# Apparent mRNA instability in *Aspergillus nidulans* and *Aspergillus terreus* of a heterologous cDNA encoding the major capsid antigen of Rotavirus

**Adela Villanueva[1,2], Andrew P. MacCabe[1], Javier Buesa[2] and Daniel Ramón[1]**

*[1]Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas y [2]Departamento de Microbiología, Facultad de Medicina, Universitat de València, Valencia, Spain*

*Summary*

Two expression plasmids designed to produce the rotaviral VP6 protein in *Aspergillus nidulans* and *Aspergillus terreus* have been constructed. In one of these plasmids the inducible *A. terreus* Gla1 glucoamylase gene promoter and Gla1 signal sequence are fused to the VP6 cDNA to enable induction and extracellular secretion of the final protein product; in the other, the strong, constitutive *A. nidulans gpdA* gene promoter has been employed. *A. nidulans* and *A. terreus* transformants containing intact copies of these plasmids have been obtained but neither intra- nor extra-cellular VP6 protein was detectable. Northern analysis indicated specific degradation of the VP6 mRNA. This lack of VP6 mRNA stability may be related to fundamental differences between the general structure of *Aspergillus* mRNA and that of rotavirus, including codon usage and AU/GC ratio.

*Key words*

*Aspergillus nidulans*, *Aspergillus terreus*, Rotavirus, VP6, Expression, mRNA instability

## Inestabilidad aparente en *Aspergillus nidulans* y *Aspergillus terreus* del mARN producido a partir de un cADN heterólogo que codifica el antígeno mayoritario de cápsida de Rotavirus

*Resumen*

Se construyeron dos plásmidos de expresión diseñados para producir la proteína VP6 de Rotavirus en *Aspergillus nidulans* y *Aspergillus terreus*. En uno de ellos el promotor inducible del gen que codifica la glucoamilasa Gla1 de *A. terreus* y su secuencia señal se fusionaron al cADN de VP6 para permitir la inducción y secreción extracelular del producto proteico final; en el otro, se empleó el promotor constitutivo fuerte del gen *gpdA* de *A. nidulans*. Se obtuvieron transformantes de *A. nidulans* y *A. terreus* conteniendo copias intactas de estos plásmidos pero no se detectó la proteína VP6 intra o extracelularmente. El análisis *northern* indicó una degradación específica del mARN de VP6. Esta falta de estabilidad del mARN de VP6 puede estar relacionada con diferencias fundamentales entre la estructura general del mARN de *Aspergillus* y Rotavirus, incluyendo el uso de codones y la relación AU/GC.

*Aspergillus nidulans*, *Aspergillus terreus*, Rotavirus, VP6, Expresión, Inestabilidad de mRNA

**Dirección para correspondencia:**
Dr. Daniel Ramón
Departamento de Biotecnología,
Instituto de Agroquímica y Tecnología de Alimentos,
Consejo Superior de Investigaciones Científicas;
Apartado 73, 46100-Burjassot, Valencia, Spain
Tel.: +34 96 3900022; Fax: +34 96 3636301;
E-mail: dramon@iata.csic.es

*Aceptado para publicación el 22 de enero de 1999*


1130-1406/99/5.00 Euros

Rotaviruses are the major cause of acute viral gastro-enteritis in children and in the young of a wide variety of mammalian and avian species [1]. The genome of these viruses consists of 11 segments of double-stranded RNA and is enclosed within a double-layered capsid that is made up of the structural proteins VP4, VP6 and VP7, each genomic segment encoding at least one protein [2]. The major structural component of virions is VP6, which is a highly antigenic and immunogenic protein. It is located on the inner capsid and contains group and subgroup antigenic determinants [2]. This protein has also been

associated with viral RNA polymerase activity and is the major protein detected in most diagnostic enzyme-linked immunoabsorbent assays [3]. These properties make VP6 an important target for gaining an understanding of the rotaviral replication process, to facilitate vaccine testing and to provide a possible means for the production of inexpensive diagnostic tests. In this regard, the gene encoding VP6 from the simian rotavirus strain SA11 [4] has already been expressed in baculovirus at high rates [5]. However, the availability of an alternative system for protein production based on fermentation by microorganisms, specifically filamentous fungi, may confer practical advantages such as greater ease of handling and protein purification.

Fungi of the genus *Aspergillus* have been employed to produce high levels of extracellular proteins (for reviews see [6,7]). Strains of the species *Aspergillus awamori* [8], *Aspergillus niger* [9] and *Aspergillus oryzae* [10] have been used successfully as industrial hosts. *Aspergillus terreus* is also a well known secretor of extracellular enzymes for which we have previously developed two transformation systems [11,12]. With regard to expression vectors, various constructs containing constitutive [9] or inducible [10,13-15] promoters have been developed. We have previously cloned the *A. terreus gla1* gene which encodes the major Gla1 glucoamylase [16], the expression of which is strongly induced by starch or maltose and repressed by glucose [16].

