Corynebacterium renaleの線毛遺伝子のクローニングとその発現

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Cloning and Expression of a Pili Gene of *Corynebacterium renale* in *Escherichia coli*

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ABSTRACT. A plasmid gene library of *Corynebacterium renale* piliated strain No. 109P$^+$ was prepared in *Escherichia coli* in order to study the chemical structure of the pili of *C. renale*. Of 3,000 recombinant clones tested, 5 reacted with anti-pili anti-serum. The gene products of these clones reacted with anti-pili monoclonal antibodies 8/4, 5/2 and B20/3 but lacked the reactivity with 13/4. SDS-PAGE analysis revealed that the expressed protein had a molecular mass of 48 kilodalton and deletion analysis showed that the encoding region for this protein was localized within a 1.4 kilobase gene including a promoter sequence. Immunoelectron microscopy showed that mouse antibodies raised to the expressed protein bound to the entire surface of the pili of *C. renale*. These results indicate that the cloned gene encodes a major structural protein of *C. renale* pili.—KEY WORDS: cloning, *Corynebacterium renale*, gene, pili, recombinant clone.

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Corynebacterium renale is a gram-positive bacterium that causes bovine pyelonephritis [12]. The bacterium possesses pili [26, 27] that mediate bacterial adhesion to the host tissue [9, 10]. The pili, therefore, may play an important role in *C. renale* infection.

There have been some reports on the chemical [16] and immunological [15] natures of the pili of *C. renale*. However, little information on the subunit structures of the pili is available since the native pili have not been successfully isolated as purified form from *C. renale*. Gene cloning techniques have made it possible to identify and to characterize putative subunits of pili from their encoding genes. These techniques have been applied to the pili of some other bacteria [6, 7, 11, 29].

The study reported here was undertaken to clone and express the pilus gene of *C. renale* in *E. coli* to provide information of the structure and formation of the *C. renale* pili.

MATERIALS AND METHODS

Bacteria and plasmids: A piliated (P$^+$) clone and non-piliated (P$^-$) clone of *C. renale* No. 109 were described previously [14]. *C. renale* No. 109P$^+$ was the source of target of DNA. *E. coli* MC1061 [8] and *E. coli* JM109 [28] were the recipient strains for recombinant plasmids. Plasmids pBR322 [2] and pUC18 [28] were used as cloning vectors.

DNA isolation and analysis: Chromosomal DNA was isolated according to the method of Donkerloot *et al.* [6]. Briefly, cells grown in LB-medium [20] containing 2% glycine were treated with lysozyme (Sigma Chem. Co., St. Louis, Mo., USA) [3]. After addition of actinase E (0.1 mg/ml, Kaken Seiyaku Co., Tokyo, Japan), they were lysed by sodium dodecyl sulfate (SDS, 1%) with a 4 hr incubation at 60°C. The viscous lysate was extracted with phenol-chloroform-isoamylalcohol (25:24:1) and chloroform-isoamylalcohol (24:1) [20].

DNA was precipitated with ethanol and then treated with RNase (2 mg/m*l*, Sigma Chem. Co.) for 30 min at 37°C. After being digested with actinase E (10 mg/m*l*) for 1 hr at 37°C, it was extracted with phenol and chloroform, and then precipitated with ethanol to remove RNase.

Closed-circular plasmid DNAs were amplified with chloramphenicol [5], extracted by SDS lysis and isolated on CsCl-ethidium bromide gradients as described by Clowell and Helinski [4]. Small-scale isolation of plasmid DNA was done as described by Brimboim and Doly [1].

DNA was digested with restriction endonuclease (Takara Shuzou Co., Kyoto, Japan and Nippon Gene, Toyama, Japan) as previously described [20] and analyzed by agarose gel electrophoresis.

Preparation of gene library: Purified chromosomal DNA was partially digested with 0.08 units of *Sau*3AI per μg of DNA for 30 min at 37°C. The digested DNA was fractionated by a 20 hr centrifugation (130,000 × g) with a 5% to 23% sucrose gradient. Fractions were collected and analyzed by electrophoresis in an agarose gel (0.7%). Fractions containing DNA of 6 to 9 kilobase (kb) size were pooled and the DNA was ligated into the *Bam*HI site of the plasmid pBR322 after dephosphorylation with calf intestinal alkaline phosphatase (Boehringer Mannheim, Mannheim, West Germany) [20]. The ligation mixture was added to *E. coli* MC1061 competent cells [19]. The cells were inoculated onto LB agar plates containing ampicillin (200 μg/m*l*) and inoculated at 37°C overnight. Colonies from ampicillin plates were transferred onto tetracycline plates (25 μg/m*l*) and confirmed to be sensitive to tetracycline.

