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NOTE

Production of the major soluble antigen of *Renibacterium salmoninarum* in *Escherichia coli* K12

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ABSTRACT: A DNA fragment containing all but the first 90 base pairs of gene *msa* encoding p57, the major soluble antigen of *Renibacterium salmoninarum*, was cloned in the plasmid vector pUC18 and subsequently a soluble fusion protein was produced using the pMAL expression vector system. The fusion protein retained major epitopes shared with the native p57 molecule and provided a source of protein suitable for further immunological analysis which was independent of *in vitro* cultures of this slow growing organism. Antiserum raised against the purified fusion protein was used to probe Western blots of cell extracts and extracellular products derived from *R. salmoninarum* cultured *in vitro*. The results show that under conditions of iron-restriction, both the production and processing of p57 are reduced.

KEY WORDS: *Renibacterium salmoninarum* · Bacterial kidney disease · BKD · Major soluble antigen

Bacterial kidney disease (BKD) is a chronic, granulomatous disease affecting farmed and wild salmonid fish worldwide (Evelyn 1993). The causative agent of BKD, *Renibacterium salmoninarum*, is a Gram-positive, slow growing, fastidious, intracellular pathogen which is difficult to culture *in vitro*. Cultures of the bacterium may take at least 6 to 8 wk and are prone to contamination. This is probably the major reason why little is known about the virulence or pathogenicity of the organism (Evenden et al. 1993).

Considerable attention has been devoted to the major soluble antigen, the predominant cell surface and extracellular protein (ECP) which has been associated with hydrophobicity and virulence (Bruno 1988, 1990). This protein, also referred to as hemagglutinin (Daly & Stevenson 1987) and p57 (Weins & Kaattari 1991), has an apparent molecular mass of 57 kilodaltons (kDa) and is produced in considerable quantities by *Renibacterium salmoninarum* within the tissues and sera of infected fish (Turaga et al. 1987). Certain characteristics of p57, such as hydrophobic and leukoagglutinating properties (Weins & Kaattari 1991) and acidic isoelectric point (pI 4.5 to 4.8) are reminiscent of other bacterial adhesins (Dubreuil et al. 1990). In addition, p57 possesses potent immunosuppressive properties which are rapidly lost with the proteolytic degradation of the protein (Rockey et al. 1991). Recent studies have demonstrated that the proteolytic degradation of p57 generates most, if not all, of the immunoreactive components of *R. salmoninarum* ECP and p57 may possess an autoproteolytic activity (Griffiths & Lynch 1991). The N-terminal sequence of p57 has been determined and gene *msa* encoding this protein has now been cloned and sequenced (Chien et al. 1992). The aim of this study was to generate a source of recombinant p57 protein in order to remove the time-consuming dependence upon *in vitro* cultures of *R. salmoninarum*. In addition, antibodies raised against the recombinant protein were used to probe Western blots of cell extracts and ECP from *R. salmoninarum* cultured *in vitro* and under iron-restricted conditions.

Materials and methods. *Escherichia coli* strains JM109 and XL1-Blue were used as host strains for plasmid vectors pUC18, pMAL-c and pMAL-p (New England Biolabs) and were cultured in Luria-Bertani medium at either 22 or 37°C. When appropriate, 100 µg ml^{-1} of ampicillin and 0.2% glucose were added to the medium. Isolates of *Renibacterium salmoninarum* including MT444 and the type strain (ATCC 33209) were cultured in Mueller-Hinton medium supplemented with 0.1% L-cysteine hydrochloride (MHCB) at 15°C and the availability of iron was restricted in cultures of *R. salmoninarum* by the addition of ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDHA) as previously described (Grayson et al. 1995).