In this article we report the construction of expression vectors for VP6 using both the inducible *gla1* and the constitutive *Aspergillus nidulans gpdA* (glyceraldehyde 3-phosphate dehydrogenase) gene promoters. These have been used to investigate VP6 production in both the genetic model organism *A. nidulans* and the more efficient secretory organism *A. terreus*.

## MATERIALS AND METHODS

*Strains and culture conditions.* *Escherichia coli* DH5α[F-, *endA1, hsdR17, gyrA96, thi1, recA1, supE44, ΔlacU169,* (*Φ80 lacZΔM15*)] was used for cloning purposes. *A. terreus* wild type (CECT 2663), obtained from the Spanish Type Culture Collection, and *A. nidulans argB*2, *metG*1, *biA*1 were used as recipient strains in transformation experiments. Both were grown in appropriately supplemented *Aspergillus* minimal medium (AMM) [17]. Transfers of mycelia transformed with pGLAVP6 (see later) which carries a maltose inducible promoter, from non-inducing (1% w/v glucose) to inducing (1% w/v maltose) conditions, were performed as described by Ventura *et al.* [16]. For transformations, *A. nidulans* and *A. terreus* protoplasts were prepared as detailed in Ventura & Ramón [11]. *A. nidulans* protoplasts were co-transformed using pILJ16 which carries the *argB*2 gene [18] as the selectable marker and subsequently selected for arginine prototrophy; *A. terreus* was co-transformed with pAN7-1 [19] and transformants selected for hygromycin resistance. A molar ratio of 10:1 of plasmids pGLAVP6 and pGPDVP6 with respect to the selectable plasmids pIJ16 and pAN7-1 was used in co-transformations (see below for details).

Simian rotavirus SA11 (ATCC VR-899) was grown and purified from infected fetal rhesus monkey kidney cells (MA104) as previously detailed [20].

*General nucleic acid manipulations.* Nucleic acid manipulations, blotting and hybridization were carried out using standard methods [21]. Rotaviral dsRNA isolation and cDNA synthesis were carried out as described by Buesa *et al.* [22]; fungal RNA isolation and northern analyses were done as detailed by MacCabe *et al.* [23]. Radiolabelled probes were prepared by the random hexanucleotide method [24].

*Construction of plasmids pGLAVP6 and pGPDVP6.* VP6 cDNA was synthesized from double stranded rotaviral RNA by reverse transcription in a final volume of 30 µl using the oligonucleotides VP6A and VP6B [22]. The VP6 oligonucleotides used for both RT and PCR were designed on the basis of the published VP6 sequence [25] and include restriction enzyme sites to facilitate cloning. VP6A (5'-CTTCAACATCCATGGCCTATAC-3') corresponds to positions -7 to +15 of the VP6 gene and includes the *Nco*I restriction enzyme site; VP6B (5'-AAAGTTGGATCCCAAGTTGTTAGC-3') corresponds to positions +1202 to +1225 of VP6 and includes the *Bam*HI restriction enzyme site (both restriction sites are underlined). One microliter of the resulting cDNA synthesis reaction mix was then subject to PCR amplification in a final volume of 50 µl using oligonucleotides VP6A and VP6B under the following cycling conditions: 1 x (94∞C for 2 min), 40 x (94∞C for 1 min, 47∞C for 2 min and 72∞C for 2 min), 1 x 72∞C for 10 min) [22]. The PCR product was subject to agarose gel electrophoresis, and the resulting 1.23 kb fragment was excised and purified with 'GeneClean' (Bio 101, La Jolla, Cal). This fragment was subsequently digested with *Nco*I and *Bam*HI.

Plasmid pGLAVP6 was constructed as follows. A DNA fragment of approximately 690 bp containing upstream sequences and the signal peptide of the *A. terreus gla1* gene [16] was obtained by PCR using 1 ng of plasmid pATGLA1 (a pBSsk$^+$ plasmid containing a 2.8 kb *Eco*RI-*Hin*dIII fragment carrying the complete *A. terreus gla1* structural gene plus 596 bp of upstream sequence [16]) as template, and the oligonucleotides M13 reverse and PGLA-2 (5'-CATCCATGGAGACTGG-3', spans positions +89 to +104 of the *gla1* gene), the latter of which contains an introduced *Nco*I site (underlined). Amplification was carried out using the following cycling conditions: 1 x (95∞C for 2 min), 40 x (95∞C for 1 min, 48∞C 2 min, 72∞C 3 min), 1 x (72∞C for 5 min). The amplified fragment was digested with *Eco*RI (a naturally occurring site in the *gla1* upstream sequence at position -596) and *Nco*I and cloned in plasmid pUCBM20 (Boehringer Mannheim, Germany), previously digested with the same enzymes, yielding plasmid pGLA1. The *trpC* terminator was obtained from plasmid pAN7-1 [19] as a 783 bp *Bam*HI-*Hin*dIII fragment. The *Nco*I-*Bam*HI VP6 fragment which contains the entire VP6 ORF and the *Bam*HI-*Hin*dIII *trpC* fragment were cloned in *Hin*dIII-*Nco*I digested pGLA1 in a triple point ligation reaction, yielding the 5.3 kb plasmid pGLAVP6 (Figure 1). Plasmid pGPDVP6 (6.9 kb) was constructed in a similar way (Figure 1): the *Nco*I-*Bam*HI VP6 fragment and the *Bam*HI-*Hin*dIII *trpC* terminator were ligated to *Nco*I-*Hin*dIII digested pPGPD. pPGPD contains 2.3 kb of *A. nidulans* sequence upstream of the glyceraldehyde-3-P-dehydrogenase (*gpdA*) coding sequence [26].

