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Production of *Vibrio cholerae* Accessory Cholera Enterotoxin (Ace) in the Yeast *Pichia pastoris*

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Accessory cholera enterotoxin (Ace) is a recently identified toxin of *Vibrio cholerae*. Preliminary studies using crude toxin extracts in animal models indicate that Ace increases transcellular ion transport, which is proposed to contribute to diarrhea in cholera. The lack of purified toxin has hindered elucidation of the mechanism of action of Ace. In this study, *ace* was cloned and was expressed in and secreted by the methylotrophic yeast *Pichia pastoris*. Secreted toxin constituted 50% of the total supernatant protein from *Pichia pastoris*. Presumed monomer and dimer forms with molecular masses of 9 and 18 kDa, respectively, were observed. The 18-kDa form predominated. Biological activity was assayed by studying ion fluxes across epithelial membranes in Ussing chambers. Among the characteristics of Ace was the unusual property of staining with silver but not Coomassie blue stain. To our knowledge this is the first report of a biologically active bacterial toxin produced with the *P. pastoris* system. The purified protein may now be used in studies of the mechanism of action of Ace in physiologic systems.

Vibrio cholerae causes the potentially lethal disease cholera through the elaboration of the intestinal secretogen cholera toxin. Over 700,000 cases of cholera have been reported in the Western Hemisphere since the beginning of the most recent epidemic in 1991, and in 1992 alone, over 100 cases of cholera were reported in the United States (2). Three toxins of *V. cholerae* that alter the potential difference (PD) and/or resistance in Ussing chambers have been identified. They include cholera toxin, Zot (zonula occludens toxin) (5), which acts by loosening tight junctions, and Ace (accessory cholera enterotoxin) (11).

We have previously reported the identification, cloning, and sequencing of the *ace* gene (11). Preliminary studies using crude toxin extracts in animal models indicated that Ace acts by increasing transcellular ion transport. In these models, Ace increased short-circuit current in Ussing chambers and caused fluid secretion in ligated rabbit ileal loops, both characteristics of a classic enterotoxin (11).

Further characterization of Ace has been hampered by the very low levels of toxin produced by *V. cholerae*. To produce more toxin, attempts were made to clone *ace* into *Escherichia coli*; however, *ace* clones in *E. coli* were unstable, producing cell lysis. To overcome this limitation, we have utilized the methylotrophic yeast *Pichia pastoris* to express the Ace protein.

MATERIALS AND METHODS

Bacterial and yeast strains and plasmids. Bacterial and yeast strains and plasmids used in this study are listed in Table 1. All media were prepared by using Invitrogen Corporation protocols.

Plasmid construction. Plasmid pMT51 was constructed by insertion of the *ace* gene engineered by PCR with compatible restriction ends into the *Eco*RI site of pPIC9. Plasmid pMT51 was constructed to create an in-frame fusion between *ace* and the α-mating factor pre-pro peptide secretion signal present on plasmid pPIC9. The *ace* gene in pMT51 was sequenced following PCR amplification to confirm its sequence and to establish that it was in frame with the pre-pro peptide secretion signal.

***P. pastoris* expression system.** The *P. pastoris* expression system uses the tightly regulated, alcohol-inducible *AOX1* promoter to express the gene of interest for heterologous protein expression. Other essential components of the expression vector include the *HIS4* gene for selection of recombinants and 5′ and 3′ sequences of the *AOX1* locus to allow integration at the *AOX1* locus in the chromosome.

Transformation of linearized plasmid DNA into yeast spheroplasts. Linearized pMT51 DNA was used to transform spheroplasts (prepared by using the Invitrogen protocol) of yeast strain GS115, a histidine auxotroph which allows transformants to be selected by growth on complete media without histidine. His^+ transformants appearing on days 3 to 7 following transformation were picked and streaked onto minimal medium with methanol (MM) and minimal medium with dextrose (MD). Differential growth rates on these two media identify whether the transformants result from integration at the *AOX1* locus (Mut^s, inability to utilize methanol as a sole carbon source, and poor growth on MM) or from integration at the *his* locus (Mut^+, ability to utilize methanol as a sole carbon source, and equal growth on MM and MD).

