Cloning and Expression in *Escherichia coli* of a Protective Antigen of *Erysipelothrix rhusiopathiae*

JORGE E. GALÁN*1,2* AND JOHN F. TIMONEY1

Department of Veterinary Microbiology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853,1 and Department of Microbiology, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 117942

Received 3 April 1990/Accepted 31 May 1990

*Erysipelothrix rhusiopathiae* is a primary pathogen of swine and turkeys and sporadic cause of disease in a variety of other hosts, including humans. A genomic library of the highly virulent strain of *E. rhusiopathiae* E1-6P was constructed in the expression-cloning vector Aγt11 and screened with serum from a pig convalescent from an *E. rhusiopathiae* experimental infection. Immuno reactive clones were screened for their ability to protectively immunized mice. Two clones, Aγt11/ersA and Aγt11/ersB, were obtained that protected mice against challenge with *E. rhusiopathiae* E1-6P. Antisera against the recombinant clones reacted with polypeptides of molecular weights 66,000, 64,000, and 43,000 in detergent-solubilized surface antigen preparations and whole-cell lysates of *E. rhusiopathiae*. These polypeptides were also the major antigens recognized by convalescent pig serum when reacted with the same preparations. Western immunoblot and Southern blot analysis revealed that the cloned genes and gene products were present in all of the *E. rhusiopathiae* strains tested.

*Erysipelothrix rhusiopathiae* is a small gram-positive rod that causes erysipelas in swine and turkeys. Erysipelas can occur as an acute septicemic or chronic disease, with development of arthritic lesions and endocarditis (31). Less frequently, it causes polyarthritis in lambs and calves (26), septicemia in ducks (18), and occasionally a variety of infections, including skin lesions and endocarditis, in humans (1, 3, 7, 16, 19). Much of the interest in *E. rhusiopathiae* has been due to the chronic and progressive character of the arthritic lesions in pigs, which superficially resemble those of human rheumatoid arthritis.

Peptidoglycan antigens form the basis for the classification of this organism into serotypes and subtypes, and at least 22 different serotypes have been identified (11, 17, 22, 32, 33). Most of the isolates from diseased animals belong to serotype 1 or 2 (32). Protection is not serotype specific, and varying degrees of cross-protection among different serotypes have been observed (23). Although protective antisera for passive immunization have been available for many years, the identity of the protective antigen(s) has not been clearly established. White and Verwey (28, 29) described a large-molecular-weight protective antigen in culture supernatants; more recently, Lachmann and Deicher (12) have described smaller-molecular-weight antigenic components by immunoblotting.

Currently available live attenuated or bacterin vaccines do not prevent the chronic form of swine erysipelas, and it has been suggested that vaccination may actually cause an increase in arthritic lesions by hypersensitizing the animal to subsequent contact with the organism (4, 31). These observations are consistent with the findings of White et al. (27), who produced synovitis in rabbits by injecting cell extracts of *E. rhusiopathiae*. If the protective antigen(s) is indeed different from the sensitizing antigen(s) of *E. rhusiopathiae*, it follows that an immunogen composed of only protective antigens would result in a safer vaccine that could protect against both the acute and chronic forms of the disease.

To better define the antigen(s) involved in protective and arthritic responses, we have cloned and expressed in *Escherichia coli* a surface protein of *E. rhusiopathiae* that induced protection in immunized mice. This protein was common to strains of different serotypes and with different levels of virulence.


**MATERIALS AND METHODS**

**Bacterial strains, bacteriophage, and growth conditions.** *E. rhusiopathiae* strains are listed in Table 1. *E. coli* Y1088, Y1089, and Y1090 (34) were used for growing and screening Aγt11 and Aγt11 recombinant clones. *E. coli* BHB2688 and BHB2690, used for preparation of packaging mixes, have been described by Hohn (9). *E. rhusiopathiae* strains were grown on brain heart infusion broth supplemented with 10% horse serum. *E. coli* strains were grown on L agar or L broth (14). When appropriate, ampicillin was added to media at a concentration of 100 µg/ml.

