Application of a charge/size two-dimensional gel electrophoresis system to the analysis of the penicillin-binding proteins of *Escherichia coli*

Juan A. Ayala, M.A. de Pedro and D. Vázquez

Centro de Biología Molecular, CSIC-UAM, Universidad Autónoma de Madrid, Facultad de Ciencias, Canto Blanco, Madrid 34, Spain

Received 23 January 1983

Abstract nor received

Penicillin-binding protein *NEPHGE* *SDS–PAGE* (E. coli)

1. INTRODUCTION

Penicillin-binding proteins (pbps) are a set of membrane proteins involved in the biosynthesis of the bacterial sacculus, and, therefore, in the processes of cell elongation and cell division [1–4]. In *Escherichia coli*, as in many other bacterial species, the pbps are minor components, present only in a small number of copies per cell [5].

Since the publication of the original work in [6], the analysis of pbps has been almost exclusively based on the fractionation of the membrane proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) as in [7], after specific labelling of the pbps with [^{14}C]benzylpenicillin or any other radioactive β-lactam [8]. Although this is still the method chosen for routine work, in some instances the availability of other analytical techniques might be desirable.

Based on the method in [9,10] for two-dimensional gel electrophoresis, we have developed a system for analysis of the pbps of *E. coli* making use of non-equilibrium pH gradient electrophoresis (NEPHGE) as the first dimension and SDS–PAGE as the second.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and growth conditions*

The *E. coli* strains: W7 (dap A, lys A) [11]; Ax655/λ^+ [sep 2158^{ts} thr, leu, thi, arg, proA, his, gal, xyl, ara, mtl, lac, rps L (λ^+)] and the dilysogen derivative of the later Ax655/λ^+, λsep$^+$82 [12] were used. Cells were grown in batch cultures at 30, 37 or 42°C under vigorous aeration in L-broth [13].

2.2. *Two-dimensional gel electrophoresis*

Preparation of cell envelopes and binding of labeled β-lactams to them were performed as in [5]. Purified envelopes were resuspended in 50 mM phosphate buffer (pH 7.0) at 18 mg protein/ml and stored at −70°C. The protein content was estimated as in [14].

Labeling of pbps was carried out using the ampicillin derivative, *N*-(3-{4-hydroxy-5-[^{125}I]iodophenyl}propionyl)-ampicillin (^{125}I-ampicillin, spec. act. 74 TBq/mmol) [15]. The binding mixtures containing 100 μl cell envelope suspension and 20 μl ^{125}I-ampicillin were incubated for 10 min at 37°C. The reaction was stopped by the addition of 2 μl of a 200 mg/ml solution of ampicillin.

Published by Elsevier Science Publishers B V.
00145793/84/$3 00

Labeled proteins were solubilized, making the binding mixture 1% (w/v) sodium sarkosylate. After 30 min at 25°C, the samples were centrifuged (15 min, 20000 × *g*, 25°C) and the supernatants diluted 1:1 with 10% (w/v) Nonidet P-40. Thirty min later, solid urea was added to give a final concentration of 9.5 M. Finally, β-mercaptoethanol and ampholines (LKB, Bromma, Sweden) were added to the samples to final concentrations of 5% (v/v) and 1% (w/v), respectively.

For the first dimension, NEPHGE gels were prepared in glass tubes (150 × 3 mm) as in [10], with LKB ampholines for the pH range 3.5–10. Each gel was loaded with 100 μl of sample (4×10^5 dpm bound ^{125}I-ampicillin) and overlaid with 10 μl of 9 M urea, 1% (v/v) ampholines. The upper chamber was filled with 0.01 M H_3PO_4 (cathode) and the lower chamber with 0.02 M NaOH (anode). NEPHGE was run at 500 V for different periods of time. After 2 h of equilibration in 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.3% (w/v) SDS, 80 mM Tris–HCl (pH 6.8), each first-dimension rod gel was run on a second-dimension SDS–PAGE slab gel, consisting of a 2.5 cm stacking gel (4% acrylamide) and a 13 cm running gel (8% acrylamide). A 20-μl sample of ^{125}I-ampicillin-labeled membranes, prepared as in [5], was run in the same slab to facilitate identification of the pbps. The gels were exposed to Kodak X-Omat S film at −20°C for 3 days, and the positions of the labeled proteins were determined from the autoradiograms. The pH gradients of the NEPHGE gel were determined from 2-mm sections of blank gels soaked for 2 h in distilled water.