Antibodies and antigens: Anti-pili antiserum was prepared as previously described [14]. Anti-pili monoclonal antibodies, B20/3 and 13/4 against pili of *C. renale* No. 115, described previously [15], were used. Anti-pili monoclonal antibodies, 8/4 and 5/2 against pili of *C. renale* No. 109 were prepared for the present study by the same methods.

Screening of recombinant clones: Recombinant clones were screened with anti-pili antiserum by colony immunoassay and immunodiffusion. Colony immunoassay was performed as described by Lo and Cameron [18] except that lysozyme (1%) and Triton X-100 (0.2%) were used to lyse the bacteria instead of chloroform vapor. Immunodiffusion was performed as previously described [21]. The antigen was the supernatant fluid (8,000 × g) of sonicated cell suspension.

ELISA: ELISA was performed essentially as described previously [13]. For *E. coli*, the same antigen for immunodiffusion was used at a dilution of 1:100. For *C. renale*, a suspension of 2.5 mg (wet)/m*l* of whole cells in phosphate-buffered saline (PBS, pH 7.2) treated at 100°C for 30 min was used as the antigen. Monoclonal antibodies in the form of ascetic fluid were used at a dilution of 1:200. Results were expressed as optical density at 405 nm as measured by a multichannel photometer.

Purification of expressed pili protein: A cold, saturated ammonium sulfate solution (pH 7.2) was added to the supernatant (7,000 × g) of the sonicated cell suspension to 50% saturation. The precipitate was dissolved in and dialyzed against PBS. The sample was applied to an affinity column prepared by coupling anti-*C. renale* No. 109 pili monoclonal antibody 8/4 to CNBr-activated Sepharose-4B (Pharmacia Fine Chem., Uppsala, Sweden). The column was washed with PBS containing 0.5% Tween 80 to remove unbound material, and bound antigen was eluted with 0.2 M glycine-NaOH (pH 3.0) containing 0.5% Tween 80. The eluate was dialyzed against distilled water.

SDS-PAGE and Western blotting: SDS-PAGE was done according to the method of

Laemmli [17]. The proteins were stained with Coomassie brilliant blue R-250 (Nakarai Chem., Kyoto, Japan) or transferred to a nitrocellulose membrane for Western blotting [24]. The blots were developed with anti-pili monoclonal antibody 8/4, and detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Lab., Richmond, Cal., USA).

Immunoelectron microscopy: Immunoelectron microscopy was performed as described by Weiss *et al.* [25]. Samples were negatively stained with 2% phosphotungustic acid and examined under a Hitachi HU-12A electron microscope (Hitachi Ltd., Tokyo, Japan).

Subcloning: Recombinant plasmid was digested with *Sal*I and ligated into the *Sal*I site of plasmid pUC18. These subcloned plasmids were further subcloned with exonuclease III (Takara Shuzou Co.) [22]. The plasmids were digested with *Kpn*I and then *Bam*HI. The fragments were digested with 25 units of exonuclease III per μg of DNA and then with S1 nuclease (Takara Shuzou Co.) after treatment with Klenow fragment (Takara Shuzou Co.), and transformed into *E. coli* JM109.

Southern blotting: The chromosomal DNA preparation was digested with *Sal*I and electrophoresed on 0.8% agarose gel. The DNA was transferred onto a nylon blotting membrane (Hybond-N; Amersham, Buckinghamshire, England) [20, 23]. Prehybridization was performed in 6x SSC (0.15 M NaCl, 0.015 M sodium citrate), 5x Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA fraction V) and 0.015% denatured sheared herring sperm DNA at 60°C for 1 hr. Hybridization was carried out in the same solution with an additional 5% dextran sulfate and 200 ng/ml biotinated probe DNA at 60°C for 24 hr. The membrane was washed in 2x SSC containing 0.1% SDS at room temperature for 15 min and then at 45°C for 30 min. The probe was detected with the BluGENE Nonradioactive Nucleic Acid Detection System (Bethesda Research Laboratories, Gaithersburg, Md., USA). The labeling of DNA was performed by nick-translation kit (Amersham) and biotin-11-dUTP (Bethesda Research Laboratories).

RESULTS

Identification of antigen-producing clones: Plasmid pBR322 was used as a cloning vector to establish a library of *C. renale* No. 109P$^+$ DNA in *E. coli* MC1061.

