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NOTE

Production of a *Renibacterium salmoninarum* hemolysin fusion protein in *Escherichia coli* K12

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ABSTRACT: Gene *rsh* encoding a novel hemolysin of *Renibacterium salmoninarum* was fused to the *lacZ* gene of the pAX5+ plasmid vector to facilitate production of a 160 kDa β-galactosidase fusion protein. The soluble fusion protein was produced cytoplasmically in *Escherichia coli* XL1-Blue and purified using affinity chromatography. The fusion protein retained epitopes which were identified in components present in *in vitro* cultures of *R. salmoninarum*. Western blots of *R. salmoninarum* cell extracts which were probed with an antiserum raised against the affinity purified fusion protein predominantly recognized bands of apparent molecular weights 82 and 78 kDa as well as a number of other bands which may represent breakdown products of the native protein. No components were detected in extracellular products and iron-restricted culture conditions did not obviously affect the production of these components.

KEY WORDS: *Renibacterium salmoninarum* · Bacterial kidney disease · BKD · Hemolysin · Fusion protein

Renibacterium salmoninarum is an obligate pathogen of salmonid fish species worldwide (Evelyn 1993). This Gram-positive, intracellular bacterium is the etiologic agent of a chronic infection typified by granulomatous lesions within the host's kidney and referred to as bacterial kidney disease (BKD). BKD has proved to be a particularly difficult disease to control using either antibiotics or immunoprophylaxis. In addition, the bacterium is very slow growing, having a generation time of 24 to 48 h, and many studies have been constrained by the lack of defined or minimal media. Consequently, little is known of the biochemistry, pathogenicity or virulence mechanisms of *R. salmoninarum* (Evenden et al. 1993).

The application of molecular methods to studies of many other bacterial pathogens has led to outstanding advances in our understanding of pathogenicity and virulence mechanisms (Findlay 1993). Recently, some of these methods have been applied to the study of *Renibacterium salmoninarum*, and the genes encoding the major soluble antigen (*msa*) and a previously undescribed metalloprotease (*hly*) have been cloned and sequenced (Chien et al. 1992, Grayson et al. 1995a). Furthermore, Evenden et al. (1990) reported the cloning and expression of a gene from *R. salmoninarum* which encoded a hemolytic product that was not detected on Western blots probed with antisera raised against *R. salmoninarum* products derived from *in vitro* cultures of the bacterium. The gene, designated *rsh*, has subsequently been sequenced and found to encode a novel protein of unknown function (Evenden 1993).

The purpose of this study was to provide a source of this hemolytic protein which could be used for further research and which was not dependent upon time-consuming and contamination-prone cultures of *Renibacterium salmoninarum*. A β-galactosidase fusion protein was produced in *Escherichia coli* and purified by affinity chromatography. Antiserum was raised against the purified fusion protein and used to probe Western blots of *R. salmoninarum* cell extracts and extracellular products (ECP). Because many pathogens produce components which are vital to pathogenesis when iron is no longer freely available (Bullen & Griffiths 1987), cultures of *R. salmoninarum* were grown under iron-restricted and iron-sufficient conditions. Epitopes of the fusion protein were present in cell extracts but not ECP of 7 strains of *R. salmoninarum*, and restricting the availability of iron *in vitro* did not affect the expression of gene *rsh*.

Materials and methods. *Escherichia coli* XL1-Blue was used as host strain and was cultured in Luria-Bertani medium at 37°C. When appropriate, 100 μg ml^{-1} of ampicillin, 15 μg ml^{-1} of tetracycline and 0.2% v/v glucose were added to the medium. Plas-

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mid expression vector pAX5+ (6.201 kbp) was used for the construction of the *lacZ-rsh* gene fusion (Markmeyer et al. 1990). Isolates of *Renibacterium salmoninarum* including the type strain (ATCC 33209) were cultured for 3 wk in Mueller-Hinton medium supplemented with 0.1% L-cysteine hydrochloride (MHCB) at 15°C. The availability of iron was restricted in cultures of *R. salmoninarum* by the addition of 200 µM ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDHA) as previously described (Grayson et al. 1995b).