PCR analysis of *Pichia* integrants. The *Pichia* integrants were analyzed by PCR to confirm the site of integration of plasmid pMT51. Genomic DNA was isolated from the *Pichia* integrants by using a standard protocol (Invitrogen). The genomic DNA was used as template in PCR using primers 5′ *AOX1* and 3′ *AOX1* (Invitrogen), corresponding to sequences present upstream and downstream, respectively, of the *ace* gene on the yeast-integrating plasmid pMT51. These primers are also present in the wild-type *AOX1* (alcohol oxidase) yeast gene. PCRs using *Taq* polymerase (1.25 U) were performed in 50-μl solutions containing yeast genomic DNA (1 μg), primers (500 ng), deoxynucleotide triphosphates (500 μM), and $MgCl_2$ (2 mM). The amplification cycle employed a hot start at 94°C for 2 min followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min to complete the cycle.

Expression of recombinant *Pichia* strains. The *Pichia ace* integrant clones were analyzed for expression of the Ace protein by using the following protocol. One hundred milliliters of minimal glycerol medium in a 1-liter baffle flask was inoculated with an integrant clone and incubated at 27 to 29°C with vigorous shaking (250 rpm) until the optical density at 600 nm (OD_{600}) was 10.0 to 14.0 (36 to 48 h). The cells were harvested by centrifugation for 10 min at room temperature at 4,000 × *g*. The supernatant liquid was discarded, and the pellet was resuspended in 20 ml of MM in a 100-ml baffle flask. The flask was covered with two layers of sterile gauze instead of a cap to allow maximal aeration. The flasks were incubated at 27 to 29°C with vigorous shaking (250 rpm) for an additional 3 days. The cells were induced with fresh methanol every 24 h to a final

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TABLE 1. Bacterial and yeast strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
P. pastoris strains		
GS115	*HIS4*	Invitrogen
GS115/His$^+$ Mut$^-$ albumin	GS115 *AOX1*::*ALB*	Invitrogen
MT22	GS115 ΩpMT51 *HIS4 AOX1* Ace^{+a}	This study
MT24	GS115 ΩpMT51 *HIS4 AOX1* Ace^{+a}	This study
MT27	GS115 ΩpMT51 *HIS4 AOX1* Ace^{+a}	This study
MT210	GS115 ΩpMT51 *HIS4 AOX1* Ace^{+a}	This study
MT211	GS115 *HIS4 AOX1*::pMT51 Ace$^+$	This study
MT219	GS115 *HIS4 AOX1*::pMT51 Ace$^+$	This study
MT318	GS115 *HIS4 AOX1*::pPIC9 Ace$^-$	This study
Vibrio cholerae 01 strains		
CVD110	Δ*ace* Δ*zot* Δ*ctx hly*::*ctx$_B$ mer* Ace$^-$ Zot$^-$ Ctx$_A$$^-$ Ctx$_B$$^+$ Hly$^-$ Hg^r	9
E7946	El Tor Ogawa strain	8
Plasmids		
pCVD315	pBR322 Ω*galK*	7
pCVD630	pCVD315 Ω(*orfU ace*)	9
pPIC9	*E. coli–P. pastoris* shuttle vector, Ap^r His$^+$	Invitrogen
pMT51	pPIC9Ω*ace*	This study

[a] Site of integration into the yeast chromosome is unknown.

concentration of 0.5%. At the end of 3 days, the cells were again harvested and the supernatant was analyzed for the Ace protein.

Analysis of yeast clone supernatants for production of the Ace protein. Following harvest, the yeast supernatants were concentrated 10-fold with polyethylene glycol and analyzed by electrophoresis on a 16% tricine–sodium dodecyl sulfate (SDS)–polyacrylamide gel (10) followed by detection by a double-staining technique with Coomassie brilliant blue and silver stain.