3. RESULTS AND DISCUSSION

A common problem in the application of electrophoretic methods based on protein charge differences to separate membrane proteins is the difficulty of extracting the proteins from the membrane without using ionic compounds. The normal solution to this problem has been the use of non-ionic detergents and urea. However, not all membrane proteins can be solubilized by these agents. In fact, we tried to solubilize the pbps with different combinations of non-ionic detergents

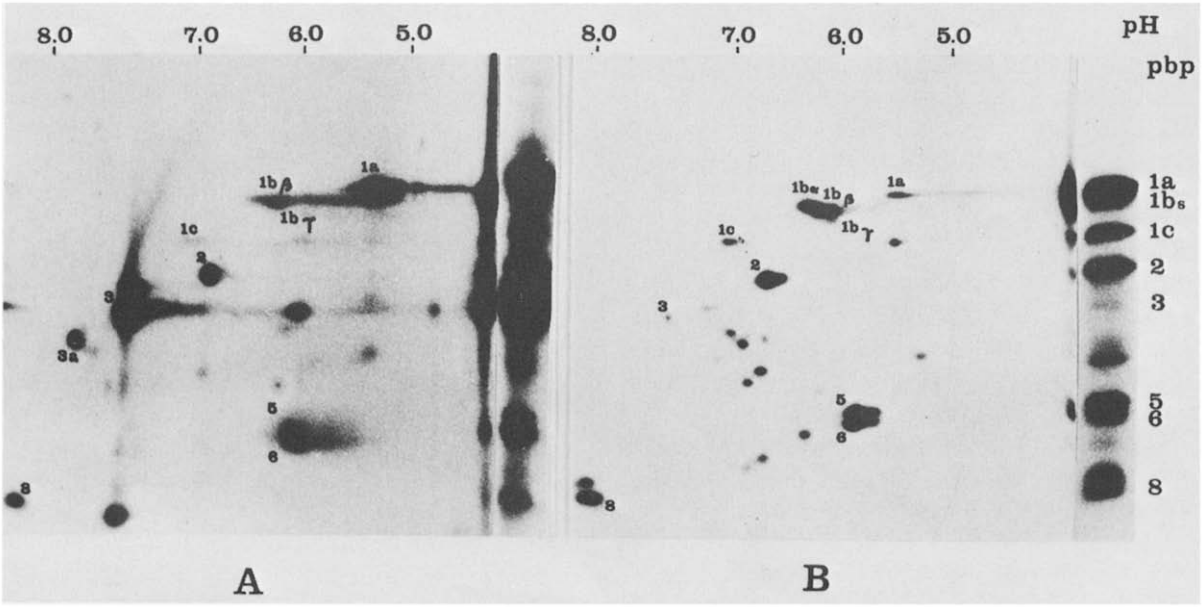

Fig.1. Autoradiograms of the pbps of *E. coli* W7 and Ax655/λ$^+$, labeled with ^{125}I-ampicillin, resolved by two-dimensional gel electrophoresis. Proteins were separated by NEPHGE for 4 h at 500 V, in the presence of ampholines for the pH range 3.5–10 in the horizontal dimension and by SDS–PAGE on 8% (w/v) polyacrylamide gels in the vertical dimension. (A) Proteins from *E. coli* W7. (B) Proteins from *E. coli* Ax655/λ$^+$.