The cloning and sequencing of gene *rsh* encoding a hemolysin has been reported (Evenden 1993). On the basis of this information a translational fusion was constructed by ligating a 1.52 kbp *Eco*47III/*Sal*I fragment to the *Nru*I/*Sal*I sites of the pAX5+ polylinker (Fig. 1). The production of the soluble fusion protein in *Escherichia coli* was optimised over a time course following induction of the *lacZ* promoter with isopropyl β-D-thiogalactopyranoside (IPTG). All the following steps were carried out either on ice or at 4°C to minimise protein degradation. At 3 h post-induction, a sample of cells was removed, resuspended in column buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 M NaCl, 10 mM β-mercaptoethanol, 1 mM NaN_3, 1 mM PMSF, pH 7.4), frozen overnight at –20°C, thawed in cold water and sonicated. The samples were centrifuged and the supernatant (crude extract) removed and retained. Samples of uninduced cells, induced cells (1, 2, 3 and 4 h post-induction), crude extract, insoluble material and soluble material bound by the affinity resin were all analyzed by SDS-PAGE and Western blotting for the presence of fusion protein. Pure β-galactosidase fusion protein was isolated using a column of *p*-aminophenyl β-D-thiogalactopyranoside (APTG) agarose resin. The crude extract, isolated as described above, was retained on ice and diluted 1:5 with column buffer. The pellet of insoluble matter was resuspended in a minimum volume of column buffer, sonicated, and pelleted as before and this supernatant was added to the first crude extract. The affinity resin was washed with column buffer and the diluted crude extract containing the β-galactosidase fusion protein was loaded onto the column and subsequently, the column was washed free of all protein other than the specifically bound fusion protein. The fusion protein was eluted with 0.1 M boric acid, pH 10.0, and fractions were pooled and mixed with an equal volume of 1 M Tris-HCl pH 7.0 in order to adjust the eluate to neutral pH.

Wistar rats were injected subcutaneously with 100 µg of the fusion protein emulsified in Freund's complete adjuvant (FCA), boosted 3 wk later with 100 µg of fusion protein emulsified in Freund's incomplete adjuvant (FIA), and then bled 3 wk after the last injection. Control sera were gathered prior to immunisation. Following SDS-PAGE (Laemmli 1970), bacterial proteins were either stained for protein using Coomassie Brilliant Blue R250 or transferred electrophoretically onto nitrocellulose (Towbin et al. 1979) and probed with either the IgG fraction of mouse anti-β-galactosidase antiserum (Sigma) at 1:1000 dilution followed by goat anti-mouse IgG peroxidase conjugate at 1:2000 (Dako) or rat anti-β-galactosidase fusion protein (1:200) followed by rabbit anti-rat IgG peroxidase conjugate at 1:1000 (Dako). Specific binding was visualised by immunoperoxidase staining.

Results and discussion. A recombinant plasmid, designated pGHLY, containing an in-frame fusion between *lacZ* and *rsh* was constructed using the pAX5+ expression vector (Fig. 2). Preliminary experi-

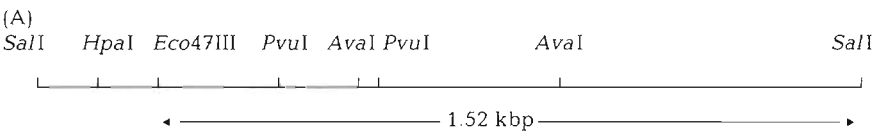

Fig. 1. Cloning strategy for the construction of a fusion of the *lacZ* gene with gene *rsh*. (A) Restriction map of the 1.67 kbp *Sal*I fragment of *Renibacterium salmoninarum* DNA from which the 1.52 kbp *Eco*47III/*Sal*I fragment of gene *rsh* to be fused was excised. The initiation codon of gene *rsh* is located 39 nucleotides to the right of the *Hpa*I site (Evenden 1993). (B) Restriction map of the multiple cloning site of pAX5+. The factor Xa cleavage site is shown. (C) Structure of the *lacZ-rsh* fusion in pAX5+