All PCR fragments were sequenced in order to ensure the absence of PCR-induced mutations. Restriction analyses were performed to confirm the structures of the recombinant plasmids and correct in-phase union of promoters with VP6 was confirmed by DNA sequencing.

*Protein analysis.* Protein extraction from mycelia was carried out as detailed by Pérez-Esteban *et al.* [27]; fractionation of insoluble material was done using the method of Wingfield *et al.* [28]. Culture supernatant analyses, including western experiments, were conducted as detailed in Ramón *et al.* [29]. Polyclonal antibodies rai-

sed against VP6 were obtained from Chemicon International Inc. (EE.UU.). Crude extracts of MA104 cells infected with SA11 rotavirus were used as a positive control in western experiments [30].

## RESULTS AND DISCUSSION

*Construction of* A. nidulans *and* A. terreus *strains containing plasmids pGLAVP6 and pGPDVP6.* Two plasmids, pGPDVP6 and pGLAVP6, were constructed as described in Materials and Methods in which the complete coding sequence of rotavirus gene 6 (encodes the viral capsid protein VP6) was placed under the control of either a constitutive or an inducible promoter (Figure 1). pGPDVP6 contains the *A. nidulans gpdA* (glyceraldehyde-3-P-dehydrogenase) constitutive promoter and pGLAVP6 contains the *A. terreus gla1* (glucoamylase) promoter which is induced in the presence of starch or maltose [16]. With regard to the latter, clustal analysis of

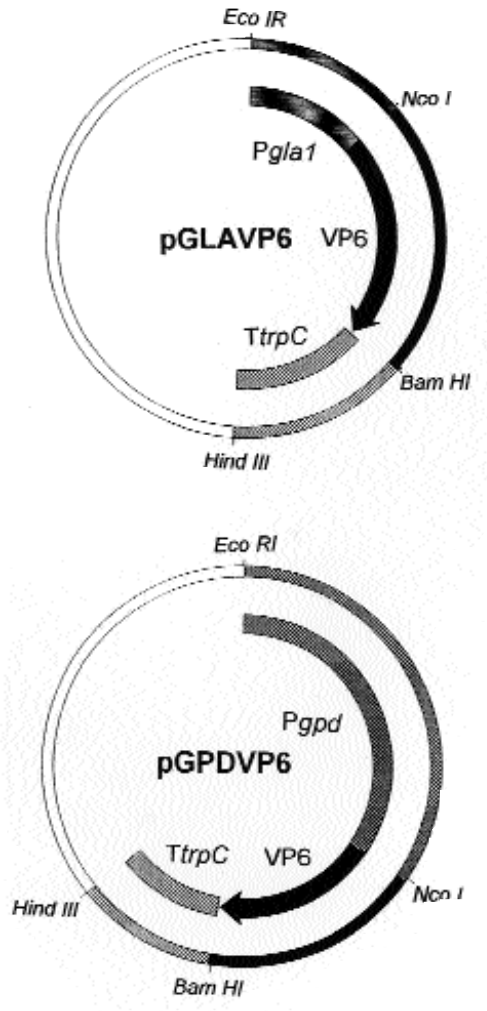


Figure 1. Restriction maps of plasmids pGLAVP6 and PGPDVP6.

the deduced amino acid sequence of Gla1 and the corresponding glucoamylase sequences deduced for a variety of fungal species indicates the occurrence of a 'pro' sequence which follows the signal peptide [31]. The sequence encoding the predicted *A. terreus* glucoamylase signal peptide and presumed 'pro' sequence was fused in phase with the VP6 ORF since previous work has shown that the *A. terreus* Gla1 glucoamylase is efficiently secreted when expressed heterologously in *A. nidulans* (Ventura and Ramón, unpublished results). The fusion protein comprising the first 32 amino acids of Gla1 plus the complete VP6 protein may thus be expected to be produced and secreted extracellularly. In the case of pGPDVP6 the absence of a signal peptide should result in the intracellular location of the heterologous protein.

