The Amazonia Variant of *Vibrio cholerae*: Molecular Identification and Study of Virulence Genes

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The pathogenic O1 Amazonia variant of Vibrio cholerae *has been shown previously to have a cytotoxin acting on cultured Vero and Y-1 cells, and to lack important virulence factors such as the cholera toxin (Coelho et al. 1995a). This study extends the molecular analysis of the Amazonia strains, detecting the presence of the* tox*R gene, with a very similar sequence to that of the El Tor and classical biotypes. The outer membrane proteins are analyzed, detecting a variation among the group of Amazonia strains, with three different patterns found. As a by-product of this work a polymerase chain reaction fragment was sequenced, reading part of the sequence of the Lon protease of the Amazonia strains. This gene was not previously described in* V. cholerae*, but its sequence is present in the TIGR database specific for this species.*

Key words: *Vibrio cholerae* - Amazonia - *tox*R - outer membrane proteins - protease - Lon

The Amazonia variant of *Vibrio cholerae* was isolated from a group of clinical O1 isolates obtained from the northwest of Brazil in 1991-1992 (Coelho et al. 1995a). The Latin American epidemic was caused by a strain of the El Tor biotype. It spread out from the Pacific coast of Latin America inwards, mainly following the Amazon river basin, reaching the northeast of Brazil and coming down up to the Rio de Janeiro region. A collection of strains from the beginning of the epidemic in Brazil was analyzed by the random amplified polymorphic DNA (RAPD) discriminative technique (Coelho et al. 1995b), and a surprising result was obtained. There was a group of strains with a different fingerprint pattern from the epidemic El Tor strains. All of these strains presented the same pattern, showing that they represented a distinct group.

These 14 strains were analyzed by various techniques. Biochemically they are undistinguishable from other *V. cholerae* strains. All of these strains were Ogawa, in contrast to the majority of strains collected at this time that were Inaba. However other El Tor strains from the same time and area were also Ogawa. The isozyme method (Salles & Momen 1991) was used on these strains, and they were classified into a new group. The same thing happened with ribotypes (Popovic et al. 1993), and they formed a new group.

The Amazonia strains were tested for the presence of the *ctx* gene (Kaper & Levine 1981, Salles et al. 1993, Kaper et al. 1994), encoding the cholera toxin, and the result was negative. The presence of other virulence genes was tested by polymerase chain reaction (PCR), and neither the ST (thermo-stable toxin) (Ogawa et al. 1990, Vicente et al. 1997a) nor the *zot* (zonula occludens) toxin (Baudry et al. 1992) were found. The *tcp*A gene, coding for the colonization pilus (Taylor et al. 1987, Rhine & Taylor 1994, Manning 1997, Vicente et al. 1997b), was not found by PCR or Southern hybridization.

When tested on rabbit ligated ileal loop, the strain did not produce an accumulation of liquid, but did show a destruction of the intestinal ephitelium, a heavy mucus production, with a large number of erythrocytes and epithelial cells embedded in it. In *in vitro* studies on cultured Vero cells, the production of a cytotoxin was detected, leading to morphological alterations of the cells, their detachment from the plastic and death.

In this paper the analysis of virulence genes of the Amazonia strain is extended, mainly with the study of the regulatory gene *tox*R (Peterson & Mekalanos 1988, DiRita & Mekalanos 1991, DiRita et al. 1991). *tox*R is considered a main regulatory gene, responsible for the recognition of en-

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vironmental stimuli for expression of a number of genes collectively denominated the ToxR virulence regulon (Skorupski & Taylor 1997, Champion et al. 1997). The major outer membrane protein of *V. cholerae*, OmpU, is directly regulated by the ToxR protein (Miller & Mekalanos 1988, Sperandio et al. 1995, Chakrabarti et al. 1996, Crawford et al. 1998). The outer membrane proteins of the Amazonia strains are also analyzed here.

MATERIALS AND METHODS

Bacterial strains - Fourteen *V. cholerae* Amazonia strains were previously described (Coelho et al. 1995a). A further group of six strains was obtained from Cholera Reference Center (Fiocruz, Brazil). A streptomycin resistant derivative of one of the original strains, 4010 was used for all the experiments described in this paper. Control classical and El Tor strains were O395 and E7946 respectively.

