 1991b). The digestion of ECP also abrogates the immunosuppressive and leukoagglutinating activity of ECP (Rockey et al. 1991b, Wiens & Kaattari 1991). The proteolytic activity is inhibited by phenylmethylsulfonyl fluoride (PMSF), and was therefore suggested to be a serine protease (Rockey et al. 1991b). Using substrate gel elctrophoresis, a band containing protease activity was identified with an approximate relative molecular mass of 100 kDa.

Recently, we reported that a 10 h incubation of *Renibacterium salmoninarum* cells at 37°C reduced cell surface associated $p57^-$ (Wood & Kaattari 1996). Immunization of chinook salmon with the $p57^-$ cells produced a 20-fold increase in antibody titers to periodate sensitive epitopes (Wood & Kaattari 1996). Here we investigate the kinetics of p57 loss and alterations in cell surface hydrophobicity using the salt aggregation assay. We demonstrate that loss of cell surface p57 and hydrophobicity is inhibited by PMSF, suggesting that elevated temperature treatment effects serine protease activity. Cellular hydrophobicity and cell surface p57 were reconstituted by incubating 37°C treated bacterial cells in ECP, suggesting that the cell surface is not irreversibly modified by the 37°C temperature treatment. From these studies we determine conditions for producing $p57^-$ bacterial cells and, in a companion paper, suggest that cells devoid of this virulence factor may be an efficacious vaccine against BKD (Piganelli et al. 1998, in this issue).

MATERIALS AND METHODS

Preparation of bacterial cells and ECP. *Renibacterium salmoninarum* ATCC 33209 and isolate D6 (originally obtained from C. Banner, Oregon Department of Fish and Wildlife, Oregon State University, Corvallis, OR) were grown in 1 l volumes in 2.5 l low form, culture flasks (VWR) with continuous shaking at 17°C. The KDM-II medium within which they were grown was prepared according to Evelyn (1977) except without serum supplementation. Bacteria were grown for 7 to 8 d to an absorbance between 0.4 and 1.0 (460 nm). Cells were pelleted at $6000 \times g$ centrifugation for 30 min and resuspended in 100 ml cold phosphate buffered saline (PBS; 0.85% NaCl, 10 mM $NaPO_4$, pH 7.2). After a second centrifugation the cells were placed in microfuge tubes for the salt aggregation assay or stored at −20°C. ECP was extracted from culture supernatants as described by Wiens & Kaattari (1989).

Salt aggregation assay. PBS washed *Renibacterium salmoninarum* cells were pelleted for 2 min at 14 000 rpm ($18\,400 \times g$) in a microfuge (Beckman, model E). The wet weight of the cells was determined after complete removal of supernatant. Cells were resuspended to a final concentration of 50 mg ml^{-1} in 10 mM phosphate buffer without saline (pH 7.2). These cells were then used in the salt aggregation assay according to the method of Lindahl et al. (1981) as described by Daly & Stevenson (1987). Briefly, cells were diluted to yield an absorbance of 0.95 (460 nm) and 25 µl of the cell suspension was mixed with 25 µl of 2-fold dilutions of ammonium sulfate (from 2.0 to 0.004 M) in phosphate buffer that had been adjusted to pH 6.8 with 1 N ammonium hydroxide. Mixtures were made in 12-well depression micro slides (Clay Adams, NJ) that were then agitated for 5 min at 100 rpm (Junior Orbit shaker, Labline Inc.). Aggregation was determined by observation of bacterial cell clumping with a dissecting scope (10× magnification) using oblique lighting. All assays were performed at room temperature. Hydrophobicity is inversely related to the concentration of ammonium sulfate required to effect aggregation.

Effects of time and temperature treatments of *Renibacterium salmoninarum* cells. *R. salmoninarum* cells (450 µl of 50 mg ml^{-1} cell suspension in 10 mM phosphate buffer without salt) were incubated in microfuge tubes for 2, 4, 6, 8 or 16 h at either −20, 4, 17, 22 or 37°C. After incubation, cells were microfuged for 2 min, and an aliquot of the cells and supernatant was retained for electrophoretic analysis.

Addition of protease inhibitors and antibiotics to *Renibacterium salmoninarum* cells. The protease inhibitor phenylmethlysulfonyl fluoride (Sigma) was added at concentrations of 2 to 15 mM in 1% ethanol. A solution of 1% ethanol was also employed as a control. Antibiotics gentamycin, chloramphenicol, ampicillin, tetracycline or sodium azide were incubated with cells at a final concentration of 2.5 mg ml^{-1} at either 4 or 37°C. After 8 h the cells were microfuged for 2 min and resuspended in PBS.