(Triton X-100, Nonidet P-40, deoxycholate, genapol) and urea. Our results were similar to those in [16], indicating that, whereas pbps 1a, 5 and 6 were extracted efficiently by a 20 min incubation at 20°C with either Triton X-100 or Nonidet P-40, the rest of the pbps remained essentially attached to the membrane, even in the presence of 8 M urea. The problem was finally solved by solubilizing the cytoplasmic membrane proteins with sodium sarkosylate (1%, 30 min at 20°C) and subsequent displacing of the ionic detergent by the non-ionic Nonidet P-40 and urea (see section 2).

The selection of the NEPHGE technique to separate the pbps in the first dimension, instead of the more common isoelectrofocusing, was due to the better resolution observed in the basic range when identical samples were analyzed in parallel by both techniques.

Three different bacterial strains were used as source of pbps. Ax655/λ^+ (an *fts* I mutant with a defective pbp 3) was analysed at 30°C as well as after a 4 h incubation at the restrictive temperature (42°C), equivalent to 3 doubling times.

Under our experimental conditions, the ^{125}I-ampicillin-labeled pbp3 in strain Ax655/λ^+, λsep82$^+$ was expressed from the wild-type allele of *fts* I present in λsep82$^+$, integrated in the chromosome of this dilysogen.

Fig.1 shows the results of an experiment in which the pbps of strains W7 and Ax695/λ^+, labeled with ^{125}I-ampicillin, were fractionated by this method. In both strains, the pbps were easily identified as well-defined spots moving into almost identical positions. All the radioactive bands resolved by one-dimensional SDS–PAGE in the normal β-lactam-binding assay seem to be homogeneous, with the possible exception of pbp 1c which gave two neatly-defined spots at pH 7.3 and 5.3 (fig.1B).

The apparent p*I* values of the individual pbps were estimated as proposed in [9], by the changes in position of the proteins in the pH gradients after NEPHGE at different voltage × hour products, as shown in fig.2 for the pbps of *E. coli* W7. Table 1 summarizes the results obtained for all the strains tested. The apparent p*I* values found for the pbps were the same, irrespective of the strain, in all instances except for pbps 1b and 1c. Pbp 1b usually appears as a 3-component band (1bα, β and γ)

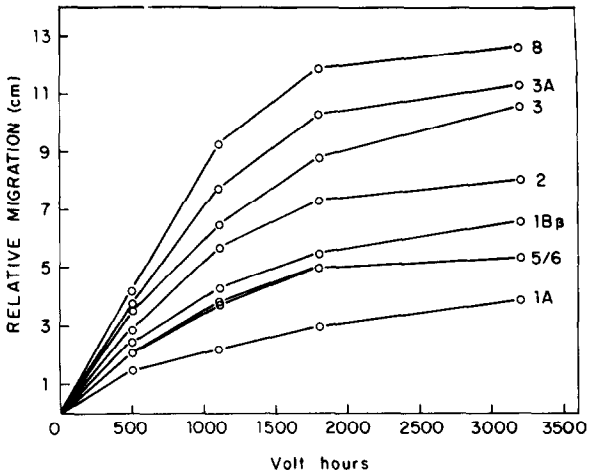

Fig.2. Relative migration of the pbps of *E. coli* W7 on NEPHGE gels run at increasing voltage × time products. ^{125}I-Ampicillin-labeled pbps from *E. coli* W7 were subjected to NEPHGE for 1–7 h at 500 V on a 3.5–10 pH gradient, followed by SDS–PAGE on 8% (w/v) polyacrylamide gels as a second dimension, to allow for identification of the individual pbps.

