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Foot-and-mouth disease virus proteinase 3C inhibits translation in recombinant *Escherichia coli*

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Abstract: Escherichia coli cultures do not survive the expression of recombinant foot-and-mouth disease virus proteinase 3C. This effect is ascribed to degradation of bacterial protein(s), as concluded from the observation of gradual cessation of gene expression upon induction of 3C expression. Most likely, translation inhibition is the cause of bacterial death, as (i) cell-free translation of the 3C gene was restored by additional bacterial ribosomes, (ii) ribosomes from proteinase 3C-producing cells differed from normal ones by a reduced content of protein S18, and (iii) transcription was not inhibited.

Key words: Foot-and-mouth disease virus; 3C Proteinase; Expression in *Escherichia coli*; Translation inhibition; EC rS18

Introduction

Foot-and-mouth disease virus (FMDV), a picornavirus, encodes a 260 kDa polyprotein in its positive-stranded RNA genome. The polyprotein is processed by the viral proteinases L, 2A and 3C to generate the mature proteins required for virus replication [1–3]. The genome organisation and cleavage sites are shown in Fig. 1A. Apart from processing virus protein, some FMDV proteinases damage the infected host cells: translation is inhibited owing to L-mediated cleavage of the eukaryotic initiation factor 4F [4], and transcription is inhibited owing to 3C-mediated truncation of the nuclear protein histone H3 [5].

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FMDV proteinase 3C, expressed from recombinant cDNA, acts in *trans* [6] and causes the death of *E. coli* cells [6,7]. However, no lethal effect was observed when active 3C enzyme of other picornaviruses, polio- and human rhinovirus 14, was produced by recombinant *E. coli* [8–10]. Although being a special property of FMDV 3C irrelevant to infection, the reason for bacterial death as a result of the production of active enzyme was analysed to see whether the lethal effect can be avoided. This would enable the use of bacteria as a cheap and safe source of the FMDV 3C enzyme.

Materials and Methods

Construction of the plasmids pVP1-52 and pVP1-Pol was described earlier [11]. The latter plasmid contains cDNA of FMDV O_1 Kaufbeuren encoding the capsid protein VP1 and all

non-structural virus proteins downstream of it. The vector was pPLc24 [12]. Removal of subfragments from the insert by the restriction enzymes *Bgl*II, *Xho*I and *Sac*I provided p^{δ}Bgl, p^{δ}Xho and p^{δ}Sac, respectively (Fig. 1B). The construct pRITD and its deletion variant pRITD$^{\delta}$ (Fig. 1C), as well as the production of antiserum to the expression product of pRITD$^{\delta}$ that recognised FMDV proteinase 3C in infected cells, was also described earlier [13]. The inserts of all constructs elongate open reading frames preceded by phage λ promoters. Use of the thermolabile λ repressor CI857 that is inactive at 42°C allows for inducible plasmid gene expression.

Plasmids were transcribed in vitro and translated in the cell-free Zubay system [14]. Briefly, the assay (30 μl volume) requires: 2 μg supercoiled plasmid DNA, 30000 g supernatant of lysed *E. coli* cells, an energy generating system, tRNA, ribonucleotides, inorganic salts and amino acids. For translation analysis, an amino acid mixture lacking methionine was used and [^{35}S]-methionine (2 μCi) was added prior to incubation at 37°C. For transcription analysis, a mixture of the non-radioactive essential amino acids was used and additional [^{32}P]-UTP (2 μCi) was added. The products of synthesis were quantitatively determined by precipitating reaction aliquots with trichloroacetic acid and counting the radioactivity contained. Qualitative protein analysis was performed by NaDodSO$_4$-PAGE of 5×10^5 trichloroacetic acid-precipitable cpm (data not shown). Transcripts were quantitatively and qualitatively analysed.

