Volume 212, number 2, 237–241 FEB 04438 February 1987

Antibody used to identify penicillin-binding protein 2′ in methicillin-resistant strains of *Staphylococcus aureus* (MRSA)

Denise M. O'Hara and Peter E. Reynolds

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, England

Received 11 December 1986

Polyclonal antibodies were raised against the membrane-bound penicillin-binding protein (PBP 2′) present in methicillin-resistant strains of *Staphylococcus aureus* (MRSA) and were used to detect its presence in membranes from strains grown under varying conditions for the expression of resistance. The antibody preparation reacted with another membrane protein from methicillin-sensitive *S. aureus* (MSSA) which migrated to the same position as PBP 2′ on SDS gel electrophoresis. Pretreatment of the antisera with membranes of sensitive strains removed antibodies recognising high-M_r proteins common to both MRSA and MSSA: the residual antibody then reacted specifically with PBP 2′. Antisera pretreated in this manner can be used as a probe for the definitive identification of MRSA strains.

Methicillin resistance; Penicillin-binding protein; Western blotting; Immunoassay; *(Staphylococcus aureus)*

1. INTRODUCTION

The introduction into clinical use of β-lactam antibiotics such as methicillin and cloxacillin that are resistant to hydrolysis by β-lactamases resulted in the appearance in the hospital environment of methicillin-resistant strains of *Staphylococcus aureus* (MRSA). These strains were first isolated in Britain [1], then in the rest of Europe in which incidences of 7–20% were reported and more recently from many parts of the world including Australia, Japan and the USA. Some of the more recently isolated strains are not only resistant to β-lactam antibiotics but also have chromosomal and plasmid-determined resistances to many other antibiotics in clinical use. In several instances vancomycin and similar compounds have been shown to be the only antibiotics to which these strains are susceptible. Naturally occurring β-lactamase-negative (p^-) strains of *S. aureus* have been isolated which retain their resistance to methicillin (m^+) confirming that production of β-lactamase is not the mechanism by which these strains have achieved their resistance to β-lactams [2].

Intrinsic resistance in MRSA strains was later attributed to the presence of a completely novel, additional PBP (termed PBP 2′ because its mobility on SDS gel electrophoresis is intermediate between those of PBP 2 and PBP 3) which is produced in large amounts [3,4], has a greatly reduced affinity for β-lactam antibiotics in comparison with the other three high-M_r PBPs [5–8] and is the sole functional PBP when MRSA strains are grown in the presence of high concentrations of β-lactam antibiotics that would kill sensitive strains [6]. Partial proteolysis of the complex of the additional PBP with radiolabelled penicillin revealed that peptides containing the active site are different from those derived from PBPs present in sensitive strains [9], indicating that the additional PBP is

Correspondence address: D.M. O'Hara, Dept of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, England

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

unlikely to have been derived from an existing PBP and that the gene encoding it has presumably been acquired from a different organism.

Furthermore, identical peptides were derived from MRSA strains isolated from several different sources, suggesting that the same additional PBP is present in all MRSA strains [9].

The detection and comparison of PBPs involve labelling of intact cells or membranes with a radiolabelled β-lactam, followed by fractionation of the proteins on slab gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and preparation of the gel for fluorography. Detection of PBPs by this technique requires several days exposure of the X-ray film and in the special case of PBP 2′ considerable expense is involved because high concentrations of radiolabelled benzylpenicillin are required to saturate the PBP because of its very low affinity for β-lactam antibiotics. An alternative method has been used to reveal PBPs in a number of different bacteria involving the use of antibodies prepared against the benzylpenicilloyl determinant [10] or against partially purified PBPs [11,12]. A rapid and sensitive method is described here for visualising PBP 2′ of MRSA strains specifically, by Western blotting and immunooverlay techniques using an antibody raised against the protein after electroelution from SDS gels.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and growth conditions*

Two isogenic strains of *S. aureus* lacking β-lactamase were used, 13136 p^-m^+ (methicillin-resistant) and 13136 p^-m^- (sensitive) [13]. The resistant strain was grown at 30°C in a rich medium [3] containing 5% NaCl and methicillin (5 mg/l), conditions favouring the expression of resistance and of PBP 2′. The sensitive strain was grown at 37°C in the absence of NaCl and of antibiotic.

2.2. *Preparation of membranes*

Exponentially growing organisms were harvested, washed in 50 mM Tris-HCl, 145 mM NaCl, pH 7.5, and resuspended at 30 mg dry wt/ml in the same buffer containing lysostaphin (20 mg/l). The suspension was incubated at 37°C for 15 min, 10 mM $MgCl_2$ and DNase (30 mg/l) were added and incubation continued for a further 15 min. Membranes were collected by centrifugation at 40000 × *g* for 20 min at 4°C, washed and resuspended in 10 mM phosphate buffer, pH 7.2. Membranes were resuspended at 10 mg protein/ml and stored at −30°C. Labelling of PBPs was carried out as described in [14].

2.3. *Electroelution of PBP 2′*

Membrane proteins were separated by SDS-PAGE on preparative slab gels and revealed by light staining with Coomassie blue. The band corresponding to PBP 2′ was carefully excised [15] and PBP 2′ electroeluted for 5 h at 100 V. The electroeluted material containing PBP 2′, any other protein having the same mobility, SDS and Coomassie blue was carefully removed from the dialysis sac and stored at −30°C.

