

Identification of the *EcoKI* and *EcoR124I* Type I Restriction–Modification Enzyme Subunits by Non-Equilibrium pH Gradient Two-Dimensional Gel Electrophoresis

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ABSTRACT. Effectively optimized and reproducible procedure for monitoring the composition of type I restriction–modification endonucleases *EcoKI* and *EcoR124I* by non-equilibrium pH gradient two-dimensional (2-D) gel electrophoresis is described. Three subunits of the enzyme complex, which widely differ from one another in their isoelectric points and molar mass, were identified in crude cell extracts of *E. coli*. For the first time all three subunits of both *EcoKI* and *EcoR124I* were detected as distinct spots on a single 2-D gel. A sensitive immunoblotting procedure was suggested suitable for routine use in determining the identity of individual subunits. Potential application of this method for detailed studies of regulation of the function and stoichiometry of the enzyme complexes is discussed.

Type I restriction-modification (R–M) systems are the most complex currently known enzyme assemblies and analysis of the genetic determinants of these systems in natural isolates of *E. coli* revealed unusual allelic diversity (Barcus *et al.* 1993). The R–M system exhibiting a high degree of sequence similarity to the type I revealed in *Mycoplasma pulmonis* was the first one described outside enteric bacteria (Dybvig and Huilan 1994) originally considered as the only host of the system. Furthermore, as the nucleotide sequences for numerous bacterial genomes have been determined, a great number of putative R–M systems were detected (Tomb *et al.* 1997). *Helicobacter pylori*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* possess genes that are predicted to encode R–M enzymes (Saunders *et al.* 1998; Murray 2000).

Type I R–M systems are classified into four distinct families, *viz.* IA, IB, IC and ID. Most of them are encoded by chromosomally located genes like the *EcoKI* (IA) system. The R–M system *EcoR124I* (IC) is a representative of plasmid-born enzymes which are encoded by three genes, *hsdR*, *hsdM* and *hsdS*. All these genes are necessary for the production of active restriction endonuclease; *hsdR* is absolutely required for restriction and is transcribed from its own promoter (P_{RES}), while *hsdM* and *hsdS* are transcribed from a separate promoter (P_{MOD}) and together are required for modification (Redaschi and Bickle 1996; Titheradge *et al.* 1996).

Classical R–M systems provide the host bacteria with immunity to infection by foreign DNA and protect cellular DNA from restriction by methylation of adenosyl residues within the sequence recognized by the restriction enzymes. The key to the success of these protective systems lies in the control of restriction *vs.* modification activities. When new R–M gene complexes enter the cell on a conjugative plasmid, the expression of the restriction enzyme is delayed compared to that of the methyltransferase so that lethal restriction of the host genome is avoided (Prakash-Cheng *et al.* 1993). In this way, R–M complexes behave as “smart parasites” because they assure the survival of the host cell (Kobayashi *et al.* 1999).

Recent years have witnessed a renaissance of interest in R–M enzymes type I. Many efforts are especially focused on elucidating the control mechanism of restriction *vs.* modification (*for review see* Firman 2000; Murray 2000).

Two-dimensional (2-D) electrophoresis in combination with immunoblotting using specific antibodies provides a powerful tool for identification of proteins in complex mixtures (Zeindl-Eberhart *et al.* 1997).

Here we report on the conditions for separation and identification of individual subunits of R–M systems *EcoKI* and *EcoR124I* on the background of complex protein mixtures of total cell extracts, which is necessary for any further proteomics studies of the system. We optimized the protocol of the 2-D electro-

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phoresis using a non-equilibrium pH gradient in the first dimension (NEPHGE) which provides high resolution for both acidic and basic proteins (O'Farrell *et al.* 1977) and ensures a simultaneous detection of all three subunits of *EcoKI* and *EcoR124I* in *E. coli* cell crude extracts.

MATERIALS AND METHODS

Chemicals. Ampholine pH 7–9 was obtained from *Pharmacia*. Ampholyte pH 3–10 and Nonidet NP-40 were from *Sigma*. Acrylamide, N,N'-methylene-bisacrylamide, SDS and ammonium persulfate were from *Serva*. TEMED and 2-D electrophoresis-protein standards were from *Bio-Rad*, benzonase was from *Merck*. PVDF membrane and ECL western-blotting kit were from *Amersham*.