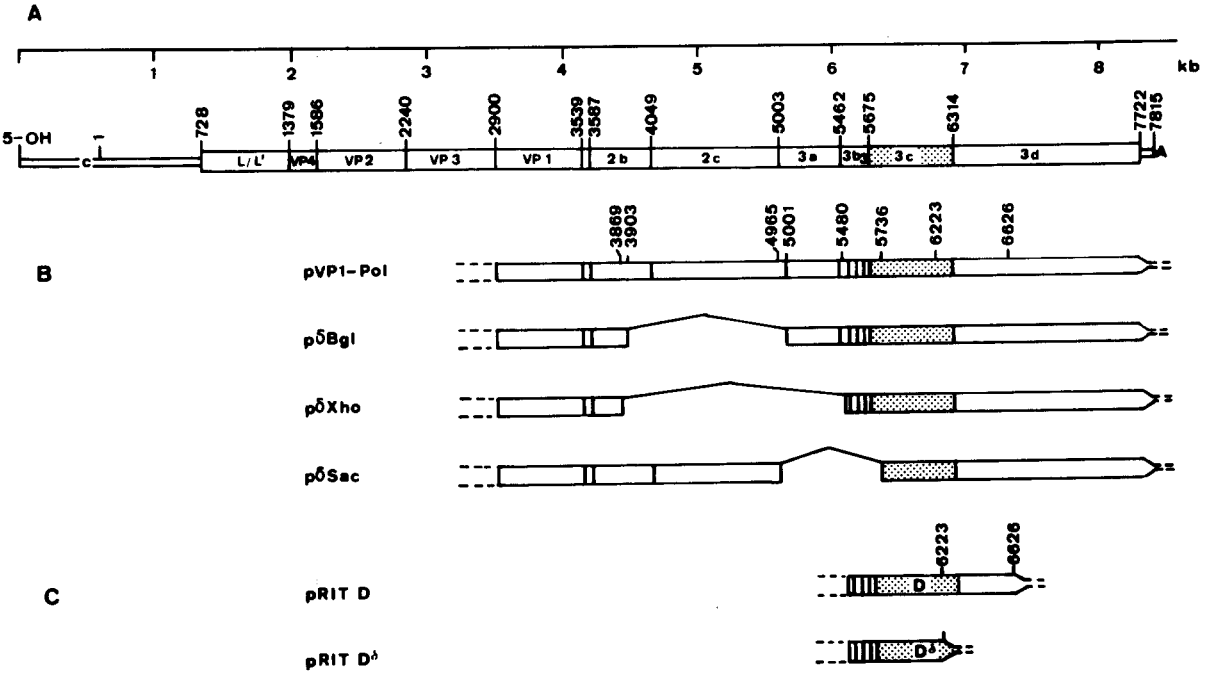


Fig. 1. Two families of inducible FMDV cDNA expression vectors. (A) Shows the FMDV genome in a kilo-base (kb) scale. The long open reading frame is indicated by the broad part of the open bar, and the encoded proteins by the map positions of their N-terminal codons. The proteinase 3C-coding sequence is dotted. (B) Shows FMDV cDNA contained in the expression vector pVP1-Pol [11] and its derivatives p^{δ}Bgl, p^{δ}Xho and p^{δ}Sac, generated by in-frame deletion of restriction fragments. The restriction sites are indicated in the map of pVP1-Pol: *Bgl*II cleaves at positions 3903 and 5001; *Xho*I at 3869 and 5480; *Sac*I at 4965 and 5736. (C) Shows the *Xho*I-*Sal*I FMDV cDNA restriction fragment (positions 5480–6626) contained in the expression vector pRITD. pRITD$^{\delta}$ was derived from pRITD [13] by deletion of a *Pst*I fragment (position 6223 and downstream in the multiple cloning site of the vector). Nucleotides are numbered according to the UWGCG data base version of the FMDV genome sequence [20].

Ribosomes and ribosomal proteins were isolated from *E. coli* HB101 according to a standard protocol [15]. Separation of ribosomal proteins by two-dimensional gel electrophoresis was performed as described by Roth and Nierhaus [16].

