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Expression of *Actinomyces viscosus* Antigens in *Escherichia coli*: Cloning of a Structural Gene (*fimA*) for Type 2 Fimbriae

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A cosmid gene library of *Actinomyces viscosus* T14V was prepared in *Escherichia coli* to examine the expression of *A. viscosus* antigens and to gain insight into the structure of *A. viscosus* type 1 and type 2 fimbriae. Out of this library of 550 clones, 28 reacted in a colony immunoassay with antibodies against *A. viscosus* cells. The proteins responsible for these reactions were identified in three clones. Clones AV1209 and AV2009 displayed nonfimbrial antigens with subunits of 40 and 58 kilodaltons, respectively. Clone AV1402 showed a 59-kilodalton protein that reacted with monospecific antibody against type 2 fimbriae and that comigrated with a subunit of type 2 fimbriae during sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This indicates that AV1402 expresses a gene (*fimA*) for a subunit of *A. viscosus* type 2 fimbriae.

Actinomyces viscosus is a gram-positive bacterium that is often found in high numbers in human dental plaque, where it may play a role in periodontal infections (reviewed in reference 14). The colonization of teeth by this microbe involves two antigenically distinct types of fimbriae (type 1 and type 2). Type 1 fimbriae selectively promote adherence to saliva-coated hydroxyapatite (9, 28). In contrast, type 2 fimbriae mediate attachment of *A. viscosus* to certain streptococci that predominate in developing dental plaque (7). This latter property depends on a lactose-sensitive lectin activity associated with type 2 fimbriae (6, 8, 25) and complementary carbohydrate-containing receptors on the streptococcal surface. Whereas the existence and function of each type of fimbriae has been clearly demonstrated, little is known about their subunits. This is largely due to their resistance to dissociation by methods that will extensively depolymerize fimbriae from gram-negative organisms (23, 28). To identify and characterize putative subunits and precursors of *A. viscosus* fimbriae, a study to clone the respective genes was undertaken.

MATERIALS AND METHODS

Strains. *Escherichia coli* HB101 (3) and *A. viscosus* T14V (6, 8) have been described previously.

Preparation of *A. viscosus* T14V chromosomal DNA. Cells were grown in 400 ml of medium and treated with lysozyme as described (5). They were suspended in 40 ml of 10 mM Tris–1 mM EDTA (pH 8.0), and lysed by adding 4.4 ml of 10% sodium dodecyl sulfate (SDS) with a 15-min incubation at 65°C. In this and subsequent steps care was taken not to shear the DNA. The viscous lysate was extracted with an equal volume of phenol saturated with 3% NaCl and then with chloroform-isoamyl alcohol (24:1 [vol/vol]). After addition of 2 volumes of ethanol, the precipitate was collected by centrifugation and dissolved in 25 ml of 10 mM Tris–1 mM EDTA–10 mM NaCl (pH 8.0). After treatment with RNase A (100 μg/ml) and RNase T1 (100 U/ml) for 1 h at 37°C, proteinase K was added (100 μg/ml), and the incubation was continued at 55°C for another hour. The solution was extracted with phenol and chloroform as described previously, adjusted to 3 M ammonium acetate, and left at 4°C for 30 min. The resulting precipitate was removed by centrifugation, and the DNA was precipitated with 2 volumes of ethanol.

Preparation of cosmid library. *A. viscosus* DNA (50 μg) was partially restricted for 10 min at 22°C with 2.5 U of *Mbo*I in 750 μl of TA buffer (24). The reaction was stopped by adding EDTA to 20 mM with a 15-min incubation at 65°C. The DNA was extracted with phenol and chloroform, precipitated with alcohol, and fractionated by a 20-h centrifugation (26,000 rpm) through a 10 to 40% sucrose gradient in a Beckman SW27 rotor as described by Maniatis et al. (22). Fractions (1.2 ml) were collected and analyzed by electrophoresis in 0.4% agarose, and those containing DNA of 20 to 45 kilobases were pooled.