Microbial techniques. Bacterial strains and plasmids used are listed in Table I. Phage buffer, complex LB medium and *in vivo* restriction assays of *E. coli* C122/pVMC3 and C122/pCP1005 were described by Hubáček and Glover (1970). The solid medium was LB with 1.5 % agar; soft-agar overlay was LB with 0.6 % agar. The antibiotics used were 100 mg/L ampicillin and 12.5 mg/L tetracycline. Transformation and manipulation of nucleic acids were done according to Sambrook *et al.* (1989).

Table I. Bacterial strains and plasmids

Name	Characteristics	Reference
Strains		
<i>Escherichia coli</i> C122	prototroph, Δ hsd	British Culture Collection strain no. 122
C3-6	<i>recA56</i> derivative of <i>E. coli</i> C122	Weiserová <i>et al.</i> 1993
C600	<i>thr leu thi</i>	Appleyard 1954
JM109(DE3)	<i>F' traΔ36 lact Δ(lacZ)M15 proAB recA1 endA1 gvrA96 (Nal^R) hsdR17 mcrA relA1 supE sbcBC thi-1 Δ(lac-proAB) λ (DE3)</i>	Yanisch-Perron <i>et al.</i> 1985
<i>Streptomyces aureofaciens</i> 84/25		Culture Collection, Institute of Microbiology, Prague
Plasmids		
pVMC3	Ap ^R , PRES- <i>hsdR</i> PMOD- <i>hsdMS</i>	Weiserová <i>et al.</i> 1993
pCP1005	<i>hsdS</i> _{124I} <i>hsdM</i> <i>hsdR</i> on pUR51	Finnan <i>et al.</i> 1985
pJS4M	a derivative of pUC119 and pET3A carrying the <i>hsdM</i> and <i>hsdS</i> genes of the <i>EcoR124I</i> R–M system under control of the P _{T7g10} promoter	Patel <i>et al.</i> 1992

Purification of *EcoKI* and *EcoR124I*. The *EcoKI* endonuclease was produced in *E. coli* C3-6 harboring recombinant plasmid pVMC3 and purified according to Weiserová *et al.* (1993). The *EcoR124I* endonuclease was reconstituted *in vitro* by mixing the purified HsdR subunit and methyltransferase in a molar ratio of 2 : 1 (Janšćák *et al.* 1998). The concentration of the endonuclease was taken as the input concentration of the methylase. The *EcoR124I* modification methyltransferase was purified according to Taylor *et al.* (1992) from strain JM109 (DE3) harboring plasmid pJS4M. Purification steps were controlled by fraction analysis on denaturing discontinuous gels (10 % SDS-PAGE) (Laemmli 1970) followed by Coomassie Brilliant Blue R250 staining.

Purification of EF-Tu. Protein synthesis elongation factor EF-Tu from *Streptomyces aureofaciens* used here as protein standard was purified to electrophoretic homogeneity according to Weiser *et al.* (1982).

Sample preparation. *E. coli* C122 cells both with and without appropriate plasmids were harvested by centrifugation at the late-exponential phase, resuspended in STE buffer (in mmol/L; Tris-HCl 10, pH 8.0; NaCl 100; EDTA 1) in the presence of 0.1 mmol/L phenylmethanesulfonyl fluoride and disintegrated by sonication. MgCl₂ was added (up to 10 mmol/L) followed by treatment with benzonase (100 U/mL) on ice for 30 min. Crude extract was cleared by centrifugation. Before adding the sample buffer, the protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard.

Non-equilibrium pH gradient electrophoresis. NEPHGE was performed according to O'Farrell *et al.* (1977) with the following modifications: Carrier ampholytes with non-linear pH 3–10 gradient were used

for the first dimension gels and pH 7–9 ampholytes in sample and overlay buffers. For the second dimension, 8 % acrylamide-slab gels were used and run at constant current 20 mA for each gel in *Bio-Rad* Mini-Protean II 2-D apparatus. Cell crude extract was mixed with sample buffer in the ratio of 1 : 5 and incubated at room temperature for 2 h. The resulting protein solution was stored in aliquots at -70°C . The optimum load was 2 μg of purified protein and 25 μg of crude-extract proteins in 15–20 μL volume.

Proteins were detected by the silver-staining technique of Rabilloud (1992), which can detect 2 ng protein per spot.