**Antigen preparation.** Surface antigens of *E. rhusiopathiae* were obtained by extraction with Triton X-100 as described by Lachmann and Deicher (12). Briefly, *E. rhusiopathiae* strains were grown on 10 ml of brain heart infusion broth supplemented with 10% horse serum. Cells were washed twice with 20 mM Tris (pH 7.6) and resuspended in 0.5 ml of 20 mM Tris (pH 7.6) containing 0.5% Triton X-100 (Sigma Co., St. Louis, Mo.). Cells were incubated at 37°C for 1 h under rotation. Samples were finally centrifuged at 9,000 × g for 5 min, and cells and supernatants were separately stored at −75°C until further analysis. Total cell lysates from *E. rhusiopathiae* were prepared by boiling the cells for 5 min in Laemml loading buffer (13). Cell debris was separated by centrifugation at 10,000 × g for 5 min. Lysates from *E. coli* lysogens were prepared as follows. Lysogens were grown at

---

* Corresponding author.
30°C to an optical density at 600 nm of 0.4 to 0.6 and then incubated at 42°C for 20 min for bacteriophage induction. At this stage, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce the lacZ promoter of Ygt11. Lysogens were further incubated with heavy aeration for an additional 3 to 5 h at 37°C. Cells were recovered by centrifugation at 5,000 x g for 10 min and lysed by repeated freeze-thawing in liquid nitrogen. DNase I (Sigma) was then added to a final concentration of 20 μg/ml, and the lysates were incubated at room temperature for 15 min. Cell debris was separated by centrifugation at 10,000 x g for 20 min, and supernatants were saved for further analysis.

Antiserum. Antiserum against *E. rhusiopathiae* was obtained from a 4-month-old pig 8 weeks after recovery from an experimental infection with an intravenous inoculation of 3 x 10⁸ CFU of strain E1-6P. To prepare antiserum against recombinant proteins, guinea pigs were inoculated subcutaneously with lysates (500 μg of protein) from *E. coli Y1089* (agt11/ersA) and Y1089 (agt11/ersB) homogenized with equal amounts of complete Freund adjuvant. Antiserum to Y1089 (agt11) was prepared by a similar procedure. To remove cross-reacting antibodies, all antiserum were extensively absorbed with a lysate of *E. coli Y1089* (agt11) absorbed onto nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) before being used in the different assays.

**DNA manipulations.** Large quantities of agt11 bacteriophage DNA were obtained from *E. coli Y1088* by procedures described by Maniatis et al. (15). Recombinant phage DNA was obtained by the method of Ivanov and Gigova (10). Plasmid DNA was isolated as described previously (2). Total cell DNA from *E. rhusiopathiae* strains was obtained as follows. Cells were grown overnight in brain heart infusion broth supplemented with 10% horse serum, recovered by centrifugation at 9,000 x g, and washed twice with TE buffer (10 mM Tris [pH 7.6], 1 mM EDTA). Cells were resuspended in 2 ml of TE buffer containing lysozyme (20 mg/ml; Sigma) and N-acetylumuramidase SG (50 μg/ml; Seikagaku Kogyo Co., Tokyo, Japan) and placed in a 10-ml ultracentrifuge tube. After 1 h of incubation at 37°C, 1 ml of TE buffer containing 10 mg of preincubated pronase (Calbiochem-Behring, La Jolla, Calif.) was added, and the mixture was incubated at 37°C for 30 min. Cells were lysed with 1 ml of 20% sodium dodecyl sulfate (SDS) solution, and the tubes were filled with TE buffer containing CsCl to give a final concentration of 1 g/ml and 0.8 ml of a 10-mg/ml solution of ethidium bromide. DNA was recovered after centrifugation to equilibrium in a CsCl gradient. Packaging mixtures of bacteriophage λ were prepared from strains BHB2688 and BHB2690 as described by Hohn (9). Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and were used as recommended by the manufacturers. Transfer of DNA to nylon membranes (GeneScreenPlus; Dupont, NEN Research Products, Boston, Mass.) was carried out according to the method of Southern (21). Hybridization was performed with ³²PdCTP-labeled probes according to standard procedures (15).