From the sequence available for *Renibacterium salmoninarum* gene *msa* (Chien et al. 1992) 2 restriction

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sites close to the initiation codon were identified as suitable for the construction of translational fusions using the pMAL system for the production of maltose-binding protein (MBP) fusions (New England BioLabs): *Hpa*I located at nucleotide 180 and *Stu*I located at nucleotide 228. The *Hpa*I truncated gene would encode a product of 513 amino acids and 49795 molecular weight, whilst the *Stu*I truncated gene would encode 497 amino acids of 47613 molecular weight. Both of these truncated proteins would include the major epitopes of p57 (Chien et al. 1992). It was decided to directionally clone either a 1.88 kb *Hpa*I/*Sal*I fragment or a 1.65 kb *Stu*I/*Xba*I fragment which had been excised from pMLG57 and ligated into the polylinker in order to enable the production of almost full length p57 fused to the carboxy-terminus of MBP (molecular mass 42.7 kDa).

Cultures of *Escherichia coli* XL1-Blue containing fusion plasmids were induced with IPTG at either 22 or 37°C and samples were removed over a time course for analysis. All of the following steps were carried out either on ice or at 4°C to minimise protein degradation. For affinity purification, at 2 h post-induction, a sample was harvested and resuspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM NaN_3, 1 mM PMSF; pH 7.4). The sample was freeze-thawed, sonicated, centrifuged and the supernatant (crude extract) removed and retained. The pellet was resuspended in column buffer and retained as a suspension of insoluble material. An aliquot of amylose resin was washed with column buffer and mixed with an equal volume of crude extract. The resin pellets were washed twice with column buffer, then resuspended in SDS-PAGE buffer. For the production of larger quantities of MBP-p57 fusion protein cultures of *E. coli* XL1-Blue containing the fusion plasmid were grown at 37°C and cells were induced with IPTG for 3 h at 37°C. Affinity purified MBP-p57 fusion protein was obtained by using an amylose resin column. The diluted crude extract containing MBP-p57 fusion protein stored on ice was loaded onto the column and subsequently, the column was washed free of all protein other than the specifically bound MBP fusion protein. MBP-p57 was eluted with column buffer containing 10 mM maltose. Protein concentrations were estimated (Bradford 1976) and yields of up to 50 µg ml^{-1} of culture were obtained.

Wistar rats were immunized subcutaneously with 100 µg of affinity-purified MBP-p57 fusion protein emulsified in Freund's complete adjuvant (FCA), and then boosted 3 wk later with 100 µg of protein emulsified in Freund's incomplete adjuvant (FIA). Control sera were gathered prior to immunisation. Rabbit anti-MBP antiserum was obtained from New England BioLabs Ltd. Immunoblot analyses of clones producing either p57 or MBP-p57 fusion protein and of *Renibacterium salmoninarum* cell lysates and culture supernatants were performed using the electrophoretic transfer procedure (Towbin et al. 1979) following SDS-PAGE in the presence of 5% β-mercaptoethanol (Laemmli 1970). Blots were probed with primary antibodies and specific binding was detected by immunoperoxidase staining of horseradish peroxidase-labelled swine anti-rabbit immunoglobulins or rabbit anti-rat IgG (Dako). The preparation of *R. salmoninarum* cell lysates and extracellular products which were used in immunoblot analyses has been described (Grayson et al. 1995).

Results and discussion. Using *Renibacterium salmoninarum* strain MT444 DNA a genomic library was constructed in the *Eco*RI site of pUC18, using *Escherichia coli* host strain JM109. Clones were picked from master plates from which duplicate plates had been immunologically screened with rabbit antisera raised against *R. salmoninarum* ECP using nitrocellulose filter discs. The phenotype was checked by probing Western blots with rabbit anti-*R. salmoninarum* ECP (Fig. 1) which demonstrated the presence of several strongly immunoreactive bands of molecular masses from about 20 to 58 kDa. The plasmid isolated from this clone was designated pMLG57 (Fig. 2). Further characterisation including restriction mapping and DNA sequencing confirmed that pMLG57 contained a 1.9 kilobase (kb) *Eco*R1 fragment of *R. salmoninarum* DNA which possessed all but the first 90 base pairs (bp) (30 amino acids) of gene *msa* fused in-frame with and under the control of the *lac* promoter.