Plasmid pHC79 was isolated from *E. coli* HB101(pHC79) by the method of Guerry et al. (15) and purified by cesium chloride-ethidium bromide centrifugation (22). This preparation was digested to completion with *Bam*HI, extracted with a mixture (1:1 [vol/vol]) of phenol-chloroform and then with chloroform, and precipitated with ethanol in the presence of sodium acetate.

For the ligation, 3.3 μg of *Bam*HI-cut pHC79 and 5 μg of *Mbo*I-digested *A. viscosus* DNA (20 to 45 kilobases) were incubated overnight at 12°C with 2 U of T4 ligase in 20 μl of ligation buffer (22). Of this mixture, 5 μl was added to an in vitro lambda packaging system (Bethesda Research Laboratories), and the DNA was packaged according to the recommendations of the manufacturer. Phage were adsorbed onto log-phase, maltose-grown, *E. coli* HB101 cells for 10 min at 30°C, diluted to 1 ml with L broth, and incubated for 40 min at 37°C. Aliquots were plated on L agar plus ampicillin (50 μg/ml). About 550 colonies were subcultured in L broth plus ampicillin and stored at 4°C to serve as the library (17).

Analysis of DNA. Cosmid clones were analyzed by the method of Birnboim and Doly (2, 22). Electrophoresis before and after *Bam*HI digestion was conducted in 0.7% agarose with 40 mM Tris–20 mM sodium acetate–2 mM Na_2EDTA as buffer (pH 7.7). Gels were stained with ethidium bromide (0.5 μg/ml), destained in water, and photographed.

Antibodies and antigens. Antibodies R32 and R66 were prepared by immunization of rabbits with *A. viscosus* wild-type and nonfimbriated mutant cells, respectively. The immunoglobulin G (IgG) was purified by DE-52 cellulose (Whatman) column chromatography (8, 9, 25). The nonfimbriated mutant was isolated from a type 2^- strain (8) by its

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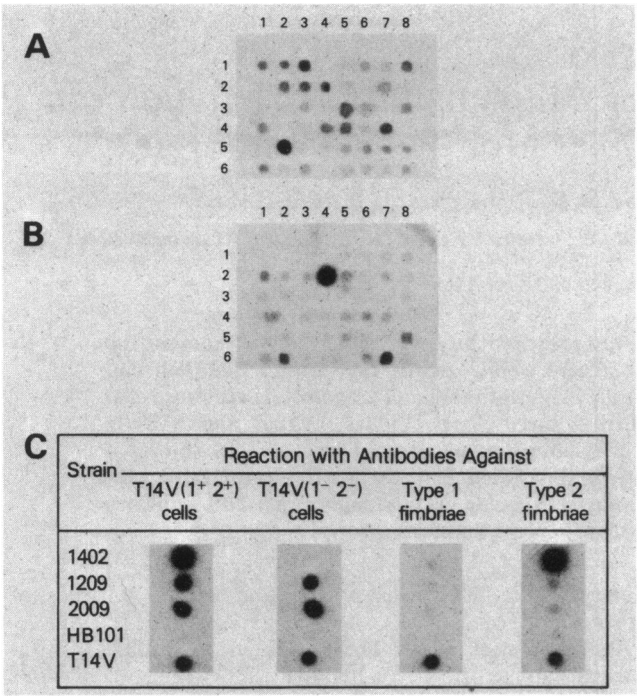

FIG. 1. Colony immunoassays. (A) The set of 42 clones selected from the initial screening (see the text) was probed with antibody R32 (10 μg of IgG per ml) against fimbriated (type 1^+, type 2^+) *A. viscosus* T14V. *E. coli* HB101(pHC79) was applied as a negative control at six locations on the filter (e.g., row 1, columns 4 and 5). (B) The same set was probed with antibody R66 against a nonfimbriated (type 1^-, type 2^-) mutant of strain T14V. (C) The three clones that reacted strongly with 1 μg of IgG per ml (see the text) were probed with the antibodies shown. For the reactions with strain T14V, 10^4 *A. viscosus* cells in 1 μl of PBS-Tween was applied to the filter just before the chloroform treatment in the colony immunoassay procedure.