Immunodetection of proteins. The proteins separated by NEPHGE were transferred to 0.45 μm poly(vinylidene) fluoride membrane using a Semi-Dry Blotter (*Sigma*). Individual subunits of endonuclease *EcoKI*, *EcoR124I* and EF-Tu were identified by polyclonal antibodies raised in rabbit against respective purified protein. The immune complexes were detected by chemiluminescence following the protocol for the ECL western-blotting system.

RESULTS AND DISCUSSION

High resolution 2-D polyacrylamide gel electrophoresis, which had been introduced by O'Farrel (1975), allows one to separate up to thousands of proteins according to charge (pI) in the first dimension and size (molar mass, M) in the second dimension. This technique has therefore a unique capacity for the resolution of complex mixtures of proteins, permitting simultaneous analysis of thousands of gene products that are under control of global regulatory networks responding to particular physiological situations. The only disadvantage of equilibrium isoelectric focusing (IEF) lies in the instability of the pH gradient in the basic region which causes a common problem, so-called "cathodic drift", when the running time is prolonged. In addition, basic proteins poorly enter the first dimension gel even when more basic ampholytes are used. This problem was overcome by NEPHGE developed later also by O'Farrel *et al.* (1977).

Our aim was the simultaneous visualisation of the three enzyme subunits with different pI and M (Table II) in a single gel. Preliminary attempts to broaden the pH range of the IEF system in order to capture all subunits have failed. The HsdR and HsdM subunits were detected within the range but the HsdS subunit has never been present in the same gel. To modify the pattern of separated subunits in relation to all other proteins in the cell extracts and thus to make their identification and quantitation possible we used the non-equilibrium system. In addition, NEPHGE could be completed within 80–90 min (*see below*), which significantly reduces the time required for 2-D electrophoresis.

Table II. Molar mass and pI of *EcoKI* and *EcoR124I* subunits

Enzyme/subunit	M , kDa	pI	Swiss-Prot accession number
<i>EcoKI</i> /HsdR	136	5.68	P08956
<i>EcoKI</i> /HsdM	59	5.06	P08957
<i>EcoKI</i> /HsdS	51	9.60	P05719
<i>EcoR124I</i> /HsdR	112	6.27	P10486
<i>EcoR124I</i> /HsdM	58	5.04	P10484
<i>EcoR124I</i> /HsdS	46	8.04	P10485

Since during NEPHGE the proteins are not focused to their isoelectric points but move at different rates across the gel, the 'volt-hour' value ($V \times h$, Vh) actually determines the protein pattern spread across the gel. It is therefore important to monitor carefully the Vh in the course of the electrophoresis to assure reproducible results (Lopez 1999). We examined the mobility of the three *EcoKI* (Fig. 1) and *EcoR124I* subunits (*results not shown*) on NEPHGE gels in dependence on Vh using purified proteins as standards. The optimum Vh values for *EcoKI* and *EcoR124I* were 533 Vh (80 min at 400 V) and 600 Vh (90 min at 400 V), respectively.

Identification of EcoKI and EcoR124I subunits in gel. We used a number of ways to identify individual subunits on 2-D gels. Figs 2A–B and 3A–B represent subtractive comparison of the protein pattern of C122/pVMC3–*EcoKI* or C122/pCP1005–*EcoR124I* with the patterns of C122 used as a control. The purified subunits were used in the second dimension as M standards. The next step of identification was immunostaining of 2-D blots with antibodies specifically cross-reacting with *EcoKI* and *EcoR124I* (Figs 2C and 3C,

respectively). Another method we have used to confirm this identification was the co-electrophoresis of the purified subunits with the cell crude extracts of the corresponding strains (*results not shown*). All these approaches helped us to identify the spots representing the three subunits of the enzymes (*boxed-in spots* in Fig. 2B and 3B).