DNA preparation, PCR reaction conditions and product analysis - Bacteria were grown overnight in alkaline peptone water (1 ml) and DNA was extracted (Silhavy et al. 1984). The program used for PCR consisted of 35 cycles, at 94°C for 1 min, 55°C for 1min 30 sec and 72°C for 1min 30 sec. The reactions included 1 µl of each primer (500ng/µl), 100ng of DNA, dNTP's 50 µM each, 5 µl reaction buffer (1.5mM $MgCl_2$ final concentration), 0.5 µl *Taq* polymerase (2.5U) (Pharmacia) and distilled water to a total volume of 50 µl. An MJ Research thermocycler (Watertown, Mass.) was used for the temperature cycling. Primers used for the *tox*R fragment of 560bp were: OL.1: 5' TCGGATTAGGA CACAACTC and OL.2: 5' CTGCGAGGGGAA GTAAGAC. DNA was analyzed on 1.4% agarose gels in TBE 1X, prepared according to Sambrook et al. (1989), and running at 100 Volts for approximately 2 hr 30 min, until the bromophenolblue reached the end of the gel.

Southern transfer and hybridization - Southern transfers to nitrocellulose were done according to Sambrook et al. 1989. The hybridization solution was 50% formamide, 6X SSC, 0.7% SDS. DNA (200ng) was labeled with the *Random Primer* kit (Life Technologies) employing aP32 dCTP.

Plasmid preparations, DNA restriction and ligation - Plasmid preparations employed Qiagen P-100 columns according to instructions of the manufacturers. Restrictions were done as described by the enzyme manufacturers (Life Technologies). A five to one proportion of insert fragment was used in the cloning experiments. Electroporation was done into the *Escherichia coli* strain DH5a.

Outer membrane proteins preparation and protein analysis on SDS-polyacrylamide gels - Outer membrane proteins were prepared from 1 ml of cells. Bacteria were spun down and treated for 10 min with 0.06M Tris HCl (pH 8.0)/ 0.2M sucrose, 0.2mM EDTA and 0.04 mg/ml lysozyme (total volume 500 µl). 10 µl of 1 mg/ml DNAse were added, and then 500 µl of Triton extraction buffer [(2%Triton X-100, 10mM $MgCl_2$, 50mM Tris-HCl (pH 8.0)] were added. Outer membrane fragments were spun down and washed with water for three times. Proteins were resuspended in SDS-PAGE sample buffer, boiled for 5 min, and loaded on 12% SDS-polyacrylamide gels with 5% stacking gels. Electrophoresis was carried out at constant current (35mA), and the gels were stained with 0.25% Coomassie blue, and destained with methanol/acetic acid.

DNA sequencing and analysis - DNA sequencing was done employing the *Thermo-sequenase* kit (Amersham) and aP33 labeled dideoxy nucleotides. Plasmid DNA or PCR amplicons were sequenced. Specific bands on agarose gels were cut, and the DNA purified with the Sephaglass kit (Pharmacia). 300 ng of DNA and 0.5 µl of the ddNTPs were used for sequencing. Standard 60 cm 6% polyacrylamide-bisacrylamide gels were used, with a glycerol tolerant buffer provided with the kit. The gels were fixed and dried and Hyperfilm was exposed for the visualization of the bands.

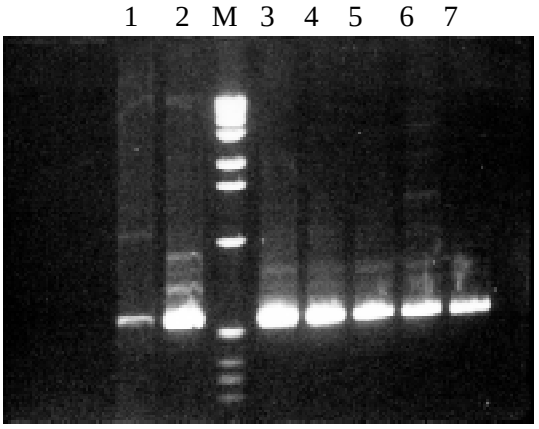
Databank searches with the sequences were made against the specific *V. cholerae* TIGR database at the Institute for Genomic Research (www.ncbi.nlm.gov/cgi-bin/BLAST/nph-tigrbl) and against the non-redundant combined database through the Blast Search (www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast?Jform=0). Individual sequences were retrieved from the Genbank (www2.ncbi.nlm.nih.gov/genbank/query_form.html).