Results and Discussion

RNA transcribed from recombinant FMDV 3C-coding cDNA in vitro and translated in reticulocyte lysate resulted occasionally in a reduced proteolytic activity of 3C [2,3,17]. Reduction of the 3C activity might minimise its toxic effect on bacteria and may be achieved by changing the genetic context of the 3C-coding cDNA. Therefore, a family of in-frame deletion variants was generated from pVP1-Pol. In p^{δ}Bgl and p^{δ}Xho (Fig. 1B), the 3C-coding portion and the 3C cleavage site between VP1 and 2A remained unmodified. p^{δ}Sac lacks, among others, 20 codons for the N-terminus of 3C. The corresponding fusion protein was expected to be proteolytically inactive.

A cDNA fragment that encodes 3C and some flanking amino acids was excised from pVP1-Pol. Subcloning of this fragment into the vector pRIT2T [18] provided pRITD (Fig. 1C). Removal of a *Pst*I restriction fragment from pRITD, encoding 30 C-terminal residues of 3C and adjacent 3D-specific residues, provided pRITD$^{\delta}$ (Fig. 1C). Virtually the same cDNA fragment as that contained in pRITD was found to express active 3C enzyme which blocked transcription on being inserted into an eukaryotic expression vector [5]. Therefore, expression of pRITD in *E. coli* was expected to provide active 3C enzyme.

The effect of FMDV cDNA expression (Fig. 1B and C) on bacterial propagation was analysed by a study of the growth kinetics. Time-dependent increase in OD_{620}, indicating cell division, was observed for cultures that expressed incomplete 3C-coding cDNA or that kept the cDNA uninduced, whereas cultures that expressed this cDNA stopped growing (data summary in Table 1). This showed that the expression of the complete 3C gene was bacteriotoxic, irrespective of the genetic context. As the expression of C- or

Table 1

Correlation of cell death and production of active 3C proteinase

E. coli containing	3C-coding sequence	Proteolytic activity [1]	Cell proliferation [2]
pVP1-Pol	complete	+	−
p^{δ}Bgl	complete	+	−
p^{δ}Xho	complete	+	−
p^{δ}Sac	incomplete [3]	−	+
pRITD	complete	n.d.	−
pRITD$^{\delta}$	incomplete [4]	n.d.	+

[1] FMDV-specific polyprotein cleavage occurred at the VP1-2A junction, as deduced from Western blotting (data not shown).

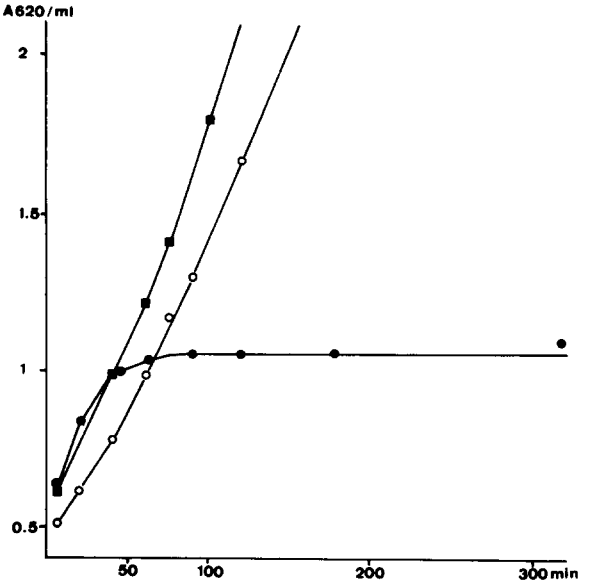
[2] deduced from OD_{620} of the cultures; examples concerning pRITD and pRITD$^{\delta}$ are provided in the figure below.

[3] 5′-end deleted.

[4] 3′-end deleted.

+ yes; − no; n.d. not detectable.

Study of the growth kinetics of *E. coli* containing pRITD or pRITD$^{\delta}$



E. coli containing pRITD were grown at 28°C, split, and either grown further at 28°C (open circles) or shifted to 42°C (closed circles). As a control, cells containing pRITD$^{\delta}$ were grown at 42°C (closed squares). Culture aliquots were removed at the indicated times and the optical density at 620 nm was measured. Results are presented as absorption (A_{620})/ml.