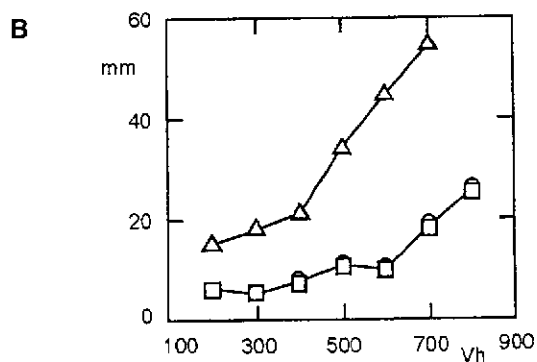
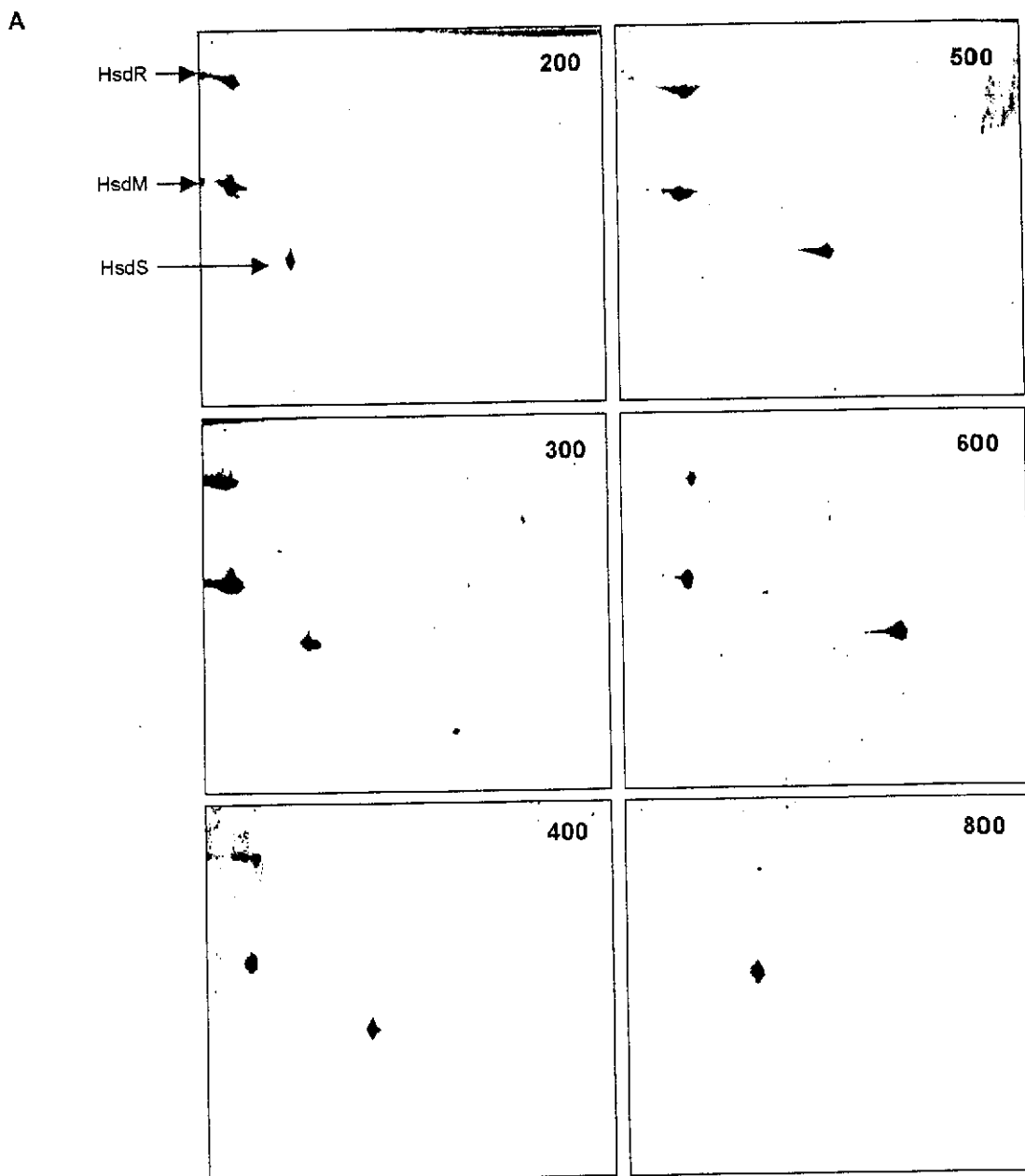


Fig. 1. A: Relative migration of the HsdR, HsdM and HsdS subunits of *EcoKI* as a function of time of electrophoresis; gels were run at 400 V; numbers (200–800) indicate the $V \times h$ values; **B:** the relative distances migrated by the HsdR (circles), HsdM (squares) and HsdS (triangles) subunits on gels (in mm) in Fig. 1A plotted as a function of Vh values of electrophoresis runs.