**Construction and screening of bacteriophage libraries.** *E. rhusiopathiae* E1-6P total cell DNA was partially digested with EcoRI, and fragments ranging from 1 to 8 kilobase pairs (kb) were isolated on a sucrose gradient. agt11 DNA was ligated overnight with T4 DNA ligase (Boehringer Mannheim), digested with EcoRI, and treated with alkaline phosphatase (Boehringer Mannheim) before being ligated to the EcoRI fragments of *E. rhusiopathiae* DNA. The ligated DNA molecules were packaged into λ phage particles as described previously (9). Recombinant phage were plated on *E. coli Y1089* to give approximately 300 plaques per plate. After incubation of the plates at 42°C for 4 to 6 h, nitrocellulose filters (82 mm; Schleicher & Schuell) previously impregnated with 10 mM IPTG were overlaid, and the plates were incubated at 37°C for an additional 8 to 12 h. Filters were then removed, washed in NET buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.25% [wt/vol] gelatin, 0.05% [wt/vol] Triton X-100 [pH 7.4]) for 2 h and incubated with *E. rhusiopathiae* E1-6P convalescent pig serum for 2 h. Filters were then washed in NET buffer for 30 min, incubated with peroxidase-labeled goat anti-pig antiserum for 2 h, and finally developed with 4-chloro-1-naphthol. Plates giving a positive signal were picked, suspended in SM medium (15), saturated with chloroform, and rescreened until all plaques on the filter gave a positive signal. Recombinant phages were then lysogenized into *E. coli* Y1089 as described previously (34). Recombinant clones were further screened for ability to induce protection in immunized mice. Groups of four 8-week-old inbred female mice of the ICR strain were used for each clone, and two doses of 500 μg of protein from lysates adsorbed to aluminum hydroxide (Alhydrogel) were given subcutaneously 15 days apart. Mice were then challenged subcutaneously with 100 50% lethal doses of *E. rhusiopathiae* E1-6P 2 weeks after the booster dose, and deaths were scored for up to 15 days after challenge. Clones showing protective abilities were further tested by immunizing a larger number of mice as described above.

**SDS-polyacrylamide gel electrophoresis and immunoblot analysis.** Electrophoresis, immunoblotting, and staining with peroxidase-conjugated antibody were performed as described previously (5, 6). Silver staining of polyacrylamide gels was done with a kit from Bio-Rad Laboratories (Richmond, Calif.) as instructed by the manufacturer.

**Statistical methods.** The Student *t* test (20) was used to compare the mean time to death and percentage of survivors of groups of mice immunized with the recombinant proteins or the negative control.

**RESULTS**

**Cloning of a protective antigen of *E. rhusiopathiae*.** Approximately 95% of the phage plaques of the agt11 library were β-galactosidase negative as determined in 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) plates and were therefore assumed to contain inserts. Approximately 10,000 plaques were screened by immunoblotting with *E. rhusiopathiae* antiserum, and 10 positive clones were found. Two clones,
TABLE 2. Mean time to death and percentage of survivors in a group of immunized mice

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>n</th>
<th>Mean time to death (days)</th>
<th>% Survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agt11</td>
<td>36</td>
<td>4.3</td>
<td>0</td>
</tr>
<tr>
<td>Agt11/ersA</td>
<td>34</td>
<td>6.4*</td>
<td>17*</td>
</tr>
<tr>
<td>Agt11/ersB</td>
<td>35</td>
<td>6.5*</td>
<td>14*</td>
</tr>
</tbody>
</table>

* P < 0.001 compared with Agt11 controls.

Agt11/ersA and Agt11/ersB showed protective abilities when further screened by immunizing and challenging groups of mice. Mice immunized with Agt11/ersA and Agt11/ersB lysates had statistically significantly higher mean time to death and percentage of survivors than did mice immunized with the negative control Agt11 (Table 2). No statistically significant differences between the protective abilities of Agt11/ersA and Agt11/ersB were detected.

Immunochemochemical characterization of Agt11/ersA and Agt11/ersB. Immunoblot analyses of Agt11/ersA and Agt11/ersB lysates are presented in Fig. 1. The recombinant clones appeared as high-molecular-weight polypeptides when reacted with pig antiserum against E. rhusiopathiae. Coomassie blue-stained SDS-polyacrylamide gels of the same preparations showed the absence of polypeptides at the expected size of the β-galactosidase and instead showed the presence of higher-molecular-weight polypeptides of similar size than those observed in Western immunoblots (data not shown). These findings, in conjunction with the fact that these polypeptides were observed only in IPTG-induced lysogens, strongly suggested that the cloned determinants in Agt11/ersA and Agt11/ersB were fused to the β-galactosidase gene of the Agt11 vector.

Reactivities of antisera against Agt11/ersA and Agt11/ersB. Figure 2 shows the reactivities of antisera against Agt11/ersA and Agt11/ersB when reacted with detergent-solubilized surface proteins (lanes B) of E. rhusiopathiae run on an SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Panels were developed with the following antisera: Agt11/ersA (a), Agt11/ersB (b), and E. rhusiopathiae E1-6P convalescent pig serum (c). Panel d is a silver stain of the same antigens. Numbers on the left indicate the positions (in thousands) of the molecular weight standards.