N-terminally truncated 3C allowed cell proliferation, the lethal effect of 3C enzyme most probably is due to its intrinsic proteolytic activity and not due to the content of sequences that are accidently toxic to *E. coli*.

The translation products of pRITD and pRITD$^{\delta}$ were compared in a growth kinetic experiment (Fig. 2). Products were identified by antibody binding to the N-terminal part of the fusion proteins, which is *Staphylococcus aureus* protein A [18]. The proteins were heterogeneous in size, probably due to degradation by bacterial proteinases. Identification of 3C-mediated proteolysis products was impossible.

The patterns differed in signal distribution. Cells that expressed pRITD for longer than 30 min did not contain primary translation product, but product fragments of decreasing size. In contrast, pRITD$^{\delta}$-expressing cells contained primary translation products at all times. A translation product pattern such as observed following pRITD expression could result from inhibited

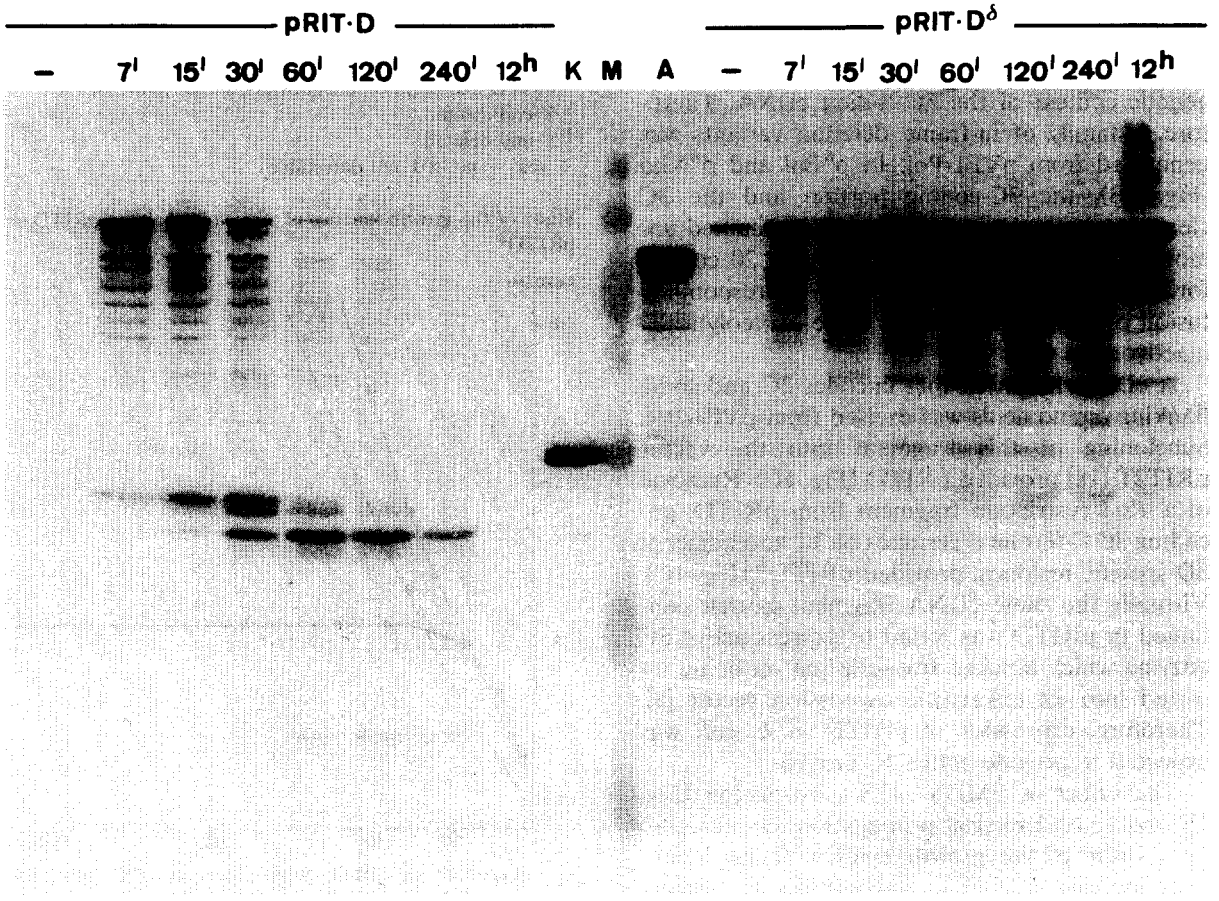


Fig. 2. Expression kinetics of *E. coli* containing pRITD and pRITD$^{\delta}$. Cultures of *E. coli* containing pRITD or pRITD$^{\delta}$ were grown at 28°C and then shifted to 42°C to induce gene expression. Culture aliquots were removed at times indicated above each slot (′, minutes; h, hours). Control samples (−) were removed before temperature shift. Total protein of all aliquots was precipitated by trichloroacetic acid. The precipitates were subjected to NaDodSO$_4$-PAGE and then transferred to a nitrocellulose membrane. The N-terminal part of the fusion proteins encoded by both vectors contains the immunoglobulin-binding domain of *Staphylococcus aureus* protein A. Products of induced vector gene expression were identified by incubating the membrane with peroxidase-conjugated immunoglobulin. Controls were native *S. aureus* protein A (lane A), and the expression product of the vector not containing any FMDV cDNA (lane K). Lane M contains prestained protein molecular mass markers.

vector gene expression and simultaneous degradation of already produced products.