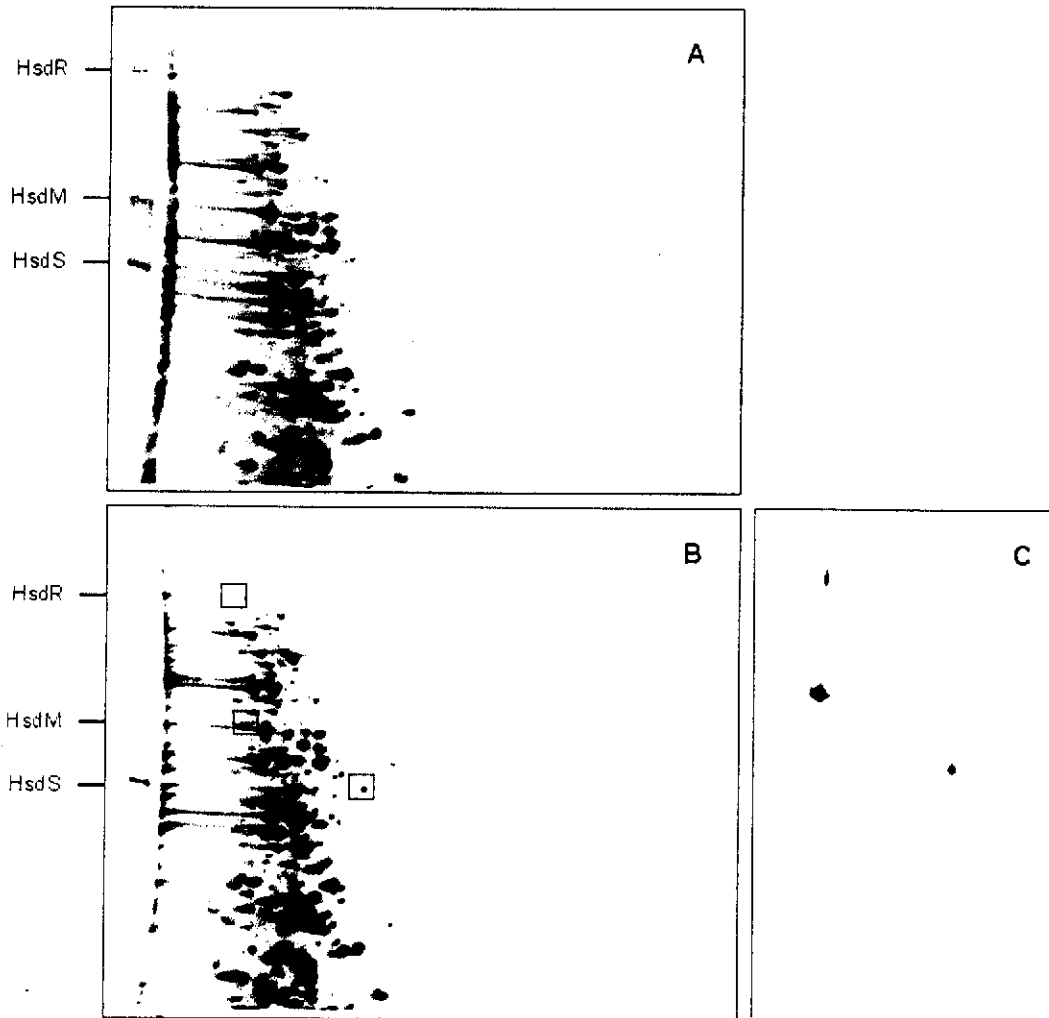


Fig. 2. Detection of *EcoKI* subunits on NEPHGE gel. **A**: Silver-stained gel of cell crude extract of *E. coli* C122 used as control; **B**: silver-stained gel of cell crude extract of C122/pVMC3 (*hsdR*, *hsdM* and *hsdS* genes); the subunits are indicated by rectangles; **C**: immunodetection of *EcoKI* subunits in C122/pVMC3 crude extract.