Recombinant plasmids, designated either pMC57HS or pMP57SX, were isolated which contained the *msa* gene inserted in-frame with *malE* as either a 1.88 kb *Hpa*I/*Sal*I fragment or a 1.65 kb *Stu*I/*Xba*I fragment in either the pMAL-c (6145 bp) or pMAL-p (6220 bp) vectors, respectively (Fig. 3). The production of MBP-p57, either within the cytoplasm (pMC57HS) or following export to the periplasmic space (pMP57SX) of *Escherichia coli* XL1-Blue, was accompanied by considerable

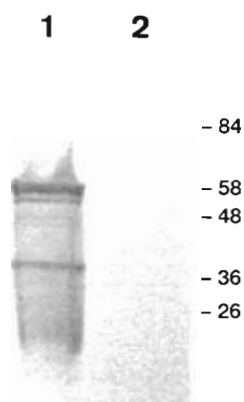

Fig. 1 Western blot of immunopositive clone isolated from a pUC18 *Eco*R1 gene bank using host strain *Escherichia coli* JM109 probed with rabbit anti-*Renibacterium salmoninarum* ECP. Track 1: *E. coli* expressing pMLG57; track 2: *E. coli* containing pUC18. The positions of protein molecular weight markers are indicated in kDa

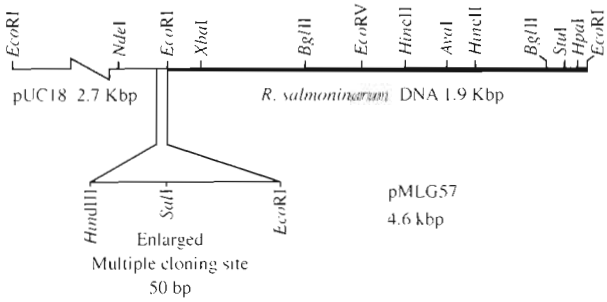

Fig. 2. Restriction map of pMLG57 containing the fragment of *Renibacterium salmoninarum* DNA from which the portion of gene *msa* to be fused was excised

degradation of the fusion protein regardless of the growth temperature and despite the care taken to carry out the purification of the fusion proteins as quickly as possible and at 4°C. Only a small proportion of each of the fusion proteins was found to possess the expected molecular weights of 90 313 (pMP57SX) or 92 495 (pMP57HS). One possible explanation for this is that the

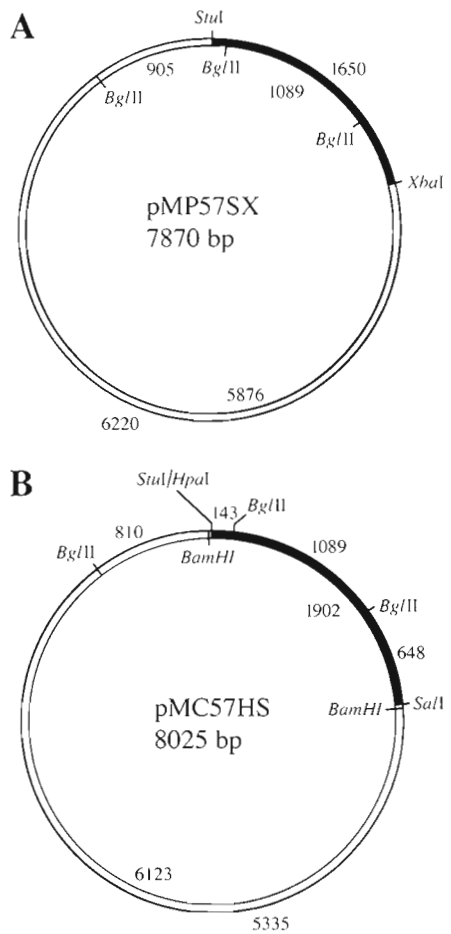

Fig. 3. Physical maps deduced for (A) pMP57SX and (B) pMC57HS. Numbers represent base pairs between neighboring restriction sites