Inhibition of gene expression must result from inhibited transcription or translation. Both functions can separately be analysed in a plasmid-dependent cell-free gene expression system [14]. The translation efficiency was quantified by determining the incorporation of [^{35}S]-methionine into newly synthesised protein. pRITD (filled squares in Fig. 3) and p$^{\delta}$Bgl (filled triangles), both containing the complete 3C gene, were inefficiently translated, whereas pRITD$^{\delta}$, containing a truncated 3C gene (dots in Fig. 3) was rapidly and efficiently translated. Translation of pRITD and p$^{\delta}$Bgl was restored by additional 70S ribosomes purified from control *E. coli* cells (open squares and triangles in Fig. 3). This ribosome preparation did not synthesize protein in the absence of plasmids (crosses). This showed that translation inhibition results from the production of active FMDV 3C enzyme.

The transcription rate of the above mentioned plasmids was not reduced in the cell-free system, and the transcripts were of expected sizes in the presence of RNase inhibitor (data not shown). Therefore, bacterial transcription was not inhibited by the production of FMDV proteinase 3C.

The results suggested that *E. coli* ribosomes were damaged by proteinase 3C. In order to identify the defect(s), ribosomes from cultures that expressed either the complete 3C gene of pVP1-Pol or did not contain a 3C gene (pVP1-52; [11]) were analysed. The isolation of ribosomes started 10 min following induction of plasmid gene expression. Ribosomes from both cultures were dissociated into subunits and then separated by sucrose gradient centrifugation. The typical pattern of adsorbance at 260 nm of 30S and 50S ribosomal subunits was obtained with material isolated from control cells. Cells that expressed pVP1-Pol contained the subunits in, however, a 1:4 instead of a 1:2 ratio (data not shown). Six to ten A_{260} units of each subunit were obtained and subjected to two-dimensional gel electrophoresis. The protein patterns of 50S ribosomal subunits of both sources were identical (data not shown), but those of 30S subunits differed in the content of protein S18. It was almost absent