Plasmids pGLAVP6 and pGPDVP6 were introduced by co-transformation into both *A. nidulans* and *A. terreus* hosts as detailed in Materials and Methods. *A. nidulans* co-transformants obtained using pGLAVP6 and pGPDVP6 were designated NGLA (one transformant) and NGPD (three transformants), respectively; *A. terreus* co-transformants arising from pGLAVP6 and pGPDVP6 were designated TGLA (eight transformants) and TGPD (ten transformants), respectively. Co-transformant genomic DNAs were digested with *Eco*RI and *Hin*dIII and analyzed by Southern blotting using a PCR-generated

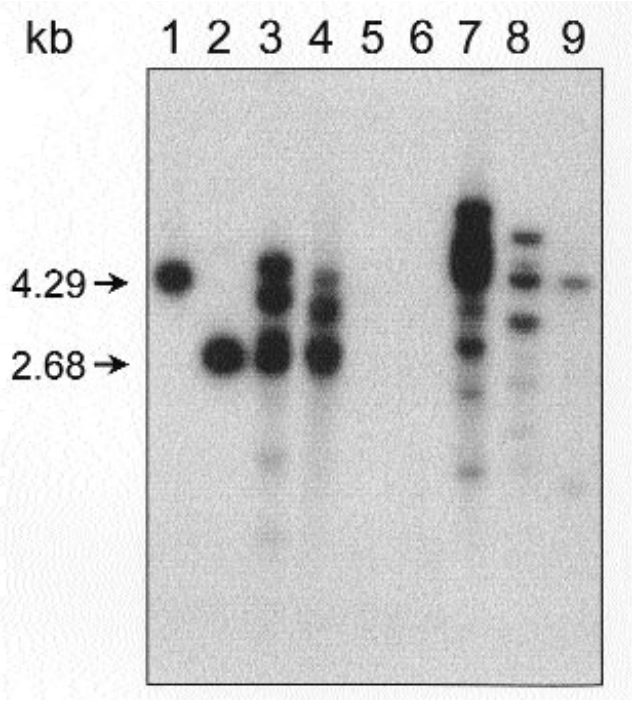


Figure 2. Southern blot analysis of transformants. Lanes 1 and 2 were loaded with 10 ng of *Eco*RI/*Hin*dIII digested pGPDVP6 and pGLAVP6, respectively, as positive controls. Lanes 3-9 were loaded with 10 µg of transformant genomic DNA digested with the same enzymes. 3: NGLA-1; 4: TGLA-1; 5: *A. terreus*-pAN7.1; 6: *A. nidulans*-pILJ16; 7: NGPD-1; 8: NGPD-2; 9: TGPD-1. Hybridizations were done using a PCR fragment (1232 bp) obtained with the oligonucleotides VP6A and VP6B as probe (see Materials and Methods for details). The *A. terreus*-pAN7.1 and *A. nidulans*-pILJ16 strains were transformed with the selection plasmids only and are included as controls.

fragment of the VP6 gene as a probe (Figure 2). Since the promoter-VP6-terminator fusion occurs on fragments that are spanned by *Eco*RI and *Hin*dIII, the occurrence of bands in digests of transformant genomic DNAs of the same sizes as those detected in the *Eco*RI/*Hin*dIII digested plasmids (pGPDVP6: 4293 bp; pGLAVP6: 2683 bp) indicates the presence of at least one intact copy of the promoter-VP6-terminator fusion region. With the exception of TGPD-1, the co-transformants contained multiple copies of the transforming DNA.

*Analysis of VP6 production in co-transformants.* Several NGLA and TGLA co-transformants (containing the maltose-inducible promoter/VP6 recombinant fragment) were grown for 24 h in AMM with 1% (w/v) gluco-

se and then transferred to AMM containing 1% (w/v) maltose as sole carbon source, conditions previously shown to result in Gla1 expression in both *A. terreus* and *A. nidulans* transformants [16]. Aliquots of culture filtrates were taken at various times (24 h, 48 h, 72 h, 96 h and 120 h) after transfer and analyzed by western blotting using a polyclonal antibody raised against VP6. In contrast to the production of Gla1 previously observed in *A. nidulans* transformants 24 h after transfer [31], no VP6-specific band was detected in media samples (either non-concentrated or concentrated) obtained from either the NGLA or TGLA co-transformants, whilst VP6 was detected in an extract of rotavirus-infected MA104 cells which was used as a positive control (data not shown).

Intracellular production of VP6 was investigated in the both the aforementioned transformants carrying the maltose-inducible VP6 recombinant and those transformants carrying the constitutive *gpd* promoter/VP6 construct (NGPD and TGPD). In the case of the former, maltose induction (transfer culture) was carried out as previously detailed whereas the latter transformants were grown for 24 h on AMM containing 1% (w/v) glucose as sole carbon source (direct culture). Mycelia were collected, disrupted and fractionated into soluble and insoluble components. No bands corresponding to VP6 were detected by western blotting of either non-concentrated or concentrated samples (data not shown).