Orientation of the mini 2-D gel. Commercial 2-D electrophoresis standards were used as external markers for calibration of 2-D gels. The standards were run either in parallel (Fig. 4B) or mixed with cell crude extracts (Fig. 4A). These standards also allowed us to estimate pI and M of the protein spots. However, for monitoring the kinetics of expression in a complex mixture of proteins, it is useful to identify protein(s) of known function as internal standard(s). The protein-synthesis elongation factor Tu (EF-Tu) was already used for this purpose (Li *et al.* 1994). This protein is easily identifiable on the gel (Fig. 4C, D) and is expressed in sufficiently large amounts during growth of *E. coli* (5–10 % total protein). In addition, the fact that it is a regular component of the translation machinery is advantageous, since the efficiency of the translation system *per se* influences the overall expression levels of individual genes.

Conclusions and perspectives. The combination of all the conditions described above gives a unique chance to follow the expression of *EcoKI* and *EcoR124I* R–M enzymes in their complexity. Our results confirm that all the subunits of both *EcoKI* and *EcoR124I* can be detected as distinct spots on NEPHGE gels. The method is also suitable for monitoring the *in-vivo* concentration of the three subunits in defined physiological situations individually as well as in relation to other proteins. These techniques, in concert with computer-aided analysis of gel images, followed by testing of the R–M activities, promise to bring more information on the currently discussed role of subunit concentration in the regulation of function of type I R–M systems.

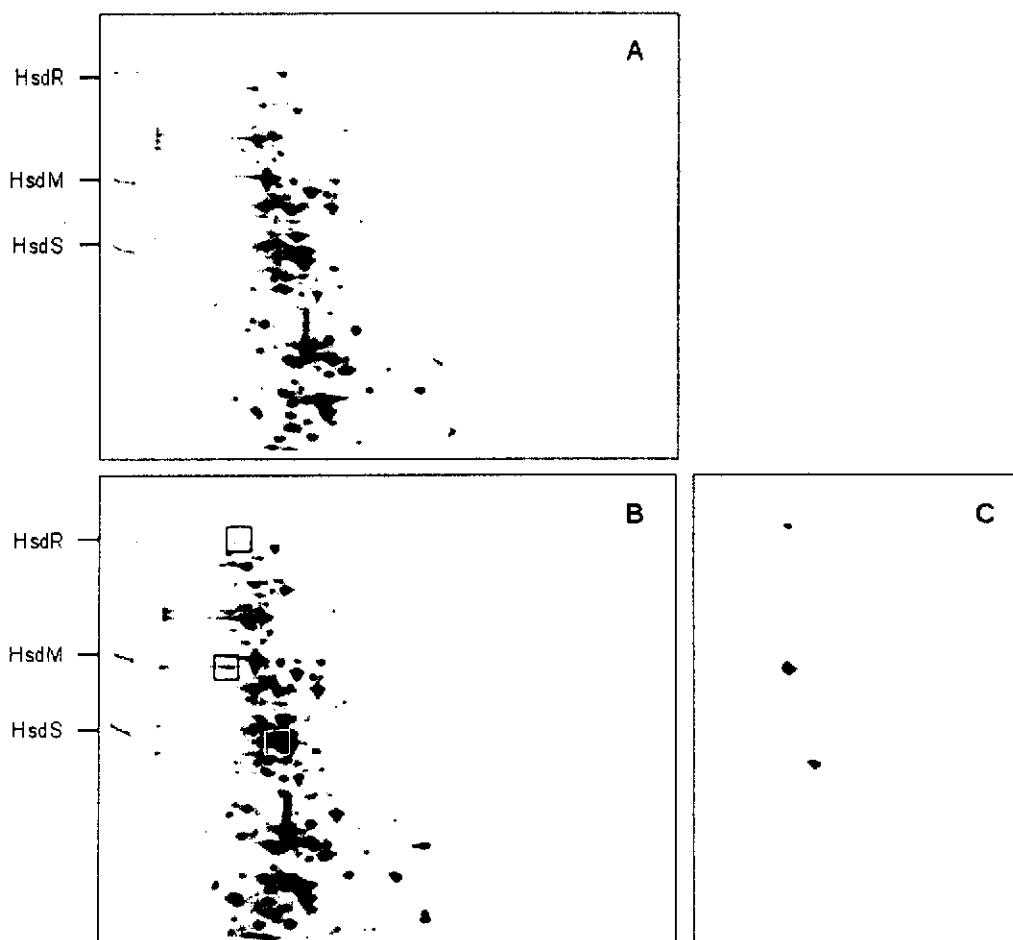


Fig. 3. Detection of *EcoR124I* subunits on NEPHGE gel. **A**: Silver-stained gel of cell crude extract of *E. coli* C122 used as control; **B**: silver-stained gel of cell crude extract of C122/pCP1005 (*hsdR*, *hsdM* and *hsdS* genes); the subunits are indicated by rectangles; **C**: immunodetection of *EcoR124I* subunits in C122/pCP1005 crude extract.