Approximately 3,000 recombinant clones were examined by colony immunoassay and immunodiffusion with anti-pili antiserum. Five independent positive clones were obtained. In immunodiffusion, the cell lysates of these positive clones formed a single precipitin line, fusing to each other and to *C. renale* No. 109P$^+$ lysate (Fig. 1). Non-piliated clone of *C. renale* No. 109P$^-$ and the recipient strain of *E. coli* MC1061 did

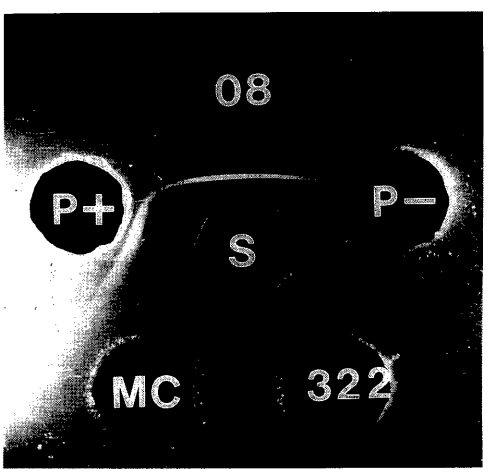

Fig. 1. Immunodiffusion of pili of *C. renale* expressed in *E. coli*. The circumferential wells contained cell lysate of recombinant clone *E. coli* AY10908 (well 08), *C. renale* No. 109P$^+$ (well P+), *C. renale* No. 109P$^-$ (well P−), receipient *E. coli* MC1061 (well MC), and *E. coil* MC1061 containing plasmid pBR322 (well 322). The central well (S) contained anti-*C. renale* pili antiserum.

Table 1. Reactivity of recombinant clones with 4 anti-*C. renale* pili monoclonal antibodies by ELISA

Bacteria	Monoclonal antibodies against pili of *C. renale*			
	No. 109		No. 115	
	8/4	5/2	B20/3	13/4
Recombinant clone				
E. coli AY10901	+	+	+	−
〃 AY10903	+	+	+	−
〃 AY10907	+	+	+	−
〃 AY10908	+	+	+	−
〃 AY10911	+	+	+	−
Control				
C. renale No. 109P$^+$	+	+	+	+
E. coli MC1061(pBR322)	−	−	−	−

The reaction was judged by optical density; + indicates value higher than 0.2, − indicates value lower than 0.1.

not give rise to any lines. In ELISA, all positive clones also reacted with anti-pili monoclonal antibodies 8/4, 5/2 and B20/3 but not with 13/4 (Table 1).

Clones AY10908 and AY10901, containing recombinant plasmids pAY10908 and pAY10901, respectively, were used for further analysis.

Characterization of the expressed proteins: The affinity-purified pili antigens expressed by cloned AY10908 and AY10901 both migrated in SDS-PAGE as one predominant band with an apparent molecular weight of 48,000 (Fig. 2A). In Western blot analysis, each band reacted with anti-pili monoclonal antibody 8/4 (Fig. 2B). In immunoelectron microscopy of the recombinant clones with monoclonal antibody 8/4, this antigen was not found on the surfaces of these bacteria (data not shown). Mouse antibodies raised to the expressed protein bound specifically to the entire surface of the native pilus of *C. renale* (Fig. 3).

Localization of the cloned gene: The restriction endonuclease maps showed that plasmids pAY10908 and pAY10901, encoding the same 48 kilodalton (kd) protein, shared a common 5.0 kb region of *C. renale* DNA (Fig. 4). Both subclones AY10921 and AY10922, from which the plasmids were prepared from pAY10908 by insertion

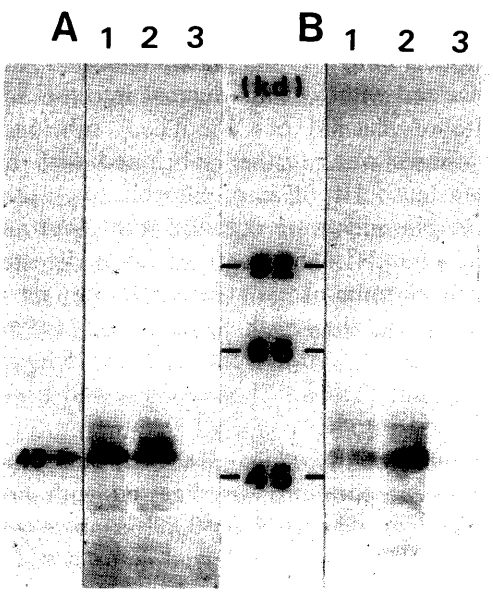

Fig. 2. SDS-PAGE and Western blot analysis of affinity-purified pili protein expressed in *E. coil*. (A) SDS-PAGE gel stained with Coomassie brillint blue. (B) Western blots developed with antipili monoclonal antibody 8/4. Lanes: 1, *E. coli* AY10901; 2, *E. coli* AY10908; 3, *E. coli* MC1061 containing plasmid pBR322.

of the same 2.7 kb region of *C. renale* DNA in reverse orientation (Fig. 5), produced the 48 kd protein, indicating that the coding region of the pili was on the 2.7 kb insert with a promoter sequence.