RESULTS

Presence of the toxR gene - Primers OL.1 and OL.2 were used in a test to check whether the *tox*R gene was present. Fig. 1A shows the presence of a strong band with an expected size of 560 bp, for various Amazonia strains. The gel was transferred and hybridized to a probe prepared from a classical strain, by amplification with the same primers. Hybridization was positive, as shown in Fig. 1B.

Cloning of a fragment of the toxR gene - The 560bp PCR fragment of the Amazonia *tox*R was cloned into the pBluescript SK vector, using *Sma*I, and producing the plasmid pMB560R. This fragment was then transferred to a second plasmid, pCVD442 (Donnenberg & Kaper 1991), a suicide plasmid that does not replicate in *V. cholerae*. *Xba*I and *Sal*I were used in the second cloning, and the plasmids obtained were denominated pCVD560R (Fig. 2). A restriction analysis of two such clones (Fig. 3A), and hybridization to the Amazonia *tox*R

A



B

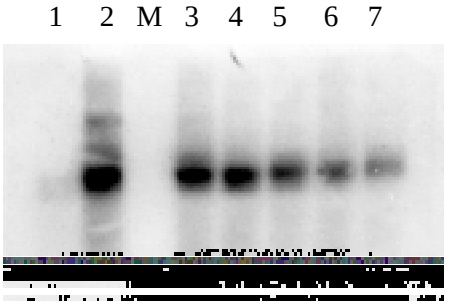


Fig. 1A: *tox*R PCR amplification from Amazonia strains. *tox*R primers were OL.1 and OL.2, which delimit a fragment of 560bp. Lanes 1 and 2 contain products from the *Escherichia coli* DH5a (negative control) and the classical strain O395, respectively. Lanes 3 through 7, PCR products from the Amazonia strains. 3, 4010; 4, 3729; 5, 3506; 6, 3439 and 7, L-34. M indicates the size marker, 1 kb ladder (Life Technologies). Fig. 1B: Southern hybridization of the gel on A with the 560bp *tox*R fragment from the O395 classical strain.

(Fig. 3B) were done, in a structural analysis of the clones.

Partial sequencing of the toxR gene - The *tox*R Amazonia 560bp fragment was sequenced, using both a universal primer for the pMB560R or internal *tox*R primers. A high similarity of the sequence (98.6%) was found to that of the classical biotypes (Fig. 4A). A translation of this sequence shows a 98.4% identity to the classical and El Tor ToxR protein (Fig. 4B). An arginine (R) for threonine 125(T) substitution in particular could cause a difference in the secondary structure of the protein.

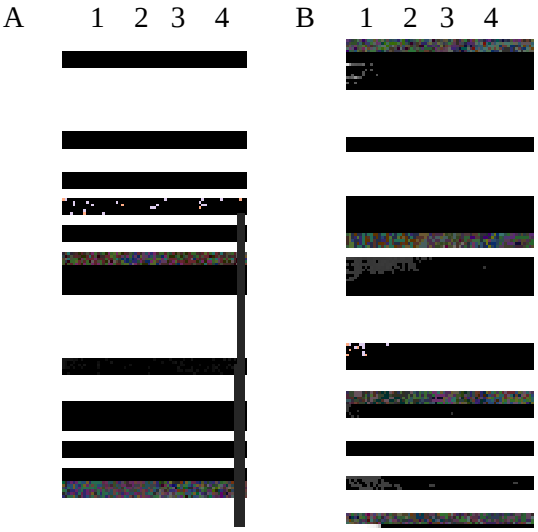


Fig. 3A: *Pst*I restriction analysis of pCVD442 and two different pCVD560R clones. Lane 1, 1kb ladder (Life Technologies); lane 2, pCVD442; lane 3, pCVD560R cl.1; lane 4, pCVD560R cl.2. Fig. 3B: hybridization of the gel on A to a 4010 Amazonia 560bp *tox*R probe.