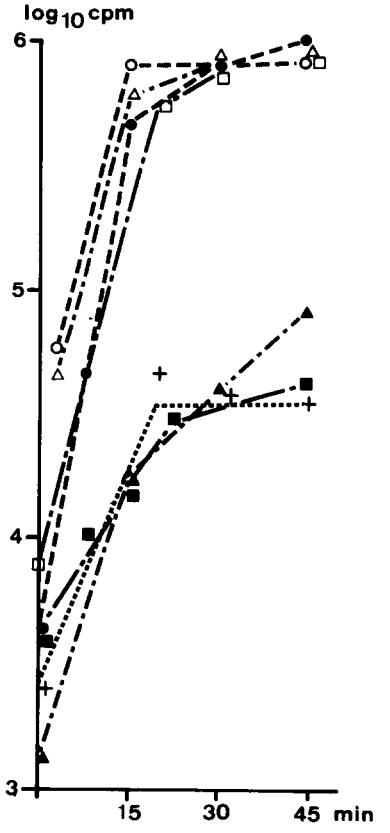


Fig. 3. Quantitative cell-free transcription/translation of FMDV 3C-coding vectors. Equal amounts of the expression vectors pRITD (filled squares), p$^{\delta}$Bgl (filled triangles) and pRITD$^{\delta}$ (filled circles) were incubated in a cell-free prokaryotic transcription/translation system containing [^{35}S]-methionine. Each reaction was repeated in the presence of additional S OD_{260} units *E. coli* 70S ribosomes (open symbols). To control for background protein synthesis ribosomes, but no plasmid, were incubated in the system (+). Aliquots were removed from each reaction at the indicated times and precipitated by trichloroacetic acid. The precipitates were loaded onto nitrocellulose filters, washed and subjected to scintillation counting. The radioactivity contained in each sample is indicated as $\log_{10}$ counts per min (cpm).

from particles extracted from pVP1-Pol-expressing cells (Fig. 4, circled), and present in the control, as expected. Additional spots in the pVP1-Pol-specific pattern represent 50S subunit proteins, possibly indicating a beginning of particle decay.

It was concluded that FMDV proteinase 3C accounted for the depletion of protein S18 from 30S ribosomal subunits, which rendered them

functionally inactive. S18, but no other of the 55 proteins that are assembled in *E. coli* ribosomes, exhibits a four-residues spanning sequence homology to the 3C cleavage site TESG, located between the FMDV proteins 3B and 3C in the viral translation product. Other ribosomal proteins exhibit, at best, sequence homologies of di- and tripeptide size [19] to 3C cleavage sites [20–22]. Therefore it is likely that S18 can be a substrate for 3C.

Protein S18 is located in the larger lobe of 30S subunits. It is accessible to antibodies directed against S18 and their binding inhibits translation [23]. Protein S18 could likewise be accessible to newly synthesised proteinase 3C and cleaved. Autocatalytic maturation of picornaviral proteinases 3C is not required for the cleavage of viral substrate [21].

With regard to the aim of the study, to see whether *E. coli* can survive the expression of recombinant DNA encoding the entire FMDV proteinase 3C, the following conclusion is drawn: to obtain large quantities of bacterially produced enzyme, a treatment of cells is required that prevents translation inhibition. It appears possible to titrate the enzyme by offering substrate in excess. This might be achieved by overproduction of protein S18 or other substrate in 3C-producing cells. Bacterial translation inhibition may also be prevented by growing the cells in the presence of proteinase inhibitors that were effective on the poliovirus 3C proteinase [8]. However, some inhibitors may be toxic for *E. coli*. Such was observed when FMDV 3C-producing cells were grown in the presence of zinc ions [6].