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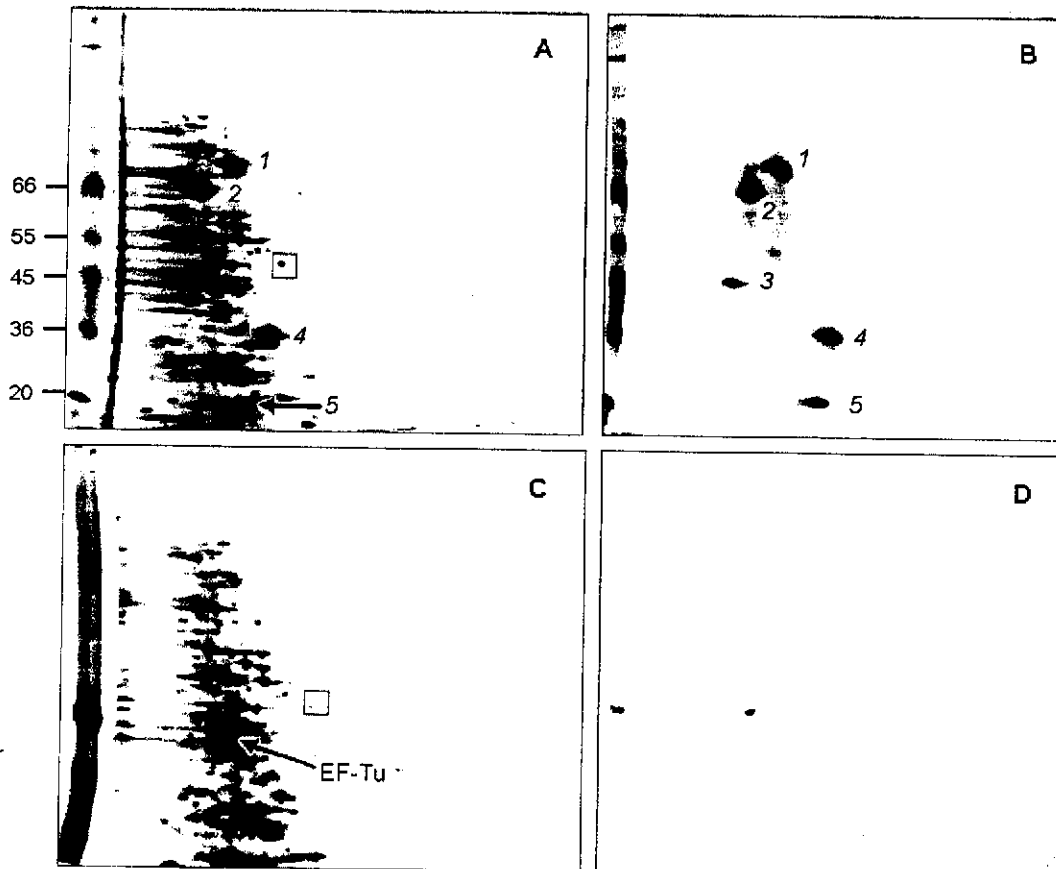


Fig. 4. Topological orientation of the NEPHGE gels using internal standard EF-Tu and commercial 2-D protein standards. **A:** Cell crude extract of C122/pVMC3-EcoKI run together with 2-D protein standards indicated by numbers, the most basic HsdS subunit (pI 9.6) is closed in a rectangle; the numbers on the left indicate M (kDa); **B:** 2-D protein standards (M/pI) run on NEPHGE; 1 - hen egg white conalbumin type 1, M 76 kDa/ pI 6.3; 2 - bovine serum albumin, 66.2/5.4; 3 - bovine muscle actin, 43.0/5.0; 4 - rabbit muscle glyceraldehyde 3-phosphate dehydrogenase 36.0/8.5; 5 - equine myoglobin, 17.5/7.0; **C:** cell crude extract of C122/pVMC3-EcoKI; run on NEPHGE, purified EF-Tu and purified EcoKI on the left were added in the second dimension as a standard; **D:** immunodetection of internal EF-Tu in cell crude extract run on gel C.

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