Purification of Ace from a yeast clone. The concentrated yeast supernatant from clone MT219 (Ace$^+$) was applied to a Bio-Rad Mini Prep cell (5-cm 16% tricine–SDS tubular gel), and the Ace protein was purified by elution according to the directions of the manufacturer.

Purification of Ace from *V. cholerae* CVD110(pCVD630). A culture of a *V. cholerae* bacterial strain [CVD110(pCVD630) (Ace$^+$)] was grown in L broth at 37°C with shaking. Culture supernatant was prepared by centrifugation followed by filtration through a 0.45-μm-pore-size filter. The filtered supernatant was then fractionated and concentrated by using Pall Filtron Omega stir cells to obtain a 5,000 to 30,000 M_r fraction. The fraction was washed and resuspended in phosphate-buffered saline (PBS) at 1/1000 of the original volume. The concentrated *V. cholerae* supernatant was applied to a Bio-Rad Mini Prep cell, and the Ace protein was purified by elution according to the directions of the manufacturer.

Analysis of the activity of recombinant Ace from the *P. pastoris* protein expression system in Ussing chambers. (i) Sample preparation for Ussing chambers. Yeast supernatants were harvested 72 h postinduction for analysis in Ussing chambers. Culture supernatants were prepared by centrifugation followed by filtration through a 0.45-μm-pore-size filter. The filtered supernatant was concentrated with an Amicon filter (M_r 3,000 cutoff) to remove the medium and then resuspended in PBS to the original volume.

Overnight cultures of *V. cholerae* CVD110(pCVD315) and CVD110 (pCVD630) were grown in L broth (containing ampicillin [100 μg/ml]) at 37°C with shaking. Culture supernatants were prepared by centrifugation followed by filtration through a 0.45-μm-pore-size filter. The filtered supernatant was concentrated with an Amicon filter (M_r 10,000 cutoff) to remove the medium and then resuspended in PBS to the original volume. All samples were stored on ice until tested in Ussing chambers.

(ii) Ussing chambers. Experiments with Ussing chambers were performed as previously described (5). Adult male New Zealand White rabbits (2 to 3 kg) were anesthetized by methoxyflurane inhalation and then sacrificed by air embolism. A segment of ileum was removed, rinsed free of intestinal contents, opened along the mesenteric border, and stripped of muscular and serosal layers. The sheets of mucosa were then mounted into Lucite Ussing chambers (1.12-cm^2 aperture) and bathed with freshly prepared Ringer's buffer. The bathing solution was maintained at 37°C and gassed with 95% O_2–5% CO_2. PD and short-circuit current (I_{sc}) were measured, and tissue conductance was calculated (6). Once the tissue reached equilibrium, 300 μl of culture supernatant was added to the mucosal side (time zero); 300 μl of the same sample was also added to the serosal side to preserve the osmotic balance. Variations in I_{sc}, PD, and tissue conductance were then recorded. At the end of each experiment, 200 μl of 0.5 M glucose was added to the mucosal side of each chamber. Only those tissues which showed increased I_{sc} in response to glucose (indicating tissue viability) were included in the analysis.

Production of antiserum raised against Ace. Concentrated yeast supernatants containing the Ace protein were emulsified with Freund's adjuvant and used for intradermal injection to immunize rabbits by standard protocols (3). Two, 6, and 10 weeks after the primary injection, the rabbits were given a booster injection of antigen in incomplete Freund's adjuvant. The rabbits were bled before the first injection and 10 days after each booster.

Analysis of Ace antiserum for neutralizing activity. The antiserum was assayed in Ussing chambers for neutralizing activity by the following protocol. Six hundred thirty microliters of crude culture supernatant of *V. cholerae* CVD110 (pCVD630) (Ace$^+$) was incubated with 70 μl of either preimmune serum or anti-Ace antiserum for 60 min at room temperature and then assayed in Ussing chambers.