Further analysis of these subclones with exonuclease III deletion using pAY1226

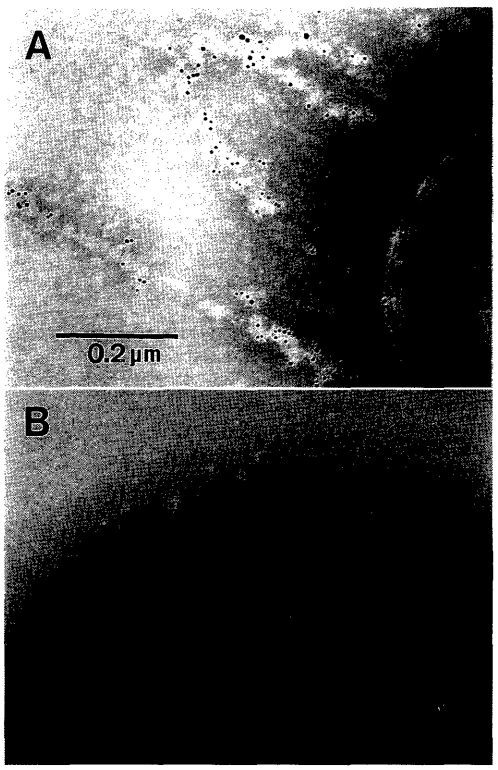

Fig. 3. Immunoelectron microscopy of *C. renale* No. 109P$^+$. Bacteria were incubated with mouse antiserum against pili protein expressed in *E. coli* (A) or normal mouse serum (B).
They were incubated with sheep anti-mouse IgG-gold (5 nm) conjugate.

In Southern blotting, the 2.7 kb fragment of *C. renale* No. 109P$^+$ chromosomal DNA showed homology with pAY10930, which was derived from pYA1226 by deleting a 1.0 kb *Sal*I-*Xho*I fragment (Figs. 5, 7).

These findings indicate that the 48 kd protein is encoded by the 1.4 kb gene derived from corynebacterial DNA.

DISCUSSION

The present results demonstrated that the gene encoding a structural protein of *C.*

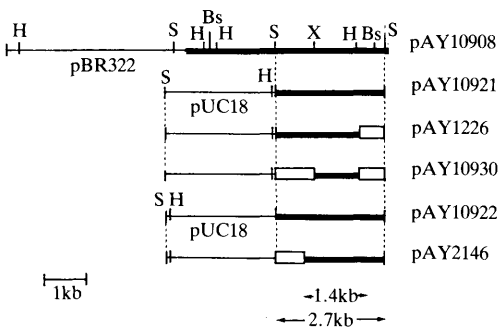

Fig. 5. Restriction endonuclease maps of deletion plasmid derived from pAY10908. Restriction sites: Ba, *Bam*HI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I. Symbols: ——, vector DNA; ■, *C. renale* DNA; □, deleted DNA.

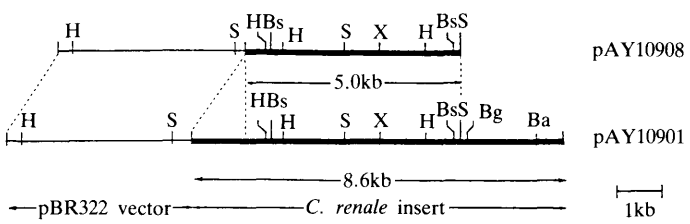

Fig. 4. Restriction endonuclease maps of recombinant plasmids pAY10908 and pAY10901. Restriction maps aligned about a shared 5.0 kb region. Restriction sites: Ba, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EIII; H, *Hin*dIII; S, *Sal*I; X, *Xho*I.

prepared from pAY10921 and pAY2146 from pAY10922 (Fig. 5) also expressed the 48 kd protein (Fig. 6). These results indicate that the gene encoding the pili protein localizes on a 1.4 kb region of the *C. renale* insert in plasmids pAY1226 and pAY2146.