That the lack of production of VP6 extracellularly in co-transformants containing pGLAVP6 is unlikely to be due to problems related to secretion processes or extracellular protease activity is indicated by the apparent lack of VP6 intracellularly in both these and the pGPDVP6 derived co-transformants.

*mRNA analysis.* To investigate whether the apparent lack of VP6 production may be related to problems concerning VP6-specific mRNA levels, mycelia from both transfer (NGLA and TGLA transformants) and direct cultures (NGPD and TGPD transformants) were prepared. Mycelia from direct cultures were collected 24 h after inoculation of spores and mycelia from transfer cultures were collected 24 h after induction by maltose. Total RNAs were extracted and analyzed by northern blotting using probes specific for VP6 and actin [32] sequences. Whereas the actin probe yielded clearly defined bands corresponding to actin mRNA, the VP6 probe yielded smears. Typical northern blot results are shown in figure 3. The actin signal reveals that little or no general mRNA degradation occurred in the samples analyzed and hence suggests that the smearing observed with the VP6 probe is due to specific instability of the VP6 mRNA in both the *A. nidulans* and *A. terreus* transformants. These observations therefore indicate that the inability to detect VP6 by western analysis is not a consequence of degradation by endogenous proteases but rather a problem related to the transcription process or its product. In this context it is noteworthy that the transformants examined contain rearranged integrations of the VP6 recombinant fragment as well as promoter-VP6-terminator fragments of the correct size (see Figure 1). The former may give rise to the synthesis of aberrant transcription products whose hybridization with the VP6 probe could account for the high molecular weight smearing seen in the northern. The nature and genomic location of the integrations may also influence the transcript profile.

Until relatively recently, most studies on heterologous protein production in filamentous fungi have focused on ways to improve yield by the use of multicopy transformants, gene fusion strategies to position strong fungal promoters and protein signal sequences directly upstream

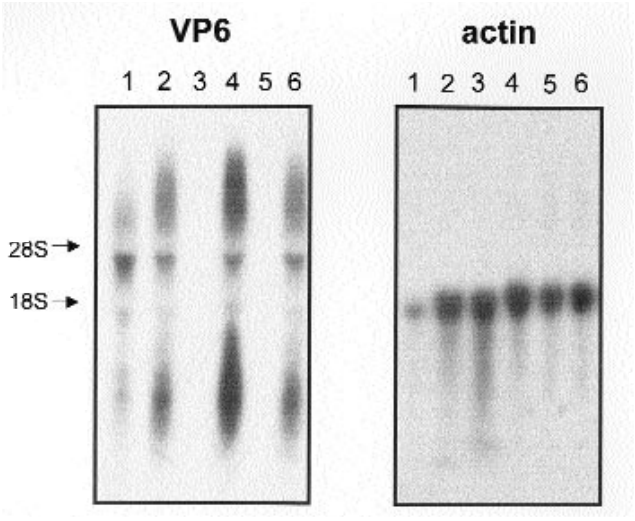


Figure 3. Northern blot analysis. All lanes were loaded with 10 µg of total RNA. A single blot was hybridized, left panel, with the VP6 probe (the same as that described in Figure 2), and subsequently re-hybridized, right panel, with the actin gene probe (a 830 nt *Kpn*I-*Nco*I DNA fragment - [33]). 1: TGLA-1; 2: TGPD-1; 3: *A. terreus*-pAN7.1; 4: NGLA-1; 5: *A. nidulans*-pILJ16; 6: NGPD-1. The *A. terreus*-pAN7.1 and *A. nidulans*-pILJ16 strains were transformed with the selection plasmids only and are included as controls.

of the heterologous gene of interest, and ways to inhibit endogenous fungal protease degradation of the heterologously produced protein. Studies which were designed to provide comparative information on both protein and mRNA production from heterologously expressed fungal and non-fungal genes in *A. awamori* [8], revealed that the levels of non-fungal mRNA produced were very considerably lower than those seen for their heterologous fungal counterparts. Of particular interest is the observation that accumulation of the *Cyamopsis tetragonoloba* αgalactosidase mRNA was influenced by codon usage such that this mRNA was detectable by northern blotting only when the sequence of the transforming gene possessed codon usage similar to that of *Saccharomyces cerevisiae*. Codon usage by *A. awamori*, *A. terreus* and *A. nidulans* is presented in table 1, as is the usage in the VP6 and wild type *Cyamopsis tetragonoloba* αgalactosidase (*aglA*) coding sequences. Whilst there are relatively small differences between the three *Aspergillus* species, the frequency of occurrence of nine codons (shaded) in VP6 is at least 25% lower than that typical of *A. nidulans*; all amino acids encoded by two codons show inverted frequencies of occurrence between *A. nidulans* on one hand and VP6 and *aglA* on the other; in many other cases, the most frequently used codons in *A. nidulans* do not correspond to those most frequently present in VP6. With a few exceptions, the codon usage of VP6 and *aglA* show reasonable levels of similarity. Given the lack of detection of full length *aglA* mRNA [8] it may not be surprising that no VP6 mRNA band can be detected. Comparison of codon usage by *A. awamori* and *A. terreus* to that presented in table 1 for *A. nidulans* reveals relatively small differences between the three species.