Agt11/ersB reacted strongly with polypeptides of molecular weights 64,000 and 43,000 and weakly with the 66,000-molecular-weight polypeptide in the same preparation (Fig. 2b). In Fig. 2c, the reactivities of the E. rhusiopathiae convalescent pig serum used for screening of the libraries are shown. This antiserum recognized several immunologically reactive polypeptides in both antigenic preparations, the most prominent being the same polypeptide bands recognized by the recombinant antiserum with molecular weights of 66,000, 64,000, and 43,000. A silver stain of the same antigenic preparations is shown in Fig. 2d. The polypeptide of molecular weight 64,000 to 66,000 appeared as one of the most abundant polypeptide species in the detergent-solubilized surface antigen preparation. An abundant lower-molecular-weight band was also present in the Triton X-100 extract. Lachmann and Deicher (12) have identified this lower-molecular-weight band as a polysaccharide. Guinea pig antiserum against Y1089(Agt11) showed no reactivities when tested against the same antigenic preparations (data not shown).

Characterization of the insert DNA fragment of Agt11/ersA and Agt11/ersB. Agarose gel electrophoresis of EcoRI digests of Agt11/ersA and Agt11/ersB DNA revealed the presence of a 5.4-kb insert (Fig. 3). Insert DNA from Agt11/ersA was subcloned into the plasmid vector pUCB8-2 (8), yielding plasmid pSB001. A restriction endonuclease site map of the insert DNA is shown in Fig. 4. No difference between the restriction maps of the inserts of Agt11/ersA and Agt11/ersB was observed. In addition, pSB001 insert DNA hybridized to Agt11/ersB insert DNA (Fig. 3), strongly suggesting that the two clones are identical.

Presence of the cloned antigenic determinants in different strains of E. rhusiopathiae. The presence of the cloned antigenic determinants of Agt11/ersA and Agt11/ersB in detergent-solubilized proteins of different strains of E. ru-
rhodopsin was determined by Western blot analysis, using the antisera against λgt11/ersA and λgt11/ersB. Antiserum against λgt11/ersA reacted to varying degrees with the 66,000- and 43,000-molecular-weight polypeptides in all strains tested (Fig. 3). Similar results were obtained with λgt11/ersB antiserum (data not shown). In addition, Southern hybridization analysis with the λgt11/ersA insert DNA fragment revealed the presence of DNA sequences sharing homology in all strains tested. The cloned DNA fragment hybridized with an EcoRI fragment of 5.4 kb in all strains except strains 2179, S-192, and NF-4, in which it hybridized with different-size fragments (Fig. 3).

DISCUSSION

We have cloned and expressed in E. coli a protective protein antigen of E. rhusiopathiae. Lysates of λgt11/ersA and λgt11/ersB protected mice against subcutaneous challenge with 100 50% lethal doses of virulent E. rhusiopathiae E1-6P. Mice immunized with the recombinant protein showed statistically significant longer times to death and higher percentage of survivors than did controls (Table 2). Nevertheless, protection was not complete, since less than 20% of the immunized mice ultimately survived the challenge. Several factors may account for these findings. It is possible that β-galactosidase is immunodominant and interferes with the response to the E. rhusiopathiae protein. A similar finding has been reported for the VP1 protein of foot-and-mouth disease virus, which induced a less effective immune response when fused to β-galactosidase (30). Moreover, the cloned protein as fused to β-galactosidase may be a defective antigen, which could account for a less efficient

![FIG. 3. Southern blot analysis E. rhusiopathiae strains. Plasmid, phage λ, and total cell DNAs were digested with EcoRI, separated on a 0.7% agarose gel, transferred to nylon membranes, and probed with [32P]dCTP-labeled λgt11/ersB insert DNA fragment. Lanes: 1, pSB001; 2, λgt11/ersB; 3, E1-6P; 4, 2192; 5, 2179; 6, SE-9; 7, S-192; 8, NF-4; 9, 422-1; 10, HC-585; 11, 2229; 12, E1-6P; 13, 1-kb ladder (Bethesda Research Laboratories). (a) Agarose gel; (b) Southern blot.](http://iai.asm.org/)

![FIG. 4. Restriction endonuclease site map of λgt11/ersA and λgt11/ersB insert DNA.](http://iai.asm.org/)

![FIG. 5. Immunoblot showing the reactivities of detergent-solubilized surface proteins of different strains of E. rhusiopathiae. Extracts were run on an SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Blots were developed with λgt11/ersA antiserum. Strains are described in Table 1. Lanes: A, S-192; B, SE-9; C, 422-1; D, NF-4; E, HC-585; F, 2229; G, 2192; H, E1-6P. Numbers on the right indicate the positions (in thousands) of the immunoreactive polypeptides.](http://iai.asm.org/)
protective immune response. The fused recombinant protein may lack determinants present in the native protein that are important in conferring full protection, or other separate antigens may be required for a high-level protective response. It is known that cellular as well as humoral immune responses (25) are involved in protection against E. rhusiopathiae, and therefore several different antigens may be involved in this process. We do not yet know which arm of the immune response mediates the protection stimulated by the recombinant antigens.