renale pili has been cloned and expressed in *E. coli*. Immunodiffusion showed that the expressed protein was antigenically the same as that of *C. renale* native pili. The expressed protein did not react with one (13/4) of the anti-pili monoclonal antibodies

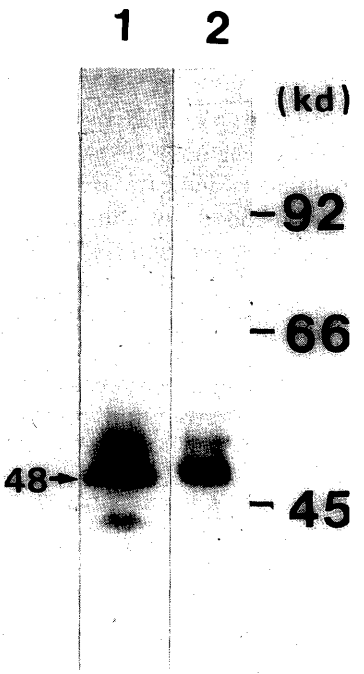

Fig. 6. SDS-PAGE analysis of pili protein expressed in subclones derived from *E. coil* AY10908. Lanes; 1, *E. coil* AY1226; 2, *E. coil* AY2146.

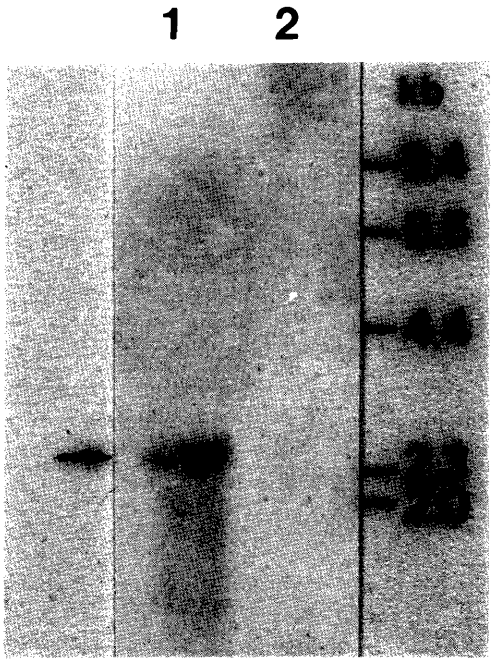

Fig. 7. Southern blot hybridization of cloned pili gene prove (pAY10903) to *Sal*I cleaved chromosomal DNA. Lanes: 1, *C. renale* 109P$^+$ DNA; 2, *E. coli* MC1061 DNA. Size markers are on the right.

to the epitope on the native pili of *C. renale* No. 109P$^+$, indicating that the protein lacks the epitope. Other gene products, therefore, also may be involved in the formation of the native pili of *C. renale*. This protein was immunogenic to mice and induced antibodies which bound to the entire surface of the pilus of *C. renale*. These findings suggest that the cloned protein is the major pilus subunit. This expressed protein may be useful to investigate on the structure and function of the pili of *C. renale* of which native form could not be purified.

In SDS-PAGE, the molecular weight of the protein expressed in *E. coli* was 48 kd, and this was a reasonable value considering the size of the 1.4 kb encoding region for this protein. In addition to this protein, some faint bands reacting with an anti-pili antibody appeared on the SDS-PAGE gel. These bands were probably due to post denaturalization of the encoding protein. Another possibility is that they result from ambiguities at the level of transcription or translation.

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要　約

Corynebacterium renale の線毛遺伝子のクローニングとその発現：阿部　聡・西藤岳彦・古賀哲文・小野悦郎・梁川　良・伊藤壽啓・喜田　宏・清水悠紀臣(北海道大学獣医学部家畜衛生学教室)——*Corynebacterium renale* の線毛の構造を調べるために No. 109株の有線毛クローン P^+ の遺伝子断片をプラスミドベクターを用いて大腸菌に導入し，線毛遺伝子のクローニングを試みた．3000個の組換え体を調べたところ，この内5個が抗線毛抗体と反応する分子量48,000の蛋白を産生していた．この蛋白は抗線毛単クローン性抗体8/4，5/2及びB 20/3と反応したが13/4とは反応しなかった．部分欠失プラスミドを用いた試験の結果より，この蛋白はプロモーターを含む1.4キロ塩基対の遺伝子によってコードされていることが明らかとなった．この蛋白で免疫したマウスの抗血清は *C. renale* 線毛の全表面と結合する抗体を含んでいた．以上の成績は，*C. renale* 線毛の構成蛋白をコードする遺伝子がクローニングされ，これが大腸菌で発現されたことを示している．