MBP-p57 fusion protein undergoes some form of autoproteolytic processing. Studies of native p57 derived from *in vitro* cultures of *Renibacterium salmoninarum* have shown that the p57 protein does possess an inherent instability (Griffiths & Lynch 1991) although this has been associated with the presence of a serine protease of molecular mass about 105 kDa by other workers (Rockey et al. 1991). Further examination of the amino acid sequence for p57 which was derived from *msa* (Chien et al. 1992) revealed the presence of a consensus serine protease motif, G-X-S-X-G, located from Gly-406 to Gly-410 (GTSIG) with Asp-323 and His-279 completing the active site (Aitken 1990). Nevertheless, the MBP-p57 fusion protein still contained major epitopes which were recognised on Western blots by rabbit anti-*R. salmoninarum* ECP (data not shown) and the soluble fusion protein was therefore considered to be of use for further immunological study. In order to obtain a maximum yield, MBP-p57 fusion protein was purified from the cytoplasm of *E. coli* XL1-Blue containing pMC57HS by the one-step affinity chromatography procedure. In this case, p57 was fused to MBP starting from Asn-45 which is 18 amino acids downstream from the N-terminal serine that is exposed following cleavage of the signal peptide (Chien et al. 1992).

Western blots showed that specific antibodies raised in rat identified the affinity purified MBP-p57 as a full length fusion protein of estimated molecular mass 90 kDa with the largest proportion of the protein degraded to 55 000 or less (Fig. 4). Western blots of *Renibacterium salmoninarum* cell extracts and ECP which were probed with antiserum to MBP-p57 recognised a series of bands which correspond to the profile of breakdown products of native p57 that have been described by previous researchers (Griffiths & Lynch 1991, Rockey et al. 1991). *R. salmoninarum* does not utilize starch or maltose and Western blots of cell extracts or ECPs which had been probed with rabbit anti-MBP showed no evi-

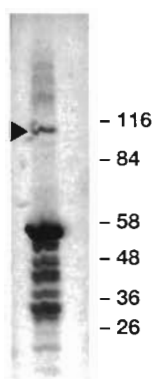

Fig. 4. Immunological detection of affinity purified MBP-p57 fusion protein by Western blot probed with rat anti-MBP-p57. The positions of protein molecular weight standards are marked in kDa. The position of the full length fusion protein is arrowed. The remaining bands represent breakdown products of MBP-p57

dence for the recognition of any cross reactive epitopes. Major epitopes were present in all strains of *R. salmoninarum* of molecular masses about 58, 45 and, to a lesser extent, 35 kDa in sonicated *R. salmoninarum* cell extracts regardless of the availability of iron (Fig. 5A, B). The ECPs derived from iron-sufficient cultures possessed major bands which were recognised by the antiserum at 25 kDa and only strains ATCC 33209 and 910019 were exceptions with a band located at about 57 kDa (Fig. 5C, D). However, ECPs derived from iron-restricted cultures showed a dramatic reduction in the degradation and production of the major epitopes located at 57 kDa, particularly in strains MT417, MT420 and MT452. Current understanding of the molecular genetics of *R. salmoninarum* is insufficient to account for this finding but it is possible that iron is involved either directly or indirectly in the regulation of the processing of p57. Interestingly, epitopes of MBP-p57 were detected in *R. salmoninarum* strains MT414 and MT417 which have previously been shown to be non-autoagglutinating and non-hydrophobic and were described as lacking a 57 kDa saline extractable cell-associated protein that has been associated with virulence (Bruno 1988, 1990). Nevertheless, this study has shown that these strains possess epitopes, both in cell extracts and ECPs, which are recognised by MBP-p57 antiserum. This finding raises the possibility that the hydrophobicity of *R. salmoninarum* may be related to aspects of the cell surface other than simply the presence of p57. Indeed, computer analysis of the published amino acid sequence (Chien et al. 1992) using DNAStar software predicts p57 to be a predominantly hydrophilic molecule (data not shown).

The availability of the cloned *msa* sequence which encodes a product that possesses the major epitopes of p57 will greatly simplify any future work, such as site-directed mutagenesis, aimed at characterising the precise role of individual amino acid residues in the biochemical and immunological properties of the protein.