Addition of ECP and herterologous proteins to 37°C *Renibacterium salmoninarum* cells. *R. salmoninarum* cells were incubated at 37°C for 12 h, washed in phosphate buffer without saline, and incubated with the following proteins: 4°C treated ECP, 37°C treated ECP, chicken ovalbumin, normal rainbow trout serum, or fetal bovine serum. All proteins were incubated with the 37°C treated *R. salmoninaurm* cells at 17°C for 1 h. The cells were then microfuged, resuspended in phosphate buffer, and subjected to the salt aggregation assay.

Polyacrylamide gel electrophoresis and western blotting. Polyacrylamide gel electrophoresis and western blotting were performed as previously described (Wiens & Kaattari 1989). Preparation of *Renibacterium salmoninarum* cells for electrophoresis was as described by Wiens & Kaattari (1991).

RESULTS

Elevated temperature decreased *Renibacterium salmoninarum* cell surface hydrophobicity and p57. Exposure of PBS washed *R. salmoninarum* cells to 37°C increased the concentration of ammonium sulfate required to cause aggregation from 0.004 to 2.0 M, indicating that the relative cell surface hydrophobicity was reduced by the elevated temperature incubation (Table 1). Loss of cell surface hydrophobicity appeared to be complete by 16 to 48 h (Table 1 and data not shown). Conversely, incubation of cells at −20, 4, 17 or 22°C for 16 h demonstrated no change in relative cell surface hydrophobicity, indicating that a temperature elevated above 22°C is critical for the cellular alteration. Examination of cell associated proteins separated by electrophoresis through 10% SDS-polyacrylamide gels (Fig. 1A) demonstrated that incubation at 37°C decreased cell associated p57 but that this was not apparent in the 17, 4, or −20°C treatments. Additional lower molecular weight proteins were observed to increase in concentration in 37°C treated cells consistent with degradation of p57 (Fig. 1A, lane 2). Some cell associated p57 appeared to remain after 16 h in agreement with our previous report that only 14% of the p57 molecule remains after a 10 h incubation at 37°C (Wood & Kaattari 1996). Removal of p57 was essentially complete by 48 h (data not shown). Interestingly, analysis of the supernatants revealed that a large amount of protein was released (Fig. 1B, lane 2) from 37°C treated cells but not from cells incubated at lower temperatures of 17, 4 or −20°C (Fig. 1B, lanes 3–5). Addition of gentamycin, choloramphenicol, ampicillin, tetracycline, or sodium azide had no effect on the loss of hydrophobicity or protein degradation profiles at 37°C, suggesting that protein synthesis or metabolism is not required (data not shown).

Table 1. *Renibacterium salmoninarum*. Minimum molar concentration of ammonium sulfate (M) required for detectable aggregation after incubation at the specified temperatures and times. Aggregation of cells was observed at 10× magnification

Time (h)	Temperature (°C)				
	37	22	17	4	−20
0.5	0.004	0.004	0.004	0.004	0.004
1.0	0.032	0.004	0.004	0.004	0.004
1.5	0.25	0.004	0.004	0.004	0.004
2.0	0.5	0.004	0.004	0.004	0.004
4.0	2.0	0.004	0.004	0.004	0.004
8.0	2.0	0.004	0.004	0.004	0.004
16.0	>2.0	0.004	0.004	0.004	0.004

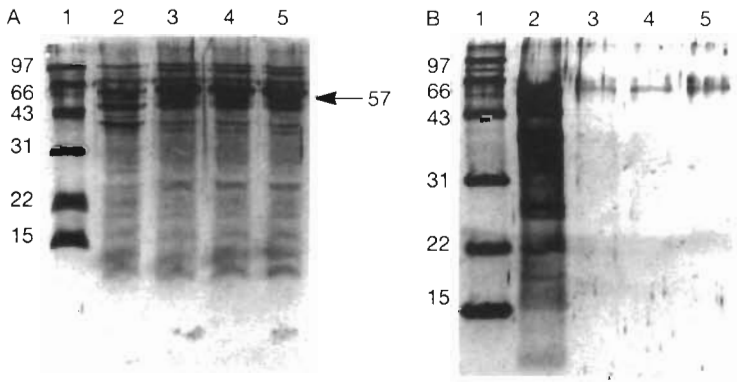

Fig. 1. *Renibacterium salmoninarum*. Total protein stains of (A) *R. salmoninarum* cells or (B) supernatants after incubation for 16 h at various temperatures. Lanes: molecular weight markers (1) and cells incubated at (2) 37°C, (3) 17°C, (4) 4°C, and (5) −20°C