2.4. *Immunisation procedure*

New Zealand White rabbits were immunised with PBP 2′ which had been electroeluted from gels. The initial injection containing 300 μg protein mixed with an equal volume of Freunds complete adjuvant and was administered subcutaneously at three different sites. Subsequent boosts were given every 2 weeks using 100 μg protein mixed with Freunds incomplete adjuvant. 20 ml blood was removed at the time of each boost and the antisera prepared and stored at −30°C in the presence of 0.02% azide.

2.5. *Western blotting and immunological detection*

Proteins were subjected to SDS-PAGE [16] under conditions giving increased resolution of the high-M_r PBPs [6]. The separated proteins were transferred electrophoretically onto nitrocellulose and visualised by an enzyme-linked immunological development [17]. Optimum transfer of proteins was achieved by blotting at 100 mA for approx. 150 V·h. Antisera was used at a dilution of 1:100 and incubated with the nitrocellulose for 2–3 h. Non-specific binding was prevented by pretreating the nitrocellulose after blotting with casein in Tris-buffered saline (TBS), pH 7.2, for 1 h; this blocking buffer was included in subsequent antibody incubations after which excess antibody was removed by several washings in TBS alone. The second antibody was a peroxidase-conjugated anti-

rabbit antibody used at a dilution of 1:1000 for 1 h. Development of the blotted proteins was achieved by soaking the nitrocellulose in TBS, pH 7.2, containing 4-chloro-1-naphthol (0.6 mg/ml), 5% methanol and 0.01% H_2O_2. The reaction was quenched by washing in distilled water.

2.6. *Fluorography*

After separation by SDS-PAGE, PBPs were visualised by fluorography [18]. Labelled PBPs bound to nitrocellulose were revealed by processing the membrane filter as in [19].

3. RESULTS AND DISCUSSION

Antisera against the high-M_r PBP 2′ present in MRSA but not MSSA strains were raised in rabbits using as antigen PBP 2′ (M_r 78000) that had been separated from the other membrane proteins by SDS-PAGE, followed by electroelution. The electroeluted material gave a single Coomassie blue-staining band on SDS-PAGE, though the large amount of PBP 2′ present could have concealed other minor protein bands having the same mobility. The antibody preparation was tested against membrane proteins of MRSA and MSSA strains, pre-labelled with [^{3}H]benzylpenicillin (30 mg/l), that had been separated by SDS-PAGE and transferred to nitrocellulose by Western blotting. Rabbit antisera produced after 6–8 weeks reacted with a protein band of 78 kDa that was present in membranes from both methicillin-sensitive and -resistant strains (fig.1(I)). It is probable that the immunogen contained two 78 kDa proteins, one of which was PBP 2′, both being electroeluted from the excised band of polyacrylamide gel. The antibody recognising the other contaminating protein that was not a PBP was removed by preadsorption of the antisera to membranes from the isogenic sensitive strain. Membranes of 13136 p^-m^- (10 mg protein) were resuspended in 500 μl antisera and incubated with shaking at room temperature for 20 min: the membranes and immunoprecipitated antibodies were removed by centrifugation and the residual antisera re-tested. After this treatment the antisera reacted only with a 78 kDa protein present in membranes from the methicillin-resistant strain and with electroeluted material used as the antigen (fig.1(II)). Preadsorption of the polyclonal antibody preparation to sensitive membranes is

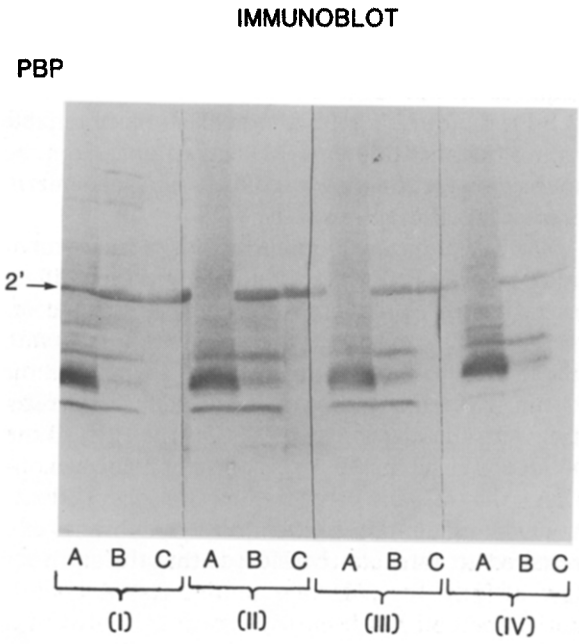

Fig.1. Immunoblot of proteins separated by SDS-PAGE, transferred onto nitrocellulose and probed with a polyclonal antibody raised against electroeluted PBP 2′. Rabbit antisera (I) were preadsorbed to sensitive membranes and the remaining supernatant tested (II); the solution containing antibodies after adsorption to sensitive membranes was followed by incubation with resistant membranes, the residual antisera being tested (IV). Antisera preadsorbed to resistant membranes only were assayed for remaining antibodies (III). All primary antibody incubations were carried out at a dilution of 1:100. Tracks A and B contained membrane proteins prepared from MSSA and MRSA respectively, whilst track C contained PBP 2′ which had been electroeluted from preparative SDS polyacrylamide gