JOURNAL OF BACTERIOLOGY, Aug. 1984, p. 683–687
0021-9193/84/080683-05$02.00/0

Vol. 159, No. 2

Cloning and Expression of a Gene Segment Encoding the Enzymatic Moiety of *Pseudomonas aeruginosa* Exotoxin A

MARK A. MOZOLA,* RUSSELL B. WILSON, ELZORA M. JORDAN, ROCKFORD K. DRAPER, AND ROYSTON C. CLOWES

Programs in Biology, The University of Texas at Dallas, Richardson, Texas 75080

Received 2 April 1984/Accepted 15 May 1984

Using the broad-host-range plasmid vector pRO1614, we cloned a segment of the gene from *Pseudomonas aeruginosa* PA103 encoding the enzymatically active part of the exotoxin A protein. Expression of the cloned gene segment has been achieved both in *Escherichia coli* and in a nontoxigenic *P. aeruginosa* host, as assayed by the production of exotoxin A-related antigen and by the ability of the gene product to ADP-ribosylate elongation factor 2. Western blot hybridization analysis revealed a series of polypeptides antigenically related to exotoxin A, the largest of which had a molecular weight of ca. 50,000.

Pseudomonas aeruginosa exotoxin A, a protein of ca. 68,000 molecular weight secreted by many strains of *P. aeruginosa* (2, 28), may be a virulence factor of this species (9, 19, 36), which in recent years has emerged as one of the most common organisms acquired by hospital infection. The mode of action of exotoxin A closely parallels that of diphtheria toxin (26), causing the arrest of protein synthesis within the cytoplasm of mammalian cells by catalyzing the covalent attachment of the ADP-ribosyl moiety of NAD^+ to elongation factor 2 (EF-2), thus inactivating EF-2 and killing the cell (14, 35). Other aspects of the mechanism of exotoxin A action are less well understood, specifically, how that part of exotoxin A comprising the active site is transported through a membrane barrier into the cell cytoplasm.

There is also a broader interest in understanding the cytocidal mechanisms of protein toxins such as exotoxin A, because they can be used to construct hybrid toxins that may have clinical applications. Hybrid toxins are prepared by attaching a natural toxin, or part of a natural toxin, to a component that binds to a specific cell surface receptor (for example, a monoclonal antibody), thereby directing the toxin to attack only those cells bearing the chosen receptor (25). We report here the cloning of a segment of *P. aeruginosa* chromosomal DNA encoding a part of exotoxin A that contains the enzymatically active site. The availability of the gene for exotoxin A should facilitate the production of structural variants of the protein that will be useful in structural-functional studies of exotoxin A action and in constructing new hybrid toxins.

MATERIALS AND METHODS

Bacterial strains and plasmids. HB101 is a *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^- F^- *Escherichia coli* K-12 strain without restriction activity (3). PA103 is a prototrophic, high-exotoxin-A-producing strain of *P. aeruginosa* (18). Several derivatives of PAO1, another wild-type toxigenic *P. aeruginosa* strain, were also used: PAO286, a *met-28 trp-6* mutant (32); PAO949, a *thr-9001 cys-59 pur-67* mutant (29); and GMA253, a *cys-5605 his15075* mutant (22, 31). The broad-host-range vector plasmid pRO1614, able to replicate in either *P. aeruginosa* or *E. coli*, is described by Olsen et al. (24); pRO1614 is a pBR322 plasmid with an approximate 1.85-kilobase-pair (kbp) segment inferred to be the replication control segment of RP1 inserted into the *Pst*I site. It thus carries the carbenicillin and tetracycline resistance determinants and cloning sites of pBR322, including the unique *Bam*HI cleavage site within the tetracycline resistance (*tet*) gene. The chromosome-mobilizing R-plasmid R68.45 used in mapping studies of *Pseudomonas* sp. has been described previously (10, 11).

Media. Nutrient agar contained (per liter) 25 g of Oxoid nutrient broth no. 2, 5 g of Difco yeast extract, and 12 g of agar. TSBD medium was prepared as described by Iglewski and Sadoff (15), deferrated with $CaCl_2$, and supplemented with 0.1 M monosodium glutamate and 1% glycerol. Solid TSBD was prepared by adding agarose (SeaKem) to 1.2%. Minimal glucose medium has been previously described (13) and was supplemented with amino acids and nucleotides, each to a concentration of 20 μg/ml. Where appropriate, disodium carbenicillin was added to a concentration of 300 μg/ml.

Isolation and mapping of the Tox^- mutant MAM2. An exotoxin A-deficient (Tox^-) mutant of *P. aeruginosa* was isolated after mutagenesis of strain PAO286 with nitrosoguanidine (1). This mutant, MAM2, was initially identified because it did not produce material that cross-reacted with anti-exotoxin A antibodies (CRM^- phenotype) as assayed by the Elek test (2), an immunodiffusion assay for secreted bacterial proteins. The mutation in MAM2 responsible for the CRM^- phenotype was mapped as described by Haas and Holloway (10, 11) as follows. The chromosome-mobilizing R-factor plasmid R68.45 was transferred to MAM2 by conjugation, and plate matings were then performed with MAM2(R68.45) as donor and various auxotrophic PAO strains as recipients. Recombinants that were prototrophic for various markers were selected on appropriately supplemented minimal glucose media, and coinheritance of the CRM^- phenotype with the selected nutritional marker was scored by the Elek test. In each cross, 100 recombinants were tested.

Cloning procedures and filter immune assays. Plasmid and chromosomal DNA was isolated as described by Olsen et al. (24). Restriction endonuclease cleavage and electrophoresis on polyacrylamide and agarose gels were carried out as described previously (6). Chromosomal DNA from *P. aeruginosa* PA103 was partially digested with *Sau*3A (New England Biolabs), and a DNA fraction enriched for 10- to 15-kbp fragments was obtained after centrifugation of the digested DNA through a 10 to 40% sucrose gradient. This DNA fraction was ligated to *Bam*HI-cleaved (Boehringer-Mannheim Biochemicals) pRO1614 vector DNA with T4 DNA ligase (New England Biolabs), using a 20:1 ratio (wt/

* Corresponding author.

wt) of chromosomal to vector DNA. *E. coli* HB101 was transformed with the ligated DNA mixture essentially as described by Cohen et al. (8). (For transformation of *P. aeruginosa* strains, the procedures of Olsen et al. [24] were used). Cb^r transformants were selected on nitrocellulose filters (HATF; Millipore Corp., 82 mm) placed on TSBD plates. Dilutions of the transformation mixture were spread to give 3,000 to 10,000 Cb^r colonies per filter. The plates were incubated for ca. 20 h at 30°C, at which time replica filters were made essentially as described by Young and Davis (37). The original and replica filters were then reincubated at 30°C for ca. 2 h to allow the colonies to regenerate, after which the colonies on the original filter were lysed in situ with sodium hydroxide (neutralized), and the proteins were fixed to the filters by baking at 60°C for 2 h, all by the methods of Meyer et al. (23). The filters were then preincubated in a solution of 3% immunoglobulin-free bovine serum albumin (BSA; Sigma Chemical Co.) in TBS buffer (150 mM NaCl, 50 mM Tris [pH 7.5]) at 37°C for 2 h, after which anti-exotoxin A immunoglobulin G (ammonium sulfate fraction of rabbit antiserum raised against purified exotoxin A as described by Vasil et al. [35]) was added to a concentration of 100 μg/ml, and incubation was continued at room temperature with gentle agitation for ca. 18 h. The filters were then washed extensively in six changes of TBS containing 0.1% sodium dodecyl sulfate (SDS) in the first wash and then again preincubated in BSA-TBS as described above. *Staphylococcus aureus* protein A (Sigma), labeled with ^{125}I by using Iodo-beads (Pierce) as described by Markwell (21), was then added to a concentration of 10^6 cpm/ml (specific activity = 9×10^6 cpm/μg), and the filters were incubated at room temperature with gentle agitation for 1 to 4 h. The filters were then washed as described above, and autoradiography was performed.

For verification of original isolates as antigen positive, areas from the replica filters carrying the putative clones were excised, and the bacteria were suspended in 0.85% NaCl. The bacterial suspensions were streaked onto nutrient agar plates containing carbenicillin and incubated overnight at 30°C. Bacteria from single colonies were then inoculated to nitrocellulose filters on supplemented TSBD plates with carbenicillin, incubated overnight at 30°C, and tested for exotoxin A-related antigen by the method described above.

Western blot hybridization analysis. Exotoxin A-related polypeptides expressed by antigen-positive transformants were examined by Western blot analysis (5). Cultures (50 ml) were grown to a density of ca. 5×10^8 cells per ml in supplemented TSBD medium plus 300 μg of carbenicillin per ml. Bacteria were pelleted by centrifugation at 4°C and suspended in 2 ml of ice-cold 50 mM Tris (pH 7.6). The suspensions were sonicated in an ice bath, and cell debris was removed by centrifugation in an Eppendorf microcentrifuge at 4°C. Next, 8 μg of DNase I (Sigma) and 24 μg of RNase A (Sigma) were added, and incubation was carried out for 10 min at 4°C. Samples (25 μl) were removed, mixed with 25 μl of 2× Laemmli (17) SDS sample buffer, boiled for 1 min, applied to a 10% polyacrylamide gel (17) containing 1% SDS with a 3% stacking gel, and electrophoresed for 8 h with 20 mA of constant current. The gel was rinsed with distilled water and soaked in a solution containing 20% methanol, 20 mM Tris, and 150 mM glycine (pH 8.3). The proteins were then transferred to a nitrocellulose sheet (BA85; Schleicher & Schuell, Inc.; 0.45 μm) in blot buffer with an E.C. Electroblot apparatus at 400 mA for 18 h at 4°C. After transfer, the nitrocellulose was rinsed with distilled water and stained with amido black (0.1% in 45% methanol–10% acetic acid) (34) for 1 min to visualize molecular weight standards (Sigma). The nitrocellulose was then rinsed with TBS buffer at room temperature until completely destained (ca. 30 min) and then preincubated in a solution of 3% immunoglobulin-free BSA in TBS at 37°C for 1 h with gentle agitation. Anti-exotoxin A immunoglobulin G was then added to the BSA solution to a concentration of 11 μg/ml, and incubation was continued at room temperature with agitation for 2.5 h. After incubation, the nitrocellulose sheet was washed three times for 15 min each with a solution of 10 mM Tris (pH 7.4), 150 mM NaCl, 0.2% Nonidet P-40, 0.1% SDS, and 0.25% sodium deoxycholate; rinsed with TBS; and again preincubated in 3% BSA for 10 min at 37°C. After preincubation, ^{125}I-labeled *S. aureus* protein A was added to a concentration of 10^6 cpm/ml, and incubation was continued for 45 min at room temperature with agitation. The nitrocellulose was washed as above and dried, and autoradiography was performed to visualize polypeptides that reacted with anti-exotoxin A antibodies.

Assay of EF-2 ADP-ribosylating activity. Bacterial cell extracts were prepared by sonication as described above. EF-2 from wheat germ was prepared according to Chung and Collier (7). ADP ribosylation of EF-2 was assayed by a modification of the procedures of Chung and Collier (7) as follows. Samples (60 μl) of bacterial extract (310 μg of protein), exotoxin A (0.2 μg), or fragment A of diphtheria toxin (7.6 μg) in 10 mM Tris buffer (pH 7.5) were mixed with 60 μl of 8 M urea in 10 mM Tris buffer (pH 7.5) plus 2% dithiothreitol. The samples were incubated for 15 min at 24°C to activate the ADP-ribosyl transferase activity of exotoxin A; 20 μl of the activated samples were mixed with 50 μl of EF-2 in 125 mM Tris buffer (pH 8.2) plus 0.2 mM EDTA and 100 mM dithiothreitol. The reaction was initiated by the addition of 25 μM [^{14}C]NAD^+ (291 mCi/mmol; Amersham Corp.). After incubation at 24°C for 1 h, the samples were placed on squares of Whatman 3MM paper that had been pretreated by soaking in 5% trichloroacetic acid and dried; paper squares containing the samples were immersed in 5% trichloroacetic acid for 20 min, rinsed in 95% ethanol, dried, and assayed for radioactivity with a liquid scintillation counter.

RESULTS AND DISCUSSION

Chromosomal DNA from *P. aeruginosa* PA103 was partially digested with *Sau*3A, and fragments of ca. 10 to 15 kbp were separated by sucrose gradient centrifugation. This chromosomal DNA fraction was ligated to *Bam*HI-cleaved pRO1614. The ligated DNA was added to competent *E. coli* HB101 cells, and the resulting mixture was spread onto nitrocellulose filters placed upon nutrient agar plates containing carbenicillin to select for transformants.

The Cb^r transformant colonies arising after overnight growth at 30°C were lysed in situ, and the proteins were heat fixed to the nitrocellulose filters. Transformants expressing exotoxin A-related antigens were identified by means of the filter immune assay, employing anti-exotoxin A antibodies and ^{125}I-labeled *S. aureus* protein A. Antigen-positive clones were obtained at a frequency of ca. 10^{-3} of transformants containing chromosomal DNA inserts (Cb^r Tc^s); one such experiment yielded clone 56C1, which was retested for the antigen-positive phenotype (Fig. 1, rows A and B) and analyzed in detail. The recombinant plasmid found in clone 56C1 has been designated pRC345.

Digestion of pRC345 with *Bam*HI produced nine fragments ranging in size from 0.16 to 6.4 kbp (data not shown). The order of the fragments (Fig. 2A) was determined from

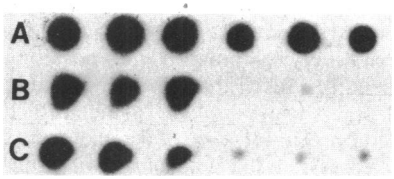

FIG. 1. Identification of antigen-positive clone 56C1 by the filter immune assay. Row A (left to right): first three spots, *P. aeruginosa* PA103; second three, *P. aeruginosa* PAO286. Row B: first three, *E. coli* HB101(pRC345); second three, HB101(pRO1614). Row C: first three, MAM2(pRC345); second three, MAM2(pRO1614).

digestion of pRC345 with *Eco*RI, or double digestion with *Eco*RI and *Bam*HI, and by *Bam*HI cleavage of the independent *Eco*RI fragments isolated by electroelution. The total size of the PA103 chromosomal DNA insert was 17.7 kbp; the largest *Bam*HI fragment was concluded to represent the pRO1614 vector. This vector DNA fragment was 0.2 kbp larger than pRO1614, since it was found to carry a chromosomal *Sau*3A fragment at one end as determined by cleavage with *Bam*HI and *Ava*I. This would be expected since ligation of *Sau*3A to *Bam*HI restriction fragments always regenerates *Sau*3A recognition sites, but only occasionally regenerates *Bam*HI recognition sites.

The nature of the immunologically reactive protein synthesized by clone 56C1 was investigated by Western blot hybridization analysis (Fig. 3). HB101 carrying pRC345 produced a collection of polypeptides antigenically related to exotoxin A. The largest of these polypeptides had a molecular weight of ca. 50,000. If this polypeptide is the primary translation product, then pRC345 apparently does not contain the entire exotoxin A coding sequence. The smaller, immunologically reactive polypeptides observed are presumed to arise from proteolyic digestion of the 50,000-molecular-weight polypeptide.

The nature of the exotoxin A-related polypeptides produced by pRC345 when present in the *P. aeruginosa* Tox$^-$ mutant MAM2 was also investigated. In addition to possessing a CRM$^-$ phenotype as assayed by the Elek test, MAM2 produced little, if any, intracellular exotoxin A-related antigen as determined by the filter immune assay (Fig. 1, row C) or Western blot hybridization. The mutation in MAM2 responsible for the CRM$^-$ phenotype appeared to be in the exotoxin A structural gene; the CRM$^-$ phenotype of MAM2 was demonstrated to be 25 and 5% linked, respectively, with the *pur-67* and *hisI* markers on the *P. aeruginosa* PAO chromosome (29) by genetic mapping with the chromosome-mobilizing R-factor R68.45. No linkage (<1%) was found between the CRM$^-$ phenotype of MAM2 and *thr-9001*. This locates the Tox$^-$ mutation in MAM2 in the same region of the chromosome as the *toxA1* mutation of Hanne et al. (12), which defines the exotoxin A structural gene.

MAM2 carrying pRO1614 showed no immunologically reactive proteins in the Western blot hybridization analysis, whereas MAM2 carrying pRC345 produced a collection of exotoxin A-related polypeptides similar to that seen in HB101 (pRC345) (Fig. 3). Presumably, the primary translation product is recognized as an abnormal protein in both *E. coli* and *P. aeruginosa* and is subject to proteolytic attack in both hosts.

The exotoxin A-related protein encoded by pRC345 possessed enzymatic activity, even though the protein expressed from the clone was not full-size exotoxin A and was apparently subject to proteolysis. Table 1 shows results of an assay for the EF-2 ADP-ribosylating activity of cytoplasmic fractions from *E. coli* or *P. aeruginosa* host cells carrying pRC345. Cytoplasmic fractions, rather than culture supernatants, were assayed for enzymatic activity since neither HB101(pRC345) nor MAM2(pRC345) appeared to secrete exotoxin A-related antigen as assayed by the Elek test (data

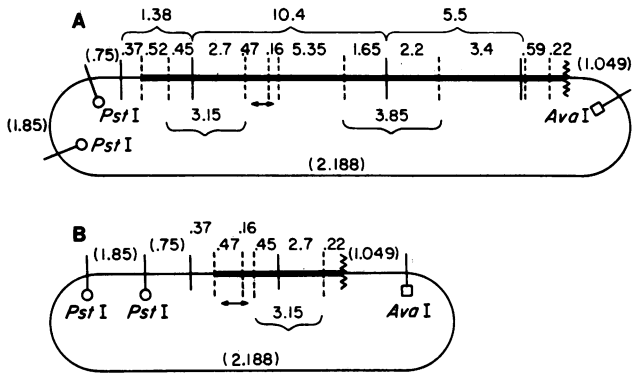

FIG. 2. *Bam*HI and *Eco*RI restriction maps of plasmids encoding exotoxin A-related polypeptides. The thin lines represent the vector pRO1614, and the thick lines represent PA103 chromosomal DNA. The symbols |, ¦, and } indicate *Eco*RI, *Bam*HI, and *Sau*3A recognition sites, respectively (only one terminal *Sau*3A site on the insert and vector DNA being shown). Restriction fragment sizes are shown in kbp and were determined from cleavage analysis as discussed in the text. The arrow between the 0.47- and 0.16-kbp fragments indicates that their relative order is not established. The location of the *Ava*I and *Pst*I sites and the sizes of fragments shown in parentheses are derived from previously published data of pBR322 (33) and pRO1614 (24). (A) pRC345; (B) pRC346.

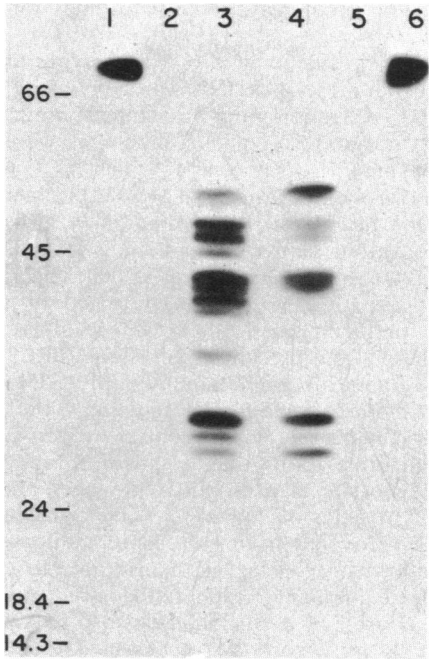

FIG. 3. Western blot hybridization analysis of exotoxin A-related polypeptides expressed by clone 56C1. Lanes: 1, purified exotoxin A (0.2 μg); 2, *E. coli* HB101(pRO1614); 3, HB101(pRC345); 4, *P. aeruginosa* MAM2(pRC345); 5, MAM2(pRO1614); 6, exotoxin A (0.2 μg). Positions of protein molecular weight standards (top to bottom: BSA, egg albumin, trypsinogen, β-lactoglobulin, and lysozyme) after electrophoresis are indicated (in thousands).

TABLE 1. ADP-ribosyl transferase activity in cell extracts[a]

Sample	[^{14}C]ADP-ribose incorported into EF-2 (cpm ± SD)
HB101(pRO1614)	256 ± 33
HB101(pRC345)	2,640 ± 500
MAM2(pRO1614)	168 ± 26
MAM2(pRC345)	2,060 ± 520
MAM2(pRC345) + anti-exotoxin A	61 ± 11
Exotoxin A	3,470 ± 145
Exotoxin A + anti-exotoxin A	55 ± 8
Diphtheria toxin fragment A	6,500 ± 380
Diphtheria toxin fragment A + anti-exotoxin A	7,500 ± 410

[a] All assays were done in triplicate. Samples treated with anti-exotoxin A antibody were incubated with the immunoglobulin G fraction (114 μg) for 10 min at 24°C before the addition of urea (see text). The specificity of the inhibitory effect of anti-exotoxin A on the activity of exotoxin A was tested in controls by measuring the effect of the anti-exotoxin A on the ADP-ribosyl transferase activity of fragment A of diphtheria toxin, which is not antigenically similar to exotoxin A but has the same enzymatic activity as exotoxin A. Since anti-exotoxin A did not interfere with the activity of fragment A, the effect of the antitoxin on the activity of exotoxin A was specific.

not shown). Extracts from HB101(pRC345) and MAM2(pRC345) possessed EF-2 ADP-ribosylating activity, whereas neither bacterial host carrying the vector plasmid pRO1614 showed significant enzymatic activity (Table 1). In addition, the enzymatic activity expressed by cells carrying the pRC345 plasmid was abolished by preincubation of the extracts with anti-exotoxin A antibodies. The component in the EF-2 fraction of wheat germ to which radioactivity was transferred was shown, by polyacrylamide gel electrophoresis, to have a molecular weight of ca. 90,000, in good agreement with previously published values for EF-2 (16) (data not shown).

To identify the region of pRC345 carrying exotoxin A-coding sequences, pRC345 DNA was subcloned by cleavage with *Bam*HI, religation, and transformation into *E. coli* HB101. Several antigen-positive subclones were identified by the filter immune assay, one of which, 8E4, was further studied. *E. coli* and *P. aeruginosa* MAM2 host cells carrying plasmid DNA from clone 8E4, when analyzed by Western blot hybridization, showed one immunologically reactive band with a molecular weight of ca. 35,000. This polypeptide did not express EF-2 ADP-ribosylating activity either with or without preincubation with urea (see above; data not shown). The plasmid present in this clone (pRC346) carries three of the *Bam*HI fragments found in pRC345, and double digestion with *Bam*HI and *Eco*RI, together with the isolation of *Eco*RI fragments and subsequent cleavage with *Bam*HI, showed their orientation to be as shown in Fig. 2B.

The catalytic site of exotoxin A has been shown to reside on polypeptides of 26,000 to 27,000 molecular weight that can be recovered from stored preparations (7, 35) or from chymotrypsin cleavage (20) of whole toxin. Also, a 48,000-molecular-weight polypeptide derived from digestion of whole toxin by *P. aeruginosa* elastase is enzymatically active (30). Recent work (M. L. Vasil, G. L. Gray, and C. C. R. Grant, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, D70, p. 62), in which the *toxA* gene was cloned, suggests that these enzymatically active fragments are derived from the C-terminal region of the exotoxin A polypeptide. Since the exotoxin A-related peptides produced by pRC345 are smaller than intact exotoxin A yet retain enzymatic activity, pRC345 is concluded to encode only a part of exotoxin A containing the C-terminal segment. This suggests that an interrupted coding sequence for exotoxin A resides adjacent to one of the two vector-insert junctions of pRC345. We have tentatively eliminated the possibility that this coding sequence resides at the right-hand vector-insert junction of the plasmids shown in Fig. 2, since pRO1614 carrying only the 0.22-kbp *Bam*HI-*Sau*3A fragment did not produce material that cross-reacted with anti-exotoxin A antibodies (data not shown). Thus, it seems likely that pRC345 carries the C terminus of the *toxA* structural gene at the left-hand vector-insert junction. In this event, we believe transcription is initiated from the P2 tetracycline resistance (*tet*) gene (4) promoter of pBR322 located on the 0.37-kbp *Eco*RI-*Bam*HI fragment, which results in transcription toward the *Bam*HI site (i.e., clockwise towards the insert DNA). Translation would thus be initiated at the start codon that begins at the nucleotide located 86 base pairs (clockwise) from the *Eco*RI site (27). If the exotoxin gene sequence is in the same reading frame, as appears to be the case, this would produce a hybrid polypeptide with 96 amino acids of the N-terminal region of the *tet* protein linked to a polypeptide carrying the antigenic and enzymatic activity of exotoxin A. Such a postulated hybrid polypeptide would include ca. 11,000 molecular weight of the *tet* protein (27); the remaining approximate 39,000 molecular weight would presumably derive from *toxA* structural gene sequences present primarily, or exclusively, on the 0.52-kbp *Bam*HI and 0.45-kbp *Bam*HI-*Eco*RI restriction fragments of pRC345. The absence of the N-terminal part of exotoxin A in a hybrid protein of this type also explains why the product is not secreted.

As stated above, subclone 8E4 produced a polypeptide of ca. 35,000 molecular weight that was immunologically related to exotoxin A. The order of the *Bam*HI restriction fragments at the left vector-insert junction is not the same in pRC346 as in pRC345, presumably due to cleavage and religation during the subcloning process (Fig. 2). This suggests that the 0.47- and 0.16-kbp *Bam*HI fragments are not part of the *toxA* coding sequence, but may provide a translation initiation codon that resulted in expression of a new hybrid polypeptide containing the *toxA* sequences present on the 0.45-kbp C-terminal *Bam*HI-*Eco*RI fragment. The fact that the exotoxin A-related polypeptide expressed from pRC346 does not possess EF-2 ADP-ribosylating activity may result from the absence of the 0.52-kbp *Bam*HI fragment in this subclone, or may be related to differences in protein conformation between the pRC345- and pRC346-encoded polypeptides.

We are presently screening new clones which we anticipate will contain the intact *toxA* structural gene. Such clones will be useful in site-directed mutagenesis experiments aimed at elucidating structural-functional relationships of exotoxin A and in the construction of hybrid toxins.

ACKNOWLEDGMENTS

We thank Bruce Holloway, George Jacoby, and Ronald Olsen for supplying bacterial strains and plasmids, Stephen Leppla for the gift of purified exotoxin A, and Beecham Laboratories for antibiotics.

This work was supported by Public Health Service grant from the National Institute of Allergy and Infectious Diseases to R.C.C. (AI-10638) and to R.K.D. (AI-17400) and was submitted in partial fulfillment of the requirements for an M.S. degree by R.B.W.

ADDENDUM IN PROOF

A recent report has appeared (Gray et al., Proc. Natl. Acad. Sci. U.S.A. **81**:2645–2649, 1984) describing the cloning and expression of the intact *toxA* gene of *P. aeruginosa*

PA103 in *E. coli*. Comparisons of restriction endonuclease cleavage and DNA nucleotide sequence data indicate that the sequences present in our clones represent the C-terminal region of the same gene as that cloned by Gray et al. and confirm our conclusions that the exotoxin A-encoding sequences found on pRC345 and pRC346 are present on the 0.45-kbp *Bam*HI-*Eco*RI and 0.52-kbp *Bam*HI restriction fragments.

LITERATURE CITED

1. **Adelberg, E. A., M. Mandel, and G. C. C. Chen.** 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. **Bjorn, M. J., M. L. Vasil, J. C. Sadoff, and B. H. Iglewski.** 1977. Incidence of exotoxin production by *Pseudomonas* species. Infect. Immun. **16**:362–366.
3. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
4. **Brosius, J., R. L. Cate, and A. P. Perlmutter.** 1982. Precise location of two promoters for the β-lactamase gene of pBR322. J. Biol. Chem. **257**:9205–9210.
5. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
6. **Chiang, S.-J., and R. C. Clowes.** 1980. Intramolecular transposition and inversion in plasmid R6K. J. Bacteriol. **142**:668–682.
7. **Chung, D. W., and R. J. Collier.** 1977. Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of *Pseudomonas aeguginosa*. Infect. Immun. **16**:832–841.
8. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. **69**:2110–2114.
9. **Cross, A. S., J. C. Sadoff, B. H. Iglewski, and P. A. Sokol.** 1980. Evidence for the role of toxin A in the pathogenesis of infection with *Pseudomonas aeruginosa* in humans. J. Infect. Dis. **142**: 538–546.
10. **Haas, D., and B. W. Holloway.** 1976. R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. Mol. Gen. Genet. **144**:243–251.
11. **Haas, D., and B. W. Holloway.** 1978. Chromosome mobilization by the R plasmid R68.45: a tool in *Pseudomonas* genetics. Mol. Gen. Genet. **158**:229–237.
12. **Hanne, L. F., T. R. Howe, and B. H. Iglewski.** 1983. Locus of the *Pseudomonas aeruginosa* toxin A gene. J. Bacteriol. **154**:383–386.
13. **Holmans, P. L., and R. C. Clowes.** 1979. Transposition of a duplicate antibiotic resistance gene and generation of deletions in plasmid R6K. J. Bacteriol. **137**:977–989.
14. **Iglewski, B. H., and D. Kabat.** 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc. Natl. Acad. Sci. U.S.A. **72**:2284–2288.
15. **Iglewski, B. H., and J. C. Sadoff.** 1979. Toxin inhibitors of protein synthesis: production, purification, and assay of *Pseudomonas aeruginosa* toxin A. Methods Enzymol. **68**:780–793.
16. **Iglewski, W. J., and H. Lee.** 1983. Purification and properties of an altered form of elongation factor 2 from mutant cells resistant to intoxication by diphtheria toxin. Eur. J. Biochem. **134**:237–240.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Liu, P. V.** 1966. The role of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. III. Identity of the lethal toxins produced *in vitro* and *in vivo*. J. Infect. Dis. **116**:481–489.
19. **Liu, P. V.** 1974. Extracellular toxins of *Pseudomonas aeruginosa*. J. Infect. Dis. **130**:594–599.
20. **Lory, S., and R. J. Collier.** 1980. Expression of enzymic activity by exotoxin A from *Pseudomonas aeruginosa*. Infect. Immun. **28**:494–501.
21. **Markwell, M.-A. K.** 1982. A new solid-state reagent to iodinate proteins. Anal. Biochem. **125**:427–432.
22. **Mee, B. J., and B. T. O. Lee.** 1969. A map order for *hisI*, one of the genetic regions controlling histidine biosynthesis in *Pseudomonas aeruginosa*, using the transducing phage F116L. Genetics **62**:687–696.
23. **Meyer, T. F., N. Mlawer, and M. So.** 1982. Pilus expression in *Neisseria gonorrhoeae* involves chromosomal rearrangement. Cell **30**:45–52.
24. **Olsen, R. H., G. DeBusscher, and W. R. McCombie.** 1982. Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. J. Bacteriol. **150**:60–69.
25. **Olsnes, S., and A. Pihl.** 1982. Chimeric toxins. Pharmacol. Ther. **15**:355–381.
26. **Pappenheimer, A. M., Jr.** 1977. Diphtheria toxin. Annu. Rev. Biochem. **256**:69–94.
27. **Peden, K. W. C.** 1983. Revised sequence of the tetracycline resistance gene of pBR322. Gene **22**:277–280.
28. **Pollack, M., N. S. Taylor, and L. T. Callahan III.** 1977. Exotoxin production by clinical isolates of *Pseudomonas aeruginosa*. Infect. Immun. **15**:776–780.
29. **Royle, P. L., H. Matsumoto, and B. W. Holloway.** 1981. Genetic circularity of the *Pseudomonas aeruginosa* PAO chromosome. J. Bacteriol. **145**:145–155.
30. **Sanai, Y., K. Morihara, H. Tsuzuki, J. Y. Homma, and I. Kato.** 1980. Proteolytic cleavage of exotoxin A from *Pseudomonas aeruginosa*: formation of an ADP-ribosyltransferase active fragment by the action of *Pseudomonas* elastase. FEBS Lett. **120**:131–134.
31. **Soldati, L., R. Crockett, J. M. Carrigan, T. Leisinger, B. W. Holloway, and D. Haas.** 1984. Revised locations of the *hisI* and *pru* (proline utilization) genes on the *Pseudomonas aeruginosa* chromosome map. Mol. Gen. Genet. **193**:431–436.
32. **Stanisich, V. A., and B. W. Holloway.** 1969. Conjugation in *Pseudomonas aeruginosa*. Genetics **61**:327–339.
33. **Sutcliffe, J. G.** 1979. Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. **43**:77–90.
34. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.
35. **Vasil, M. L., D. Kabat, and B. H. Iglewski.** 1977. Structure-activity relationships of an exotoxin of *Pseudomonas aeruginosa*. Infect. Immun. **16**:353–361.
36. **Young, L. S.** 1980. The role of exotoxins in the pathogenesis of *Pseudomonas* infections. J. Infect. Dis. **142**:626–630.
37. **Young, R. A., and R. W. Davis.** 1983. Efficient isolation of genes using antibody probes. Proc. Natl. Acad. Sci. U.S.A. **80**:1194–1198.