Finally, it has recently been noted [33] that high AU content can lead to incorrect transcript processing in *A. awamor*i. The VP6 coding sequence has an AU content of 61.9% whereas the overall AU content of *A. nidulans* coding sequences is estimated at 46.76%. This, in combination with the differences in codon usage noted above, may result in specific instability of the VP6 mRNA and hence account for the lack of heterologous production of VP6 protein by any of the *Aspergillus* transformants

**Table 1**. Comparison of codon usage. The percentage usage of each codon for each amino acid is presented. Codons whose frequency of use in the VP6 CDS is less than 25% of overall use in *A. nidulans* are shaded. The columns for *A. nidulans*, *A. terreus* and *A. awamori* are data calculated from 229 CDS (138870 codons), 8 CDS (6076 codons) and 5 CDS (2444 codons), respectively. The CDS data presented were obtained from the DNA Information and Stock Center, Japan: http://www.dna.affrc.go.jp/~ nakamura/CUTG.html.

| AMINO ACID | CODON | % *A.nidulans* | % *A. terreus* | % *A. awamori* | % VP6 | % *aglA* |
|---|---|---|---|---|---|---|
| Ala | GCA | 18.9 | 17.4 | 15.0 | 55.6 | 51.4 |
| | GCC | 32.6 | 41.0 | 31.5 | 7.4 | 8.6 |
| | GCG | 20.4 | 23.5 | 16.0 | 3.7 | 0 |
| | GCU | 28.1 | 18.1 | 37.5 | 33.3 | 40.0 |
| Arg | AGA | 9.7 | 8.2 | 9.6 | 68.0 | 50.0 |
| | AGG | 9.4 | 3.8 | 6.4 | 24.0 | 25.0 |
| | CGA | 16.4 | 11.0 | 19.1 | 4.0 | 12.5 |
| | CGC | 27.6 | 35.7 | 27.7 | 4.0 | 0 |
| | CGG | 16.9 | 24.3 | 17.0 | 0 | 0 |
| | CGU | 20.0 | 17.0 | 20.2 | 0 | 12.5 |
| Asn | AAC | 63.7 | 65.9 | 65.6 | 32.4 | 24.0 |
| | AAU | 36.3 | 34.1 | 34.4 | 67.6 | 76.0 |
| Asp | GAC | 52.9 | 56.1 | 51.8 | 44.4 | 25.9 |
| | GAU | 47.1 | 46.9 | 48.2 | 55.6 | 74.1 |
| Cys | UGC | 63.2 | 60.0 | 60.6 | 33.3 | 28.6 |
| | UGU | 36.8 | 40.0 | 39.4 | 66.7 | 71.4 |
| Gln | CAA | 37.8 | 26.6 | 29.0 | 77.8 | 90.0 |
| | CAG | 62.2 | 63.4 | 71.0 | 22.2 | 10.0 |
| Glu | GAA | 41.1 | 39.5 | 44.6 | 61.1 | 68.0 |
| | GAG | 58.9 | 60.5 | 55.4 | 38.9 | 32.0 |
| Gly | GGA | 21.5 | 23.1 | 17.1 | 57.9 | 57.9 |
| | GGC | 34.8 | 45.3 | 41.2 | 15.8 | 10.5 |
| | GGG | 14.9 | 14.8 | 7.5 | 10.5 | 10.5 |
| | GGU | 28.8 | 16.8 | 34.2 | 15.8 | 21.0 |
| His | CAC | 54.5 | 60.3 | 55.2 | 16.7 | 44.5 |
| | CAU | 45.5 | 39.7 | 44.8 | 83.3 | 55.5 |
| Ile | AUA | 10.8 | 2.4 | 10.3 | 28.6 | 34.8 |
| | AUC | 52.4 | 65.2 | 50.5 | 14.3 | 13.0 |
| | AUU | 36.8 | 32.4 | 39.2 | 57.1 | 52.2 |
| Leu | CUA | 9.5 | 4.2 | 8.5 | 25.7 | 16.1 |
| | CUC | 25.6 | 24.7 | 25.6 | 11.4 | 0 |
| | CUG | 23.1 | 39.4 | 33.5 | 5.8 | 16.1 |
| | CUU | 21.2 | 13.2 | 18.2 | 11.4 | 12.9 |
| | UUA | 5.9 | 2.3 | 2.3 | 20.0 | 35.5 |
| | UUG | 14.7 | 16.5 | 11.9 | 25.7 | 19.4 |
| Lys | AAA | 32.4 | 24.2 | 32.9 | 75.0 | 52.6 |
| | AAG | 67.6 | 75.8 | 67.1 | 25.0 | 47.4 |
| Met | AUG | 100 | 100 | 100 | 100 | 100 |
| Phe | UUC | 65.1 | 76.0 | 71.6 | 30.8 | 50.0 |
| | UUU | 34.9 | 24.0 | 28.4 | 69.2 | 50.0 |
| Pro | CCA | 22.2 | 13.2 | 15.0 | 71.4 | 42.9 |
| | CCC | 26.7 | 32.7 | 29.0 | 4.8 | 7.1 |
| | CCG | 22.8 | 35.1 | 23.0 | 14.3 | 0 |
| | CCU | 28.3 | 19.0 | 33.0 | 9.5 | 50.0 |
| Ser | AGC | 19.0 | 18.5 | 26.5 | 3.8 | 18.4 |
| | AGU | 11.3 | 11.7 | 12.3 | 14.8 | 26.3 |
| | UCA | 14.1 | 8.7 | 6.4 | 40.7 | 28.9 |
| | UCC | 20.3 | 27.2 | 20.6 | 7.4 | 10.5 |
| | UCG | 16.6 | 18.3 | 14.2 | 14.8 | 0 |
| | UCU | 18.7 | 15.6 | 20.0 | 18.5 | 15.8 |
| Thr | ACA | 21.3 | 10.2 | 10.9 | 46.4 | 47.7 |
| | ACC | 32.7 | 42.9 | 48.1 | 3.6 | 9.5 |
| | ACG | 20.7 | 27.8 | 13.8 | 7.1 | 9.5 |
| | ACU | 25.3 | 19.1 | 27.2 | 42.9 | 33.3 |
| Trp | UGG | 100 | 100 | 100 | 100 | 100 |
| Tyr | UAC | 63.2 | 71.8 | 77.0 | 40.0 | 15.4 |
| | UAU | 36.8 | 28.2 | 23.0 | 60.0 | 84.6 |
| Val | GUA | 9.2 | 3.5 | 6.0 | 29.7 | 16.7 |
| | GUC | 38.2 | 45.4 | 33.1 | 14.8 | 12.5 |
| | GUG | 23.7 | 36.0 | 35.0 | 33.3 | 16.7 |
| | GUU | 28.9 | 15.1 | 25.9 | 22.2 | 54.2 |