Table 2. *Renibacterium salmoninarum*. Minimum ammonium sulfate concentration required to produce aggregation of cells after incubation with concentrations of PMSF at 37°C for 12 h. +: aggregation, –: lack of aggregation of cells (10× magnification)

$(NH_4)_2SO_4$ (M)	PMSF (mM)				
	0	2	5	10	15
0.004	–	–	–	+	+
0.032	–	–	–	+	+
0.25	–	–	+	+	+
0.5	–	–	+	+	+
2.0	+	+	+	+	+

Loss of *Renibacterium salmoninarum* cell surface hydrophobicity and p57 are prevented by incubation with the protease inhibitor PMSF. Incubation of 5 mM PMSF partially blocked the temperature-induced reduction of cellular hydrophobicity, while concentrations greater than 10 mM completely blocked loss of hydrophobicity (Table 2). These concentrations of PMSF were also able to block the degradation of cell associated p57 as determined by total protein stain and western blot (Fig. 2). Partial protection of p57 was observed using 2 mM PMSF (Fig. 2A, lane 4) while complete protection of p57 was seen using 10 and 15 mM PMSF (Fig 2A, lanes 6 and 7). The protection of p57 loss was more clearly observed by western blotting (Fig. 2B, lanes 5–8). Note that the 37°C treatment for 12 h removed all cell associated 4D3 immunoreactivity (Fig. 2B, lane 3). Interestingly, a small amount of higher molecular weight immunoreactive bands were more prevalent in preparations of cells containing PMSF, suggesting aggregation or multimerization of p57 in the presence of protease inhibitor. The presence of higher molecular weight forms of p57 was also enhanced by formalin treatment of bacterial cells (Fig. 3B, lane 3).

Relative cell surface hydrophobicity could be restored by the addition of 1.0 mg ml^{-1} ECP to 37°C treated cells (Table 3). The restoration of hydrophobicity correlated with the reassociation of p57 with the bacterial cell surface as observed by total protein stain (Fig. 3A, lane 4) and the identity confirmed by western blot (Fig. 3B, lane 5). While p57 was the predominant component that was visualized by total protein stain, we do not exclude the possible contribution of other unidentified components present in ECP. These data suggest that the proteolytic activity did not irreversibly modify the cell surface, at least with respect to p57 reassociation. Treatment of ECP at 37°C for 12 h inhibited this restorative function (Table 3), consistent with proteolytic degradation of p57 in ECP (data not shown). Restoration of hydrophobicity was specific to ECP because the heterologous proteins bovine serum albumin, hen egg albumin, and rainbow trout serum were unable to completely restore cell surface hydrophobicity (Table 3).

DISCUSSION

Cell surface hydrophobicity is an important bacterial property which facilitates phagocytic engulfment, as well as attachment to substrates (Finlay & Falkow 1989). A number of investigators have documented the hydrophobic nature of *Renibacterium salmoninarum* by the salt aggregation assay, adherence to hydrocarbons, and binding to nitrocellulose filters (Daly & Stevenson 1987, Bruno 1988, Bandin et al. 1989). *R. salmoninarum* isolates lacking a hydrophobic cell sur-