failure to agglutinate with monospecific antibody R56 against type 1 fimbriae (manuscript in preparation). Monospecific antibodies R55 and R56 against type 2 and type 1 fimbriae, respectively, have been described previously (8). All IgG preparations were adsorbed with washed *E. coli* HB101(pHC79) cells and with an affinity gel prepared by coupling a sonic extract of this strain to CNBr-activated (12) Sepharose CL-4B (Pharmacia Fine Chemicals). Type 2 fimbriae of *A. viscosus* T14V were purified as described previously (8, 25) by elution with 3 M sodium thiocyanate from an affinity column prepared by coupling monoclonal antibody 2A (6) to CNBr-activated (12) Sephacryl S1000 (Pharmacia).

Colony immunoassays. One microliter of culture was spotted on a nitrocellulose filter placed on L agar containing 50 μg of ampicillin per ml. After 16 to 24 h of incubation at 37°C, the filters were processed by the procedure of Helfman et al. (16), except that phosphate-buffered saline (PBS; 0.9% NaCl, 0.02% sodium azide, 0.02 M phosphate [pH 7.2]) containing 0.05% Tween 20 (PBS-Tween) was used after the overnight lysis for all remaining steps (1). Filters were incubated 2 to 4 h with antibody (1 or 10 μg of IgG per ml), washed, incubated overnight with 2×10^6 cpm of ^{125}I-labeled protein A (35 mCi/mg; Amersham Corp.) in 25 ml of PBS-Tween, washed, dried, and autoradiographed.

Immunoblotting. Washed cells from 10 ml of L broth were suspended in 1 ml of sample buffer (20) and kept at 100°C for 10 min. After a 15-min centrifugation at 148,000 × *g*, 50 μl of the supernatant fluid was subjected to SDS electrophoresis in 10% polyacrylamide gels according to the method of Laemmli (20). ^{14}C-labeled protein standards (Amersham) were run on each gel, and the M_r values supplied by the manufacturer were used to calculate molecular masses. The proteins were transferred electrophoretically to nitrocellulose for 15 h at 10 V (0.35 A) in 50 mM sodium phosphate (pH 7.5) (1). After blocking for 2 h with 0.5% bovine serum albumin in PBS-Tween, the nitrocellulose sheets were further processed as described above.

RESULTS

Construction of an *A. viscosus* gene library. Cosmid cloning with pHC79 as vector (17) was used to establish a library of the *A. viscosus* T14V genome in *E. coli* HB101. About 550 presumptive recombinant colonies were picked from the ampicillin selection plates and subcultured. Analysis of 18 randomly selected clones showed that all contained a plasmid ranging in size from ca. 40 to 50 kilobases, and treatment with *Bam*HI revealed that these plasmids had distinct restriction patterns (data not shown). Thus, strictly on a statistical basis (10), it is likely ($P > 0.99$) that the collection of 550 clones represents the entire genome of *A. viscosus* T14V. Nevertheless, a strong bias probably exists against *A. viscosus* DNA sequences that express proteins deleterious to *E. coli*.

Expression of *A. viscosus* antigens. The entire cosmid library was first probed with antibody (R32) prepared against fimbriated (type 1^+, type 2^+) *A. viscosus* T14V cells to obtain a minimum estimate of the frequency of *A. viscosus* antigen expression in *E. coli*. Based on the results of these initial colony immunoassays, 42 clones were selected for further testing. Twenty-eight of these gave signals of varying intensity on autoradiograms after growth on nitrocellulose filters, in situ lysis, and sequential incubation with antibody (10 μg of IgG per ml) and ^{125}I-labeled protein A (Fig. 1A). Several of these clones were also labeled by antibody R66 against a nonfimbriated (type 1^-, type 2^-) mutant of strain T14V, whereas others reacted weakly or not at all (Fig. 1B). At a 10-fold lower antibody concentration (1 μg of IgG per ml), only one clone still gave a strong response with R32, and two clones gave a strong response with R66. These were examined further. AV1209 and AV2009 reacted both with R32 and R66 IgG, and not with monospecific antibodies against type 1 and type 2 fimbriae, which indicates that they express nonfimbrial antigens (Fig. 1C). In contrast, AV1402 reacted with antibody prepared against fimbriated bacteria as well as with monospecific antibody against type 2 fimbriae (R55), but not with antibody directed against nonfimbriated bacteria or type 1 fimbriae. None of the other clones shown in Fig. 1A reacted with monospecific antibodies against type 1 or type 2 fimbriae (data not shown).

Characterization of immunoreactive proteins. To identify the proteins responsible for the strong signals in the colony immunoassays, SDS extracts of clones AV1209, AV1402, and AV2009 were electrophoresed in duplicate, and the separated proteins were transferred to nitrocellulose sheets. When probed with antibodies against fimbriated and nonfimbriated cells, clones AV1402, AV1209, and AV2009 showed immunoreactive proteins with apparent molecular masses of 59, 40, and 58 kilodaltons, respectively (Fig. 2). In agreement with the colony immunoassays, the 59-kilodalton protein from AV1402 reacted only with IgG against fimbriated bacteria, whereas the antigens from AV1209 and AV2009 were readily detected with antibody directed against nonfimbriated cells.

The previous experiments indicated that the 59-kilodalton protein expressed by AV1402 was cross-reactive with type 2 fimbriae from *A. viscosus*. The relationship between this protein and type 2 fimbriae was further assessed by comparing SDS extracts of AV1402 and purified type 2 fimbriae. After electrophoresis and transfer to a nitrocellulose sheet, probing with monospecific antibody against type 2 fimbriae revealed that the fimbrial preparation contained one component indistinguishable in size from the immunoreactive gene product of AV1402 (Fig. 3). In similar experiments, the 59-kilodalton protein was also detected by its reaction with monoclonal antibodies (results not shown) known to bind repeating epitopes of type 2 fimbriae (6). In addition to this component, a number of larger polypeptides, including one of 85 kilodaltons and a faint one of ca. 34 kilodaltons, were observed in the sample of partially dissociated type 2 fimbriae (Fig. 3, lane 4). These components were not seen in the AV1402 preparation.

DISCUSSION

The present study demonstrates that molecular cloning can be used to gain insight into the molecular architecture of *A. viscosus* fimbriae. One clone, AV1402, was identified that expressed a structural gene for a subunit of type 2 fimbriae. This conclusion is based on the reaction of monospecific and monoclonal antibodies with similarly sized proteins from AV1402 and type 2 fimbriae (Fig. 3). At this stage it is not known whether the 59-kilodalton protein expressed by AV1402 is identical to the fimbrial subunit, contains a leader sequence, or is modified in some other way. Although preliminary results indicate that the *fimA* gene product occurs in the cytoplasm of AV1402, its exact distribution within the cell is unknown.

In addition to the 59-kilodalton species, many higher-molecular-mass polypeptides, including one of ca. 85 kilodaltons, were detected in the preparation of type 2 fimbriae (Fig. 3). This may indicate that these fimbriae are composed of nonidentical subunits. The 85-kilodalton species could be derived from the 59-kilodalton protein and a second, smaller, component. In this regard, an immunoreactive *A. viscosus* protein of ca. 34 kilodaltons has been observed as a faint band in partially dissociated fimbriae (Fig. 3, lane 4), and more clearly in extracts of *A. viscosus* cells (unpublished data). Studies to identify the gene for this protein are in progress.