Table 1

Apparent isoelectric point of the pbps from different strains of *E. coli* estimated by NEPHGE

Pbp	W7	Ax655/λ^+ (30°C)	Ax655/λ^+ (42°C)	Ax655/λ^+, λsep82$^+$
1a	5.3	5.3	5.3	5.3
1bα	–	6.4	6.4	6.4
1bβ	6.2	6.2	6.2	6.2
1bγ	ND	6.1	6.1	6.1
1c	7.3	7.3	5.3	7.3
	5.3	5.3		5.3
2	6.9	6.9	6.9	6.9
3	7.9	–	–	7.9
3a[a]	8.0	–	–	8.0
4[b]	–	–	–	–
5	6.0	6.0	6.0	6.0
6	6.0	6.0	6.0	6.0
8	8.3	8.3	8.3	8.3

[a] Pbp 3a is detectable only with ^{125}I-ampicillin. Its absence in the *fts* I mutant Ax655/λ^+ suggests a possible relation to pbp 3

[b] Pbp 4 cannot be accurately detected with ^{125}I-ampicillin

The apparent p*I* values for the different pbps were deduced from experiments as in that shown in fig.2; data are the mean values from at least 3 independent experiments. The standard error was in all instances lower than 5%. ND, not determined

after SDS–PAGE. However, in W7, only two bands could be detected with apparent p*I* values corresponding to those of pbps 1bβ and 1bγ.

The behavior of pbp 1c was also rather different from the others. This protein was resolved into two components with apparent p*I* values of 7.3 and 5.3 in strains W7; Ax655/λ^+, λsep82$^+$ and Ax655/λ^+ grown at the permissive temperature (30°C). However, in strain Ax655/λ^+ grown at 42°C, only the acidic component was detected.

The results in table 1 indicate that most pbps are neutral or slightly acidic, with apparent p*I* 6–7. The extreme positions are occupied by pbp 1a as the most acidic (p*I* = 5.3) and pbps 3 and 8 as the most alkaline (p*I* = 7.9 and 8.3, respectively). The experimental value obtained for pbp 3 (7.9) agrees rather well with that calculated (8.2) from its amino acid composition reported in [17].

Our data indicate that the NEPHGE/SDS–PAGE two-dimensional system is applicable to the analysis of the pbps and, probably to other minority membrane proteins, provided that an appropriate detection method is available.

REFERENCES

[1] Spratt, B.G. (1975) Proc. Natl. Acad. Sci. USA 72, 2999–3003.
[2] Ishino, F. and Matsuhashi, M. (1981) Biochem. Biophys. Res. Commun. 101, 905–911.
[3] Botta, G.A. and Park, J.T (1981) J. Bacteriol. 145, 333–340.
[4] Spratt, B.G. (1977) J. Bacteriol. 131, 293–305.
[5] Spratt, B.G. (1977) Eur. J. Biochem. 72, 341–352
[6] Spratt, B.G. and Pardee, A.B. (1975) Nature 254, 516–517.
[7] Laemmli, U.K. (1970) Nature 227, 680–685.
[8] Wientjes, F.B., Deijhoek, T.J.M., Schwarz, U. and Nanninga, N. (1983) J. Bacteriol. 153, 1287–1293.
[9] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[10] O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) Cell 12, 1133–1142.
[11] Hartmann, R., Holtje, J.V. and Schwarz, U. (1972) Nature 235, 426–429.
[12] Fletcher, G., Irwin, C.A., Henson, J.M., Fillingim, C., Malone, M.M. and Walker, J.R. (1978) J. Bacteriol. 133, 91–100.
[13] Lennox, E.S. (1955) Virology 1, 190–206.
[14] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Schwarz, U., Seeger, K., Wengenmayer, F. and Strecker, H. (1981) FEMS Microbiol. Lett. 10, 107–109.
[16] Nakagawa, J., Matsuzawa, H. and Matsuhashi, M. (1979) J. Bacteriol. 138, 1029–1032.
[17] Nakamura, M., Maruyama, I.N., Soma, M., Kato, J., Suzuki, H. and Hirota, Y. (1983) Mol. Gen. Genet. 191, 1–9.