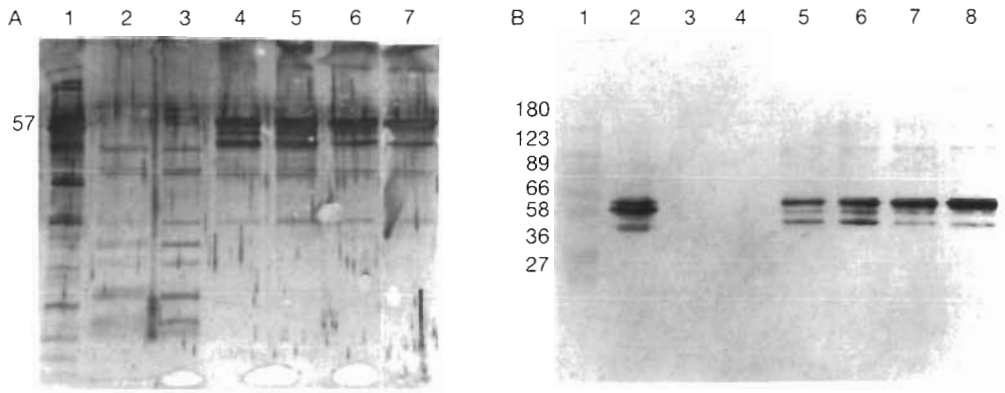

Fig. 2. *Renibacterium salmoninarum*. (A) Total protein stain and (B) western blot of cells incubated with or without PMSF at either 37 or 4°C for 12 h. Lanes in (A) *R. salmoninarum* cells incubated at (A) 4°C, (2) 37°C, (3) 37°C in 1% ethanol diluent, (4) 37°C in 2 mM PMSF, (5) 37°C in 5 mM PMSF, (6) 37°C in 10 mM PMSF, and (7) 37°C in 15 mM PMSF. Lanes in (B): molecular weight markers (1) and *R. salmoninarum* cells incubated at (2) 4°C, (3) 37°C, (4) 37°C in 1% ethanol control diluent, (5) 37°C in 2 mM PMSF, (6) 37°C in 5mM PMSF, (7) 37°C in 10 mM PMSF, and (8) 37°C in 15 mM PMSF. Western blot was probed with anti-p57 monoclonal antibody 4D3 (Wiens & Kaattari 1989)

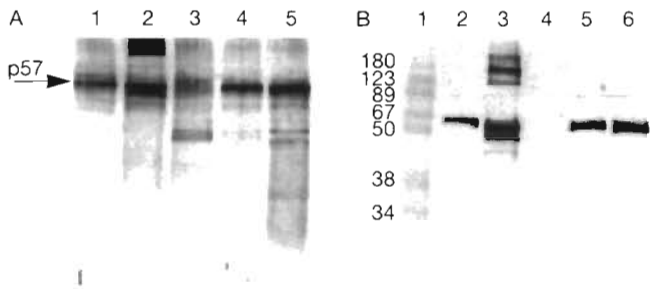

Fig. 3. *Renibacterium salmoninarum*. Reconstitution of p57 onto 37°C incubated cells. (A) Total protein stain and (B) western blot. Lanes in (A): (1) 4°C control, (2) cells incubated at 4°C followed by formalin fixation, (3) 37°C incubated cells, (4) 37°C incubated cells were washed with phosphate buffer without saline, and ECP was added back; (5) ECP alone. Lanes in (B): (1) molecular weight markers, (2) *R. salmoninarum* cells, 4°C control, (3) cells incubated at 4°C followed by formalin fixation, (4) 37°C incubated cells, (5) 37°C incubated cells were washed with phosphate buffer without saline, and ECP was added back, (6) ECP alone. Western blot was probed using the anti-p57 monoclonal antibody 4D3

Table 3. *Renibacterium salmoninarum*. Reconstitution of relative cell surface hydrophobicity to cells by ECP. *R. salmoninarum* cells were incubated at 4 or 37°C for 12 h, washed, and incubated with the concentrations of protein indicated. The minimum concentration of ammonium sulfate required to

Protein added	Temperature treatment of *R. salmoninarum* cells (°C)	Salt aggregation [$(NH_4)_2SO_4$, M]
Buffer alone[a]	4	0.062
Buffer alone	37	2.0
1.0 mg ml^{-1} ECP 4°C[b]	37	0.004
0.1 mg ml^{-1} ECP 4°C	37	1.0
0.01 mg ml^{-1} ECP 4°C	37	1.0
1.0 mg ml^{-1} ECP 37°C[c]	37	1.0
0.1 mg ml^{-1} ECP 37°C	37	1.0
0.01 mg ml^{-1} ECP 37°C	37	1.0
1.0 mg ml^{-1} OVA[d]	37	0.5
0.1 mg ml^{-1} OVA	37	1.0
0.01 mg ml^{-1} OVA	37	1.0
1.0 mg ml^{-1} BSA[e]	37	2.0
1/10 RBT serum 56°C[f]	37	2.0
1/10 FBS serum 56°C[g]	37	0.5