Immunoblot analysis showed that antisera to the recombinant protein recognized proteins of molecular weights 66,000 (occasionally resolved as two closely spaced bands) and 43,000 in whole-cell extracts and Triton X-100-solubilized surface proteins of E. rhusiopathiae (Fig. 2a). Antisera to \( \lambda g1/er5A \) and \( \lambda g1/er5B \) showed slightly different reactivities (Fig. 2b). Nevertheless, we have been unable to detect differences between these two clones by using Southern hybridization and restriction analysis. Most likely, the guinea pigs immunized with each lysate may have responded differently to the immunogen, not an unexpected finding considering the outbred nature of these animals.

The three proteins in the extracts of E. rhusiopathiae recognized by the recombinant protein antisera appeared among the most reactive proteins when the immunoblots were developed with convalescent pig antiserum (Fig. 2c). In addition, convalescent pig antiserum strongly reacted with a 70,000-molecular-weight protein in both whole-cell extracts and Triton X-100 preparations. Lachmann and Deicher described several immunologically reactive proteins in similar preparations of E. rhusiopathiae, using a rabbit antiserum against heat-killed E. rhusiopathiae (12). The 66,000- and 43,000-molecular-weight proteins were the most prominent antigens reacting with the rabbit antiserum. In the same work, these authors established the surface location of these proteins. As shown in the silver stain of Fig. 2d, the 66,000-molecular-weight protein appears as the most abundant detergent-solubilized surface protein of E. rhusiopathiae. Although insufficient information is available regarding the relationship between the 66,000-, 64,000-, and 43,000-molecular-weight proteins, it is possible that the lower-molecular-weight proteins are processed or degraded forms of the larger molecule. Lachman and Deicher found that rabbits immunized with heat-killed E. rhusiopathiae made antibodies to a low-molecular-weight polysaccharide that is abundant in Triton X-100 preparations of this organism (12). Our immunoblots showed no reactivity of convalescent pig antiserum to such a polysaccharide although it was present in large amounts in the preparations used in the analysis shown in Fig. 2d.

Peptidoglycan antigens form the basis of the classification of E. rhusiopathiae into at least 22 serotypes. The degree of relatedness of the protein antigens among the different serotypes is not known, although varying degrees of cross-protection have been observed (23). We determined the distribution of the cloned protein among different serotypes of E. rhusiopathiae of veterinary importance by using the antisera against the cloned determinants. Figure 5 shows a Western blot of detergent-solubilized proteins of different strains of E. rhusiopathiae developed with the \( \lambda g1/er5A \) antiserum. The 66,000- and 43,000-molecular-weight peptides were present in varying amounts in all strains tested. Strains of low or moderate virulence showed less reactivity than virulent strains, although the significance of these findings is still unclear. There was no correlation between serotype and amount of reactivity. Southern hybridization analysis revealed DNA sequences sharing homology with the cloned fragments in all strains tested regardless of virulence or serotype, although some restriction fragment length polymorphism was apparent among the different strains (Fig. 3).

A lack of correlation of serum antibody titers with protection against E. rhusiopathiae has been reported (24). This finding may reflect the inadequacy of the antigen used in the tests (either whole cells for the agglutination test or crude cell extracts for the enzyme-linked immunosorbent assay). The availability of a protective antigen free of other nonrelevant antigens will help in the development of improved in vitro methods for assessing immune status.

Although we have cloned and expressed in E. coli a protein of E. rhusiopathiae that stimulates partial protection of mice against challenge with a highly virulent strain of this organism, immunization studies are needed to evaluate the protective ability of this recombinant protein in the pig. None of the immunized mice that survived the challenge with the virulent strain of E. rhusiopathiae developed arthritic lesions; nevertheless, additional experiments in pigs will be necessary to determine whether the cloned determinant can sensitize these animals to subsequent contact with the organism and therefore predispose them to development of arthritic lesions. The results of such studies will establish the feasibility of using protective epitopes on this protein in a subunit vaccine against erysipelas.

ACKNOWLEDGMENT

We thank R. L. Wood for providing bacterial strains.

LITERATURE CITED