analyzed.

*This work was supported by grant 108994/1030 from IMPIVA (Generalitat Valenciana). A.P. MacCabe was the recipient of an EC Biotechnology Program fellowship (BIO2-CT94-8136).*

## References


1. Kapikian AZ, Chanock RM. Rotaviruses. In: Fields BN, Knipe DM, Chanock RM, Melnick T, Roizman B, Shope RE (Eds.) Virology. New York, Raven Press, 1990; 863-906.
2. Estes MK. Rotaviruses and their replication. In: Fields BN, Knipe DM, Howley PM, *et al.* (Eds.) Fields Virology. Philadelphia, Lippincott-Raven Publishers, 1996; 1625-1655.
3. Beards GM, Campbell AD, Cottrell NR, *et al.* Enzyme-linked immunosorbent assays based on polyclonal and monoclonal antibodies for rotavirus detection. J Clin Microbiol 1984; 19: 248-254.
4. Malherbe HH, Harwin R, Ulrich, M. The cytopathic effect of vervet monkey viruses. South Afr Med J 1963; 37: 407-411.
5. Estes MK, Crawford SE, Penaranda ME, *et al.* Synthesis and immunogenicity of the rotavirus capsid antigen using a baculovirus expression system. J Virol 1987; 61: 1488-1494.
6. van den Hondel CAMJJ, Punt PJ, van Gorcom RFM. Heterologous gene expression in filamentous fungi. In: Bennet JW, Lasure LL (Eds.) More gene manipulations in fungi. San Diego, Academic Press, 1991; 396-428.
7. van den Hondel CAMJJ, Punt PJ, van Gorcom RFM. Production of extracellular proteins by the filamentous fungus *Aspergillus*. Ant van Leeuwen 1992; 61: 153-160.
8. Gouka RJ, Punt PJ, Hessing JGM, van den Hondel, CAMJJ. Analysis of heterologous protein production in defined recombinant *Aspergillus awamori* strains. Appl Environ Microbiol 1996; 62: 1951-1957.
9. Broekhuijsen MP, Mattern IE, Contreras R, Kinghorn JR, van den Hondel CAMJJ. Secretion of heterologous proteins by *Aspergillus niger*: production of active human interleukin-6 in a protease deficient mutant by KEX2-like processing of a glucoamylase-hIL6 fusion protein. J Biotechnol 1993; 31: 135-145.
10. Ward PP, Lo JY, Duke M, May GS, Headon DR, Conneely OM. Production of biologically active recombinant human lactoferrin in *Aspergillus oryzae*. Bio/technology 1992; 10: 784-789.
11. Ventura L, Ramón D. Transformation of *Aspergillus terreus* with the hygromycin B resistance marker from *Escherichia coli.* FEMS Microbiol Lett 1991; 82: 189-194.
12. Ventura L, Ramón D, Pérez JA. Isolation of an *Aspergillus terreus* mutant impaired in arginine biosynthesis and its complementation with the *argB* gene from *Aspergillus nidulans.* FEMS Microbiol Lett 1992; 99: 187-192.
13. Gwynne DI, Buxton FP, Williams SA, Garven S, Davies RW. Genetically engineered secretion of active human interferon and a bacterial endoglucanase from *Aspergillus nidulans*. Bio/technology 1987; 5: 713-719.
14. Tsuchiya K, Tada T, Gomi K, *et al.* High level expression of the synthetic human lysozyme gene in *Aspergillus oryzae*. Appl Microbiol Biotechnol 1992; 38: 109-114.
15. Hintz WE, Lagosky PA. A glucose-derepressed promoter for expression of heterologous products in the filamentous fungus *Aspergillus nidulans*. Bio/technology 1993; 11: 815-818.