[a]Phosphate buffered saline as described in 'Materials and methods'
[b]*R. salmoninarum* ECP previously incubated at 4°C for 12 h
[c]*R. salmoninarum* ECP previously incubated at 37°C for 12 h
[d]Chicken ovalbumin (OVA)
[e]Bovine serum albumin (BSA)
[f]Rainbow trout serum (RBT) obtained from stock trout held at OSU diluted 1/10 in buffer and incubated at 56°C for 30 min to eliminate any complement activity
[g]Fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) diluted 1/10 in buffer and incubated at 56°C for 30 min to eliminate any complement activity

face appear to be less virulent (Bruno 1988) and are reported to lack the 57 kDa protein (Bruno 1990). Daly & Stevenson (1987) found that cell surface hydrophobicity was sensitive to trypsin or protease K treatment, suggesting the contribution of a proteinaceous component that was later identified to be the 57–58 kDa protein (Daly & Stevenson 1990). In this manuscript we demonstrate that the 37°C treatment modulates *R. salmoninarum* cellular hydrophobicity. Incubation at 37°C also reduced the amount of cell-associated p57 as determined by total protein stain and western blot. The loss of cell surface hydrophbicity is likely due to the loss of cell surface p57, as hydrophobicity and p57 could be specifically restored to the cell surface by incubating cells in ECP in agreement with the previous results of Daly & Stevenson (1990). In addition, the reconstituting factor was sensitive to a 12 h 37°C incubation, a condition that results in degradation of p57 in ECP (Rockey et al. 1991b). However, we cannot exclude the possibility that other unidentified components, which are temperature sensitive and are not detectable by 10% SDS-PAGE, may contribute to or be responsible for the restoration of hydrophobicity. Interestingly, the reconstitution of p57 to the cell surface suggests that the elevated temperature treatment does not irreversibly alter the cellular ultrastructure. However, whether cell surface alterations or conformational changes of the reassociated p57 have occurred requires further investigation.

We demonstrate that subjecting *Renibacterium salmoninarum* cells to heat treatment (37°C) resulted in a significant decrease by 16 h, and essentially complete digestion by 48 h, of cell associated p57 as determined by western blot and total protein stain. These findings are consistent with our previous publication in which we reported that by 10 h most cell associated p57 was degraded (Wood & Kaattari 1996). The loss of cell associated p57 correlated with endogenous serine protease activity, as proteolysis could be partially blocked with the addition of 2 mM PMSF and totally inhibited at 15 mM PMSF. The characteristics of the proteolytic activity are similar to serine protease activity present in culture supernatant that we and others have previously described, as its activity is increased at 37°C and inhibited by PMSF and p57 is a substrate (Griffiths & Lynch 1991, Rockey et al. 1991b). We have previously demonstrated that proteolytic activity migrated at ~100 kDa, consistent with Barton et al.'s (1997) recent observations. However, they note that not all isolates produce the same high molecular weight proteolytic activity and further suggest that p57 or fragments of p57 may be responsible for the observed serine protease activity (Barton et al. 1997). It is important to note that *R. salmoninarum* isolates may vary in cell-associated proteolytic activity and that our studies

have been restricted to the ATCC 33209 and D6 isolates of *R. salmoninarum*.

At present the *in vivo* function of the secreted and cell-associated proteolytic activity is unclear, as the temperature of highest activity, 37°C, is not physiologically relevant for either *Renibacterium salmoninarum* or the salmonid host. It is important to note that we have investigated a relatively restricted set of temperatures and that detailed characterization of the effects of temperature on proteolytic activity and hydrophobicity awaits further investigation. It is possible that a low level of activity is present at physiological temperatures (below 20°C) and that this activity may function to modulate or regulate levels of p57 during infection. We hypothesize that proteolytic modulation of cell surface p57 may change adherence or invasive properties or may alter the antigenic characteristics recognized by the salmonid immune system. In support of this latter possibility we have recently demonstrated that immunization of chinook salmon with 37°C treated cells resulted in a 20-fold increased antibody titer as compared to the titer of fish immunized with *R. salmoninarum* cells containing p57 (Wood & Kaattari 1996). Clearly, further purification of serine protease activity and characterization of antigens unmasked or created during 37°C incubation are required for further elucidation of the *in vivo* significance of p57 modulation. In an accompanying report we describe the heat treatment of *R. salmoninarum* cells as a novel vaccine preparation and the incorporation of treated cells into a cellular vaccine in pH protected, enteric-coated antigen microspheres (Piganelli et al. 1998).

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