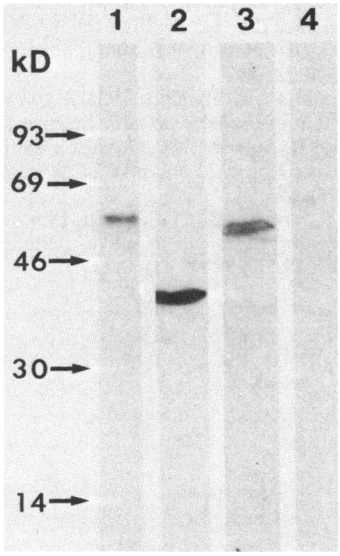

FIG. 2. Identification of immunoreactive *A. viscosus* proteins expressed in *E. coli*. Lane 1, AV1402 probed with R32 IgG against wild-type (fimbriated) strain T14V; lane 2, AV1209 probed with R66 IgG against a nonfimbriated mutant of strain T14V; lane 3, AV2009 probed with R66 IgG; lane 4, *E. coli* HB101(pHC79) probed with either R32 or R66 IgG. Arrows indicate the molecular mass markers.

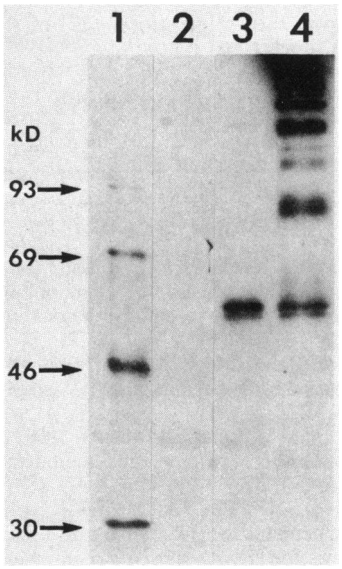

FIG. 3. Comparison between type 2 fimbriae and the immunoreactive protein expressed by AV1402. Preparations were subjected to SDS-polyacrylamide gel electrophoresis, and the proteins were transferred to nitrocellulose and probed with monospecific antibody (R55) against type 2 fimbriae. Lane 1, Standards with their molecular masses indicated in the left-hand margin; lane 2, *E. coli* HB101(pHC79); lane 3, AV1402; lane 4, purified type 2 fimbriae from *A. viscosus* T14V.

Certain genes from gram-positive organisms belonging to genera such as *Streptococcus* (18, 19, 26), *Bacillus* (4, 11, 13), and *Lactobacillus* (21) have been found to express in *E. coli*. However, the genus *Actinomyces* is only distantly related to these genera, and no information was available on the frequency of expression of *Actinomyces* genes in *E. coli*. To answer this question, the gene library was first screened with antibody that recognizes a number of *A. viscosus* antigens, including the fimbriae. At the antibody concentration used for screening, 5% of the clones gave an autoradiographic signal. At a 10-fold lower antibody concentration, only 1% of the clones still gave a visible signal. Only these latter clones (AV1209, AV1402, and AV2009) contained proteins that could be readily detected after electrophoresis and immunoblotting. This result could be due to the high level of antigen in these clones or the high antibody titers to these proteins. On the other hand, repeated screening of the library has not revealed a clone reacting with antibody directed against type 1 fimbriae. Reasons that could account for this include (i) the gene does not express in *E. coli*, (ii) the appropriate sequence has not been cloned in an uninterrupted fashion, and (iii) expression is deleterious to the host. Other cloning methods are currently being explored to circumvent these problems. Within this context it should be noted that some of the genes that did express may be transcribed from a promoter in the pHC79 vector. In fact, ongoing experiments suggest that *fimA* expression in AV1402

is dependent upon the P2 promoter (27) of the tetracycline resistance gene in pHC79.

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