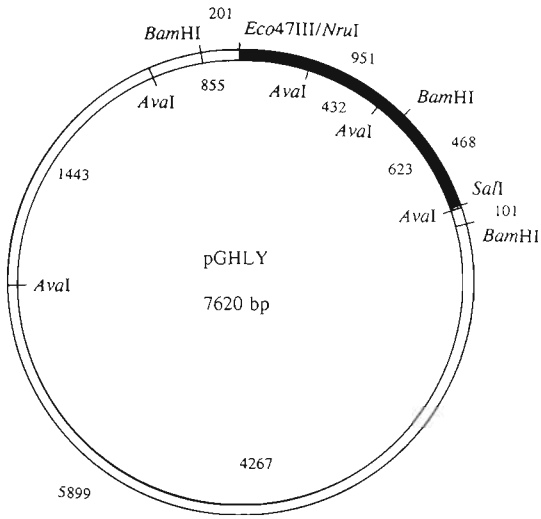

Fig. 2. Physical map deduced for pGHLY. Numbers represent base pairs between neighbouring restriction sites

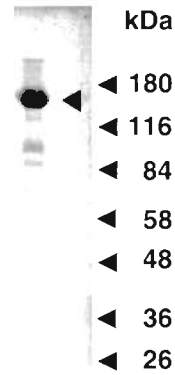

Fig. 3. Immunological detection of purified β-galactosidase-RSH fusion protein by Western blot probed with rat anti-β-galactosidase-RSH. The positions of protein molecular weight standards are marked in kilodaltons. The position of the full length fusion protein is arrowed. The remaining bands represent breakdown products of the full length molecule

ments showed that the β-galactosidase-RSH fusion protein was expressed at a high level and mainly in a soluble form. The β-galactosidase-RSH protein was purified from the cytoplasm of *Escherichia coli* XL1-Blue by using a 1-step affinity chromatography method. Degradation of the β-galactosidase-RSH protein was minimised by ensuring that samples, glassware, buffers and the affinity column were kept cold throughout. The purified β-galactosidase-RSH protein migrated as a single band on reduced SDS-PAGE with a calculated molecular weight of 160 000, which was close to the predicted size of 159 500 (Fig. 3). Gene *rsh* encodes a protein consisting of 416 amino acids with a molecular mass of 47 600 daltons (Evenden 1993). The construction of the gene fusion would yield a truncated gene product of 381 amino acids and 43 442 molecular weight fused to the β-galactosidase protein. RSH was fused to β-galactosidase starting from Leu-36 which is close to the carboxy terminal end of the putative membrane-spanning portion of RSH located at Ala-37 (Evenden 1993).

Seven strains of *Renibacterium salmoninarum* which have been shown to differ in their virulence by previous research (Bruno 1988, 1990) were probed with antibodies raised against the affinity purified β-galactosidase-RSH fusion protein. Cell extracts or