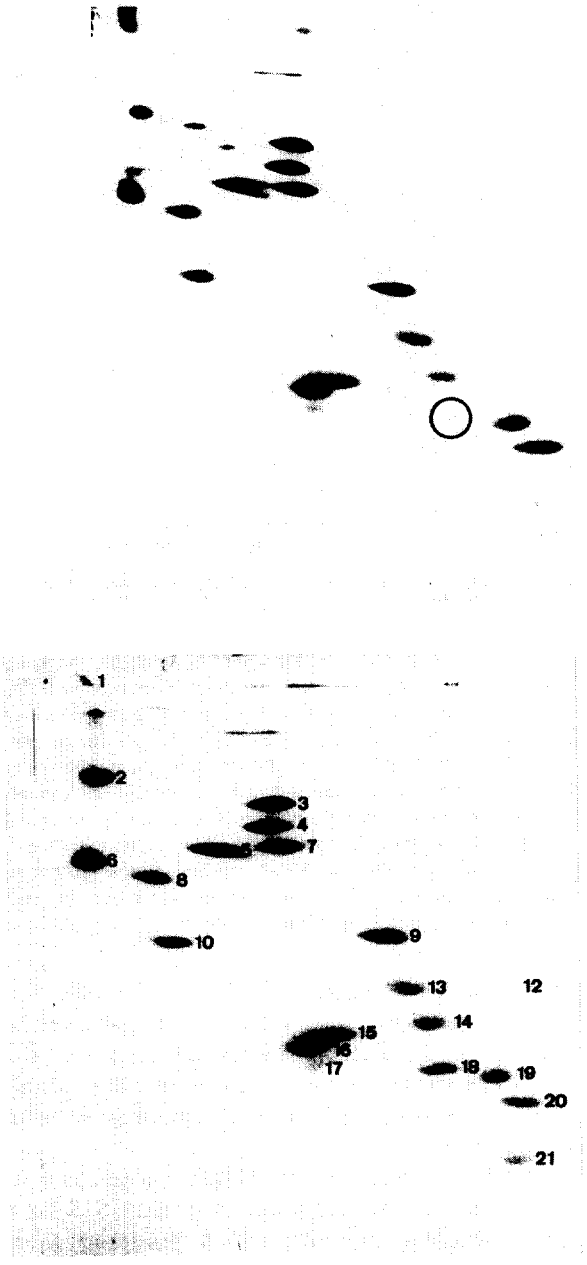


Fig. 4. 30S ribosomal proteins extracted from FMDV proteinase 3C-producing (top) and non-producing (bottom) *E. coli*. *E. coli* harbouring the plasmids pVP1-Pol or pVP1–52 [11] were grown at 28°C in 400 ml medium to 0.5 OD_{620}, pelleted by centrifugation and resuspended in 400 ml medium prewarmed to 42°C. Following further incubation at 42°C for 10 min, cells were collected and disrupted. Cell debris was removed by centrifugation first at low speed and then at 35000 × *g*. The supernatant was loaded on 30% sucrose and centrifuged at 100000 × *g*. The pellet was resuspended and ribosomes contained therein were dissociated into 50S and 30S subunits and separated by conventional sucrose gradient centrifugation. The gradients were fractionated and the ratio of 260/280 nm was measured for each fraction. The peak fractions containing 6–10 OD_{260} of 30S or 50S material, respectively, were subjected to two-dimensional gel electrophoresis. Proteins in the gels were stained by amido black and photographed. The patterns of 50S ribosomal proteins were identical (not shown), whereas those of 30S subunits differed in the content of protein S18 (circled).

An alignment of FMDV 3C-coding sequences of several subtypes of three serotypes showed amino acid conservation of more than 99% [24]. This suggests similar proteolytic specificity of the enzyme, irrespective of the virus strain used as a source. The 3C sequences of different picornaviruses are, however, poorly conserved [25]. This must account for their different substrate specificities [21]. The poliovirus enzyme, for example, is strongly restricted to substrates containing QG. This explains why it is feasible to produce this enzyme in *E. coli* [8,10].

Picornaviral proteinases are structurally similar to serine proteinases [26,27], but differ from these by the requirement of cysteine in the active centre close to the C-terminus [9,28]. The other residues that form the catalytic triad are not unequivocally identified [29].

Like FMDV proteinase 3C, the proteinase of human immuno-deficiency virus is toxic to recombinant *E. coli* [30]. The bacterial protein that is the putative target of the latter enzyme is not yet identified. Both viral proteinases differ with regard to their cleavage sequences. One would like to exploit the different activities of viral proteinases for protein analysis.

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