RESULTS

Transformation of linearized plasmid DNA into yeast spheroplasts. The pMT51 plasmid containing *ace* under the control of the alcohol-inducible promoter *AOX1* was digested with *Bgl*II to generate linearized DNA for transformation. From 2 to 10 μg of digested DNA was used to transform yeast strain GS115 spheroplasts. His$^+$ transformants appeared on days 3 to 7 following transformation. In two experiments we identified 64 and 72 His$^+$ transformants. Nineteen and 20 were of the Mut$^-$ phenotype (29.7 and 27.8%, respectively) (the manufacturer suggests an expected frequency of 5 to 35%). To further confirm the site of integration, six of these Mut$^-$ strains were analyzed by PCR.

PCR analysis of *Pichia* integrants. Six *Pichia* integrants were analyzed by PCR (Fig. 1). From all six a 740-bp band was amplified (291 bp from *ace* plus 449 bp from the pPIC9 vector). In two integrants this was the only band amplified, indicating integration at the *AOX1* gene. In addition, a 2.2-kb band (wild-type *AOX1* gene) was amplified from two integrants, suggesting integration of *ace* in a location outside the alcohol oxidase

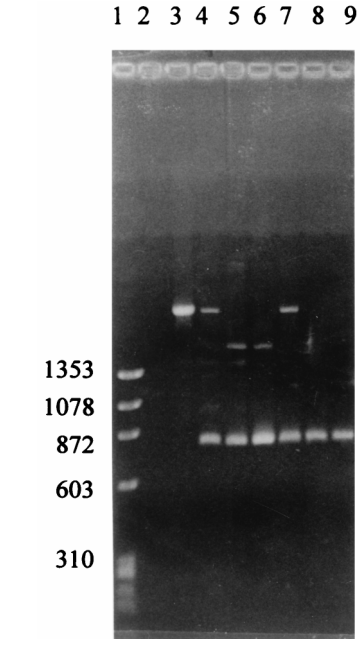

FIG. 1. PCR amplification from the chromosomes of yeast clones (1% agarose gel). Lane 1, molecular weight markers. lane 2, GS115/His$^+$ Mut$^-$ albumin (no *AOX1*, no *ace*); lane 3, GS115 (no *ace*); lanes 4 to 9 (Ace$^+$ yeast strains), MT22, MT24, MT27, MT210, MT211, and MT219, respectively.