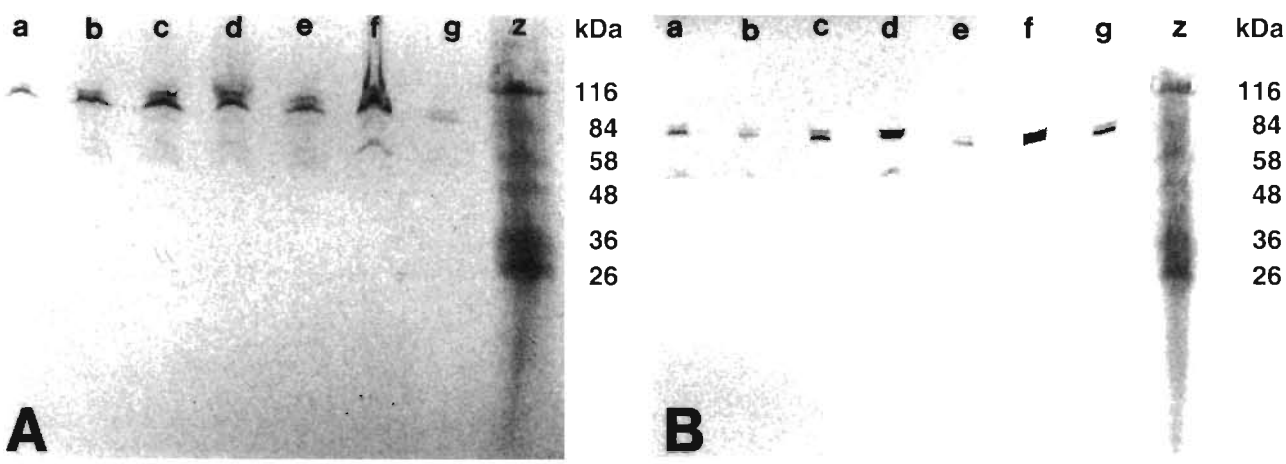

Fig. 4. Detection of epitopes of β-galactosidase-RSH in cell extracts of 7 *Renibacterium salmoninarum* strains. Preparations were separated by SDS-PAGE using a 10 to 15% gradient gel, transferred onto nitrocellulose, and then probed with a rat antiserum raised against β-galactosidase-RSH fusion protein. Cultures were grown in either (A) MHCB or (B) MHCB supplemented with 200 μM EDDHA. Tracks were: (a) strain 910019; (b) strain MT452; (c) strain MT425; (d) strain MT420; (e) strain MT417; (f) strain MT414; (g) strain ATCC33209; (z) protein molecular weight standards (marked in kDa)

ECPs were isolated from cells which had been cultured under either iron-restricted or iron-sufficient conditions, separated by SDS-PAGE and blotted onto nitrocellulose. Cell extracts of all strains of *R. salmoninarum* were found to possess epitopes recognised by rat antiserum to β-galactosidase-RSH regardless of the availability of iron. Two major bands of 82 000 and 78 000 were found to be present together with a number of weaker bands between 78 000 and 56 000 and one very weak band at 25 000 which is not discernible in the figure (Fig. 4A, B). No epitopes of β-galactosidase-RSH were detected on Western blots of ECPs. Evenden (1993) has predicted that the RSH protein is anchored to the cell wall and is exposed on the surface of the bacterium. This may explain the absence of detectable quantities of the protein in the ECPs. In addition, sequence analysis by Evenden (1993) has shown that the *rsh* gene encodes a protein of 47 600 molecular weight which is smaller than many of the bands identified on Western blots in this study. Some explanations for this include the possibility that the native protein may possess carbohydrate or lipid moieties which alter its migration on SDS-PAGE. In this respect, Evenden (1993) has identified at least 3 possible carbohydrate attachment sites (Asn-X-Ser/Thr) on the RSH protein and the molecule is rich in proline (8.65%), which is known to retard the migration of polypeptides on SDS-PAGE gels (Hames & Rickwood 1990). Alternatively, other molecules produced by *R. salmoninarum* may share epitopes which are present on the β-galactosidase-RSH fusion protein. In relation to this possibility it is worth noting that *R. salmoninarum* has been shown to be negative for β-galactosidase activity (Goodfellow et al. 1985), and Western blots of *R. salmoninarum* cell extracts which were probed with mouse anti-β-galactosidase antiserum showed no evidence for the recognition of cross reactive epitopes.

Given the paucity of knowledge relating to *Renibacterium salmoninarum* this study represents progress towards a better understanding of the pathogen. The availability of gene *rsh* and an antiserum directed against epitopes of the native molecule will greatly facilitate further studies of the functional significance of this protein, in particular, the immunological significance of RSH in the pathogenesis of BKD. The RSH protein has been previously shown not to be recognized on Western blots probed with rabbit antiserum raised against products derived from *R. salmoninarum* cultured *in vitro* (Evenden 1993). The reasons for this are as yet unclear but may be related to either the immunodominance of the p57 antigen or the presence of immunosuppressive components in *R. salmoninarum* products. These questions will be addressed by future research.

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