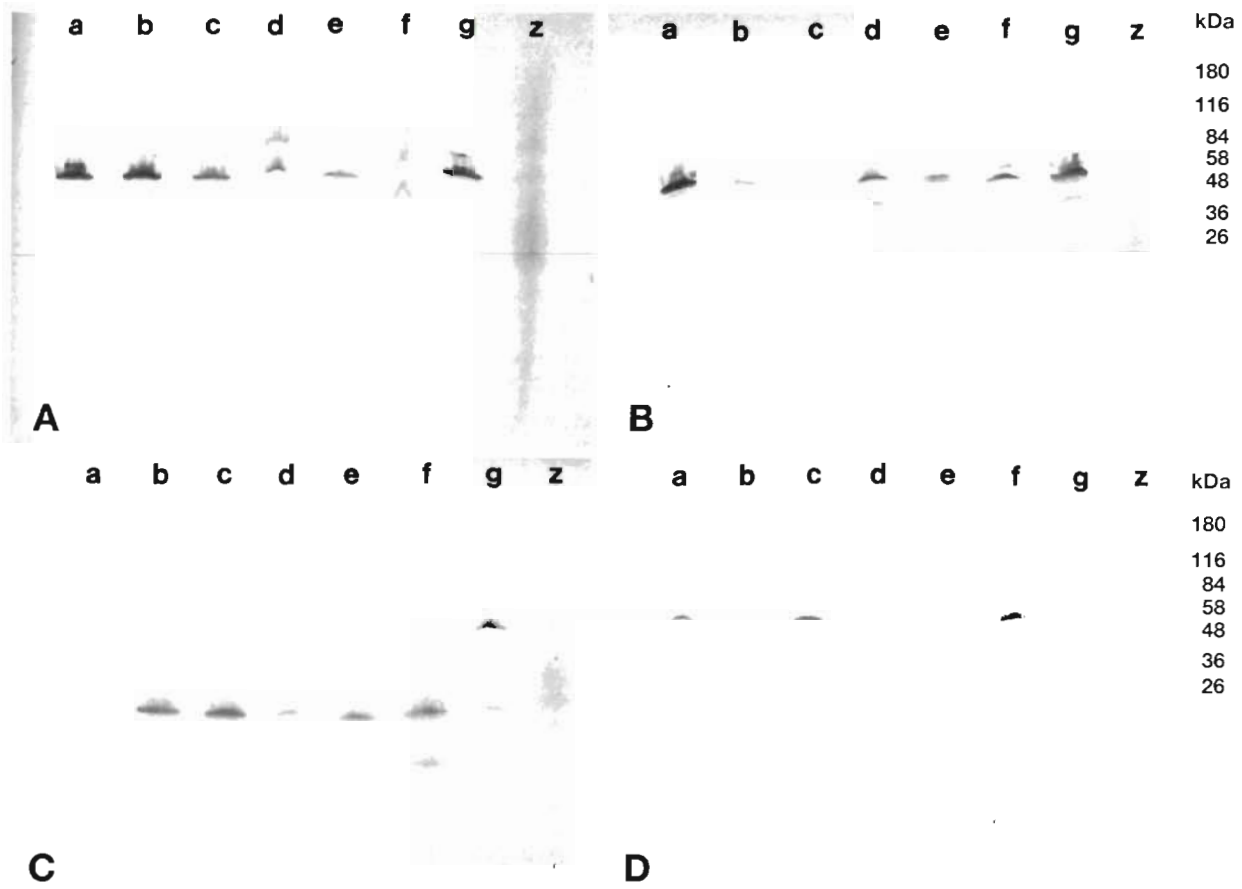

Fig. 5. The immunological detection of epitopes of affinity purified MBP-P57 in cell extracts and ECPs of 7 *Renibacterium salmoninarum* strains which had been cultured under conditions of either iron restriction or iron sufficiency. Western blots were probed with rat anti-MBP-p57. (A) and (B) are whole cell extracts of cultures grown in either MHCB or MHCB supplemented with 200 µM EDDHA, respectively. (C) and (D) are ECPs derived from cultures grown in either MHCB or MHCB supplemented with 200 µM EDDHA, respectively. Tracks: (a) strain 910019; (b) strain MT452; (c) strain MT425; (d) strain MT420; (e) strain MT417; (f) strain MT414; (g) strain ATCC 33209. The positions of protein molecular weight standards are indicated in kDa

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