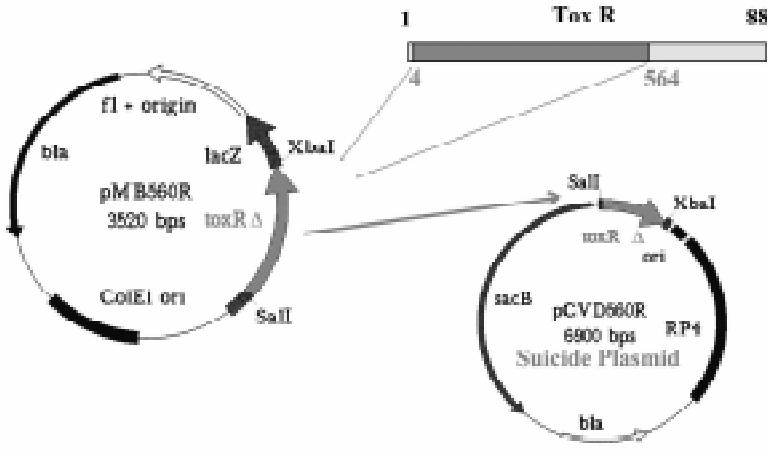


Fig. 2: cloning of the 560bp PCR fragment of the Amazonia strain 4010Sm^r into the *Sma*I site of pBluescript SK, producing plasmid pMB560R and further cloning, with *Xba*I and *Sal*I, into pCVD442, to yield plasmids pCVD560R.

A

B

Fig. 4A: DNA sequence of the *tox*R fragment from the Amazonia strain 4010 compared to the sequence of the classical 569B strain. Numbers correspond to the number of nucleotides. * are used to mark the same nucleotide as in the previous line. Fig. 4B: aminoacid comparison between a translation of the DNA sequence of the Amazonia strain 4010, compared to the sequence of the ToxR protein of classical strain 569B and El Tor strain E7946. * are used to mark the same aminoacid as in the previous line.

The major outer membrane proteins of Amazonia strains - The outer membrane proteins of the Amazonia strains were analyzed on polyacrylamide gels (Fig. 5). Three types of patterns were obtained. Some strains present one major protein with a size of 38kDa, as the 38kDa OmpU protein of the El Tor and classical strains. Other strains present one smaller protein of 35kDa. A third group presents both of these proteins. Different media were used for growth, to check whether this major proteins would vary according to growth conditions (data not shown), but it seems that the pattern is strain determined.

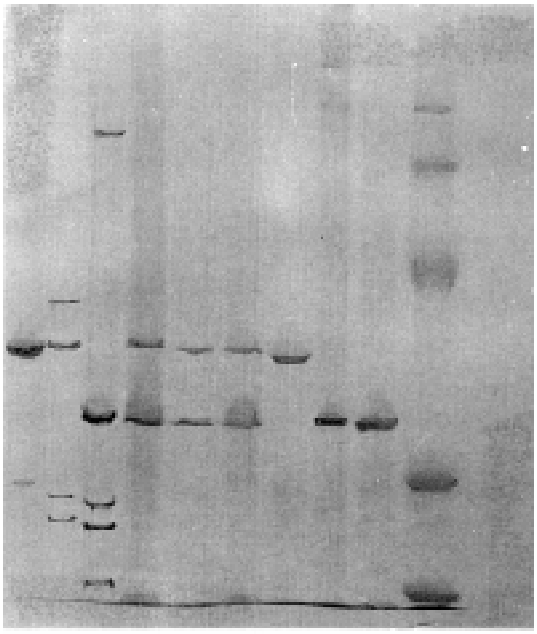


Fig. 5: SDS- polyacrylamide gel electrophoresis of outer membrane proteins. Lane 1: classical strain O395; 2-6, Amazonia variant strains (2, L-34; 3, 3218; 4, 3439; 5, 4010; 6, 3506); 7, El Tor (607); 8 and 9, Amazonia variant strains (8, 3501; 9, 4132); 10, protein molecular weight markers.