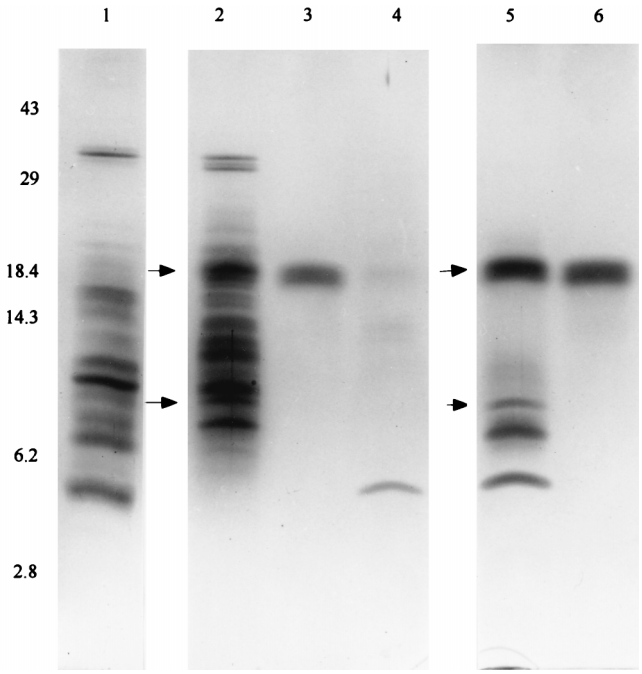

FIG. 2. Electrophoretic pattern of Ace protein in crude supernatants of yeast and *V. cholerae* clones (Coomassie blue and silver-stained 16.5% tricine–SDS–polyacrylamide gel). Lane 1, CVD110(pCVD315) (Ace^-, *V. cholerae*); lane 2, CVD110(pCVD630) (Ace^+, *V. cholerae*); lane 3, purified *V. cholerae* Ace protein; lane 4, MT318 (Ace^- yeast clone) induced; lane 5, MT219 (Ace^+ yeast clone) induced; lane 6, purified recombinant Ace protein from yeast. Arrows to the left of lanes 2 and 5 indicate the two physical forms of Ace. The sizes of the molecular mass standards are shown on the left, in kilodaltons.

gene (most commonly the *his* gene), and a 1.7-kb band was amplified from two integrants for which we can offer no explanation. Thus, of the strains identified as Mut^- by plating on the differential media, one-third were found to have integration at the *AOX1* gene by PCR.

Expression of recombinant *Pichia* strains. To determine whether the *Pichia ace* integrant clones produced Ace protein, one of the six clones (MT219) identified by PCR as containing the *ace* gene integrated at the *AOX1* gene was tested for expression of the Ace protein in comparison to the Ace^- control strain (MT318). We examined expression of the Ace protein in buffered complex medium and minimal medium. We found that expression of a methanol-induced protein was produced in a stable pattern in the minimal medium. In minimal medium, the Ace protein was visible in culture supernatants analyzed on tricine-SDS-polyacrylamide gels by 48 h post-methanol induction. Protein production appeared maximal at 72 h postinduction (data not shown). In contrast, in buffered complex medium there appeared to be a breakdown of the Ace protein, with a smear of low-molecular-mass products from 3,000 to 6,000 Da.

Two new protein bands were visible following double staining with Coomassie brilliant blue and silver stain in the induced Ace^+ strain (MT219) (Fig. 2, lane 5) compared to the induced Ace^- strain (MT318) (Fig. 2, lane 4) or the noninduced Ace^+ strain (MT219) (data not shown). The predominant protein had an M_r estimated to be 18,000, while the less-predominant protein had an M_r estimated to be 9,000. The sizes of the induced proteins are consistent with a monomer and dimer of the Ace protein (size predicted from sequence analysis of the *ace* gene fusion is 12,300 Da). These two induced proteins were not visible following the initial staining step with Coomassie brilliant blue. The predominant Ace protein was purified by elution from a Bio-Rad Mini Prep cell (Fig. 2, lane 6).

TABLE 2. Activity of recombinant Ace from *P. pastoris* in Ussing chambers

Strain	ΔI_{sc} ($\mu A/cm^2$)[a]	Ace expression
MT211 (Ace^+)	38.8	Yes
MT219 (Ace^+)	66.4	Yes
MT318 (Ace^-)	−44.4	No
CVD110(pCVD630) (Ace^+)	40.4	Yes
CVD110(pCVD315) (Ace^-)	−35.2	No

[a] Results are the means of two experiments. Δ, change.

Analysis of the activity of recombinant Ace from the *Pichia pastoris* protein expression system in Ussing chambers. Two Ace-producing yeast clones (MT211 and MT219) were chosen for analysis of activity in the rabbit Ussing chamber model. Yeast clone MT318, which had the pPIC9 vector alone integrated into the *Pichia* genome, was run in parallel as a negative control. Seventy-two hours postinduction, the samples were analyzed. Supernatants of the two Ace-containing yeast clones produced an increase in I_{sc} compared to the supernatant of the negative control yeast clone (Table 2). *V. cholerae* control strains CVD110(pCVD630) (Ace^+) and CVD110(pCVD315) (Ace^-) were run in parallel as additional positive and negative controls.

Analysis of Ace antiserum for neutralizing activity. The crude culture supernatant of *V. cholerae* CVD110(pCVD630) (Ace^+) was incubated with either preimmune serum or anti-Ace antiserum (obtained after the second or final boost) for 60 min at room temperature and then assayed in Ussing chambers. The toxin activity in crude culture supernatants incubated with preimmune serum remained active in the Ussing chamber; however, toxin activity in crude culture supernatants incubated with anti-Ace antiserum was inhibited (Table 3). *V. cholerae* control strains CVD110(pCVD630) (Ace^+) and CVD110(pCVD315) (Ace^-) were run in parallel as positive and negative controls.

Identification of the Ace protein in *V. cholerae*. The concentrated supernatants of *V. cholerae* CVD110(pCVD315) (Ace^-) and *V. cholerae* CVD110(pCVD630) (Ace^+) were analyzed by electrophoresis on a 16% tricine–SDS–polyacrylamide gel. Bands corresponding to the new protein bands identified in the induced Ace^+ yeast strain (MT219) were recognized in the concentrated supernatant of *V. cholerae* CVD110(pCVD630) (Ace^+) (Fig. 2, lane 2) but not in the concentrated supernatant of *V. cholerae* CVD110(pCVD315) (Ace^-) (Fig. 2, lane 1). Like the yeast-produced Ace proteins, these bands were distinguished by staining with silver but not Coomassie brilliant blue. The predominant *V. cholerae* Ace protein was purified by elution from a Bio-Rad Mini Prep cell (Fig. 2, lane 3).

TABLE 3. Analysis of Ace antiserum for neutralizing activity in the Ussing chamber

Strain	Serum	ΔI_{sc} ($\mu A/cm^2$)[a]	Ace present
CVD110(pCVD315)	None	−14.8 (2)	No
CVD110(pCVD630)	None	43.5 (2)	Yes
CVD110(pCVD630)	Preimmune	36.7 (2)	Yes
CVD110(pCVD630)	Ace antiserum	−27.4 (3)	Neutralized

[a] Results are the means of two or three experiments, as indicated in parentheses. Δ, change.

DISCUSSION

We have demonstrated the efficient expression of the Ace toxin in the methylotrophic yeast *P. pastoris*. The expression system produced 7 mg of Ace toxin per liter. We had previously been unable to express the Ace toxin in a prokaryotic expression system due to the toxicity of Ace to the *E. coli* host strain. Expression of the Ace protein was highly dependent on the composition of the growth medium and the density of the culture at the time of induction of protein expression. We initially attempted expression in buffered complex glycerol medium. This medium is buffered with a phosphate buffer, and thus the culture is maintained at a neutral pH. However, in this medium there was extensive proteolysis of the secreted protein, as evidenced by a smear of bands in the 3,000- to 6,000-Da range. This most likely was due to activity of neutral proteases which have optimal activity at a neutral pH. Expression using unbuffered minimal medium allows the pH to drop to ≤ 3, inactivating many neutral pH proteases. *P. pastoris* is resistant to low pH, so the low pH did not affect growth. In addition, we found that inducing the culture at the standard OD_{600} of 2 to 6 indicated in the manufacturer's protocol again resulted in proteolytic digestion, even if expression was in minimal medium. If, however, we continued the growth of the culture until an OD_{600} of 10 to 14 was achieved and then induced, we recorded consistent and stable production of the Ace protein. Again, this is most likely due to the pH of the medium at the time of induction. As the *P. pastoris* culture increases in density, the pH of the medium falls, thus inhibiting neutral proteases. The use of unbuffered medium was the most efficient method of expressing Ace, as the addition of protease inhibitors at the time of supernatant harvest in buffered complex glycerol medium did not prevent proteolysis.

Certain classes of proteins are distinguished by their differential chemical reactivities to silver versus Coomassie stain. In particular, some glycosylated glycoproteins, especially sialoglycoproteins, are not stained by Coomassie blue but are readily stained by silver stain (4). Additionally, some lipoproteins and very acidic proteins are poorly stained or not stained by Coomassie blue but can be stained by silver stain (4). The Ace protein did not stain with the standard Coomassie blue staining technique, but it did stain with a double-staining technique that employed Coomassie brilliant blue followed by silver stain. Ace is an acidic protein with a predicted pI of 4.26, based on DNA sequence analysis. This characteristic most likely accounts for the lack of staining with Coomassie brilliant blue. Many yeast proteins undergo glycosylation; therefore, periodic acid-Schiff (PAS) staining was performed to determine whether the Ace protein was glycosylated by *P. pastoris*. The Ace protein did not stain with the PAS stain, suggesting that it was not glycosylated (data not shown). It is unlikely that the Ace protein is a lipoprotein, since it lacks the consensus motif found in bacterial lipoproteins (Leu-Leu-Ala-Gly-Cys) (1); furthermore, it does not contain any Cys residues, which are the universally conserved +1 residues of mature lipoproteins and the site of diglyceride conjugation and N acylation.

We previously suggested that Ace may form multimers which aggregate to form a pore that is inserted into the eukaryotic membrane, thereby resulting in electrolyte movement and accounting for the changes in PD and I_{sc} observed in the Ussing chamber (11). The predominant form of the toxin produced in the yeast expression system was a protein at an M_r of 18,000. This most likely represents a dimer of the Ace toxin, consistent with a proposed mechanism of action of the Ace protein as a pore-forming toxin. The presence of a second silver-stained band at an M_r of 9,000 was also consistent with a monomer form of Ace. Although the gels were run under denaturing conditions with SDS in the gel and running buffer, the sample buffer used as a vehicle for the yeast supernatants lacked denaturing agents. The Ace protein was not soluble in sample buffer containing denaturing agents. It therefore is possible that the Ace protein was not completely denatured and persisted as a multimer in the gel.

Characterization of the yeast recombinant Ace protein in Ussing chambers showed that it retained biological activity similar to that seen with the Ace toxin produced in *V. cholerae*. To our knowledge this is the first report of a biologically active bacterial toxin produced in the *P. pastoris* system. The recombinant toxin was not only active but was effective as an immunizing agent in rabbits, producing antiserum capable of neutralizing wild-type Ace toxin from *V. cholerae*. It should be noted, however, that the antiserum was produced by using concentrated whole culture supernatants from Ace-positive yeast clones and may not be monospecific for the Ace protein.

Recently, Waldor and Mekalanos (12) have shown that the genes present in the *V. cholerae* chromosomal core region (including the genes encoding the Ace and Zot toxins) are part of a filamentous bacteriophage named CTXΦ. The gene VI, an Ace homolog, encodes a small hydrophobic protein of unknown function in the M13 filamentous phage. We had previously shown that the Ace toxin cloned as a separate open reading frame (that is, without any of the other open reading frames required for phage production) had the activity of a classical enterotoxin with an in vivo model (rabbit ileal loops) and an in vitro model (rabbit Ussing chambers) (11). In addition, we have now shown that the purified protein produced in yeast has activity in the rabbit Ussing chamber. We conclude that Ace appears to be bifunctional, having both phage function and toxin activity.

The production of the Ace protein in the *P. pastoris* system has facilitated its characterization, which in turn allowed the purification of Ace from *V. cholerae*. The *V. cholerae* Ace protein was identified in supernatants of a *V. cholerae* Ace^+ strain by identification of protein bands staining with silver but not with Coomassie brilliant blue stain. Two bands of molecular weights similar to those identified in yeast were found in the *V. cholerae* Ace^+ strain. Again, the predominant physical form was that of molecular weight 18,000, consistent with a dimer of the Ace protein, while a second protein of molecular weight 9,000, consistent with a monomer of Ace, was also identified. The quantity of Ace in wild-type *V. cholerae* E7946 was estimated to be 0.6 μg/liter, 10,000-fold less than that produced in the yeast expression system. We attempted to determine the N-terminal sequence of the native Ace protein, but analysis at two different protein sequencing facilities failed. The availability of purified toxin will make it possible to investigate the mechanism of action of Ace in physiologic systems.

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