16. Ventura L, González-Candelas L, Pérez-González JA, Ramón D. Molecular cloning and transcriptional analysis of the *Aspergillus terreus gla1* gene encoding a glucoamylase. Appl Environ Microbiol 1995; 61: 399-402.
17. Pontecorvo G, Roper JA, Hemmons LJ, MacDonald KD, Bufton AWJ. The genetics of *Aspergillus nidulans*. Adv Genet 1953; 5: 141-238.
18. Johnstone IL, Hughes J, Clutterbuck AJ. Cloning an *Aspergillus nidulans* developmental gene by transformation. EMBO J 1985; 4: 1307-1311.
19. Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Hondel CAMJJ. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 1987; 56: 117-124.
20. Offit PA, Clark HF, Plotkin SA. Response of mice to rotavirus of bovine or primate origen assessed by radioimmunoassay, radioimmunoprecipitation, and plaque reduction neutralization. Infect Immun 1983; 42: 293-300.
21. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual (2nd. ed). New York, Cold Spring Harbor Laboratory Press, 1989.
22. Buesa J, Colomina J, Raga J, Villanueva A, Prat J. Evaluation of reverse transcription and polymerase chain reaction (RT/PCR) for the detection of rotaviruses: applications of the assay. Res Virol 1996; 147: 353-361.
23. MacCabe AP, Fernández-Espinar MT, de Graaff L, Visser J, Ramón D. Identification, isolation and sequence of the *Aspergillus nidulans xlnC* gene encoding the 34-kDa xylanase. Gene 1996; 175: 29-33.
24. Feinberg BP, Vogelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 1984; 137: 266-267.
25. Mansell E, Ramig R, Patton J. Temperature-sensitive lesions in the capsid proteins of the mutants tsF and tsG that virion assembly. Virology 1994; 204: 69-81.
26. Ganga A, González-Candelas L, Ramón D, Pérez-González JA. Glucose tolerant expression of *Trichoderma longibrachiatum* endoglucanase I, an enzyme suitable for use in wine production. J Agric Food Chem 1997; 45: 2359-2362.
27. Pérez-Esteban B, Orejas M, Gómez-Pardo E, Peñalva MA. Molecular characterisation of a fungal secondary metabolism promoter: transcription of the *Aspergillus nidulans* isopenicillin N synthetase gene is modulated by upstream negative elements. Mol Microbiol 1993; 9: 881-895.
28. Wingfield PT, Palmer I, Liang SM. Purification of recombinant proteins. In: Coligan JE, Dunn BM, Ploegh HL, Speicher DW, Wingfield PT (Eds.) Current protocols in protein science. 1995; 1: 6.0.1-6.5.27.
29. Ramón D, van den Veen P, Visser, J. Arabinan degrading enzymes from *Aspergillus nidulans*: induction and purification. FEMS Microbiol Lett 1993; 113: 15-22.
30. Estes MK, Graham DY, Smith EM, Gerba CP. Rotavirus stability and inactivation. J Gen Virol 1979; 43: 403-409.
31. Ventura L. Modificación Genética del Ascomycete *Aspergillus terreus.* 1996, Ph.D. Thesis.
32. Fidel S. Doonan JH, Morris NR. *Aspergillus nidulans* contains a single actin gene which has unique intron locations and encodes γ-actin. Gene 1988; 70: 283-293.
33. Gouka RJ, Punt PJ, van den Hondel CAMJJ. Glucoamylase gene fusions alleviate limitations for protein production in *Aspergillus awamori* at the transcriptional and (post)transcriptional levels. Appl Environ Microbiol 1997; 63: 488-497.