Presence of the lon gene - In the process of looking for virulence related genes, we obtained a spurious 800bp PCR amplicon that was partially sequenced. This sequence corresponds to the Lon protease of various bacteria. This is one of the main proteases, responsible for the degradation of misfolded proteins (Gottesman 1996). In Fig. 6 we compare the sequence of the Amazonia strain putative Lon protein to a sequence found in the TIGR databank for *V. cholerae* and to the other bacteria and archae.

DISCUSSION

The pathogenic O1 Amazonia *Vibrio cholerae* may be considered a new human pathogen. It was discovered with the use of discriminative molecular techniques (Coelho et al. 1995b, 1997). This strain belongs to the same species as the epidemic strains but seems to cause diarrhea by a different mechanism. The lack of the cholera toxin, and the presence of a cytotoxin leading to intestinal tissue damage in rabbits strongly suggest a different route for the disease.

The *tox*R gene was found in the Amazonia strains, and its sequence is highly homologous to that of the El Tor and classical biotypes. A *tox*R homologous gene has been described in *Vibrio parahaemolyticus*, *Vibrio fischeri* and a *Photobacterium* sp., with much lower DNA homology values, all below 70%.

The presence of the regulatory gene *tox*R, in the absence of various genes that it normally regulates, leads to the question of its further role. One possibility is the regulation of the outer membrane

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E. coli              EQQIPFSASLTFEQSYSEVDGDSASMAELCALISALADVPVNQSIAITGSVDQFGRAQPV
                       ::     ::::::  :::::::::: ::  ::    :  : ::::::   ::  :
V. cholerae          TARIPLTTTITFEQSYGGVDGDSASMAEFCAIVSAFSKQPNRQDIAITGSMNHFGESQAI
                           ::::::::::::::::::::  ::::::::::::::::::::
V.chol. Amazonia           TTITFEQSYGGVDGDSASMA?LCAIVSAFSKQPNRQDIAITGRLR?K
                             ::::::  :::::: :  :  :::    :  : :::::
Hae. inf.            PSQLPFSASLVFEQSYGEIDGDSASLAIFCVLVSALADLPLPQHIAITGSIDQFGLVHSV
                             :   :    :::::  :     ::    :  :  : :::    : :  :
Arch.fulgidus         DISNMDVHIQFVGTYEGVEGDSASISIATAVISAIEGIPVDQSVAMTGSLSVKGEVLPV
                      :::: : ::::   : :::::::: : ::::::: : :::::::: :::::  : ::::
Meth.thermo.         TDISNYDIHIQFLQAYDGVEGDSASVSVATAVISALEEIPVDQSVALTGSLSIRGDVLPV
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Fig. 6: aminoacid sequence comparison of the Amazonia putative Lon protease and the corresponding protein in other bacteria and archae. Bacteria: *Escherichia coli*, *Vibrio cholerae*, *Haemophilus influenzae.* Archae: *Archaeoglobus fulgidus* and *Methanobacterium thermoauxotrophicum*.

proteins. It is known that *tox*R directly regulates the OmpU protein, which has been proposed as an adhesin with a role in virulence (Sperandio et al. 1995, 1996). The Amazonia strain, as in the case of El Tor and classical strains, presents major outer membrane proteins, which could have functional homologies to OmpU. Further developments in this work will include studies on the presence of other regulatory genes such as genes of the tcp cluster, like the *tcp*P and *tcp*H (Carroll et al. 1997, Manning 1997, Häse & Mekalanos 1998) and other putative virulence genes (Karaolis et al. 1998, Tacket et al. 1998). It will also aim at the construction of *tox*R mutants to evaluate the role of this gene in the pathogenicity of the Amazonia strain, including an analysis of its adhesion to cells, cytotoxicity, and behavior in rabbit ileal loops. These comparative studies may shed light not only on the virulence mechanisms involved in the Amazonia strain but also on *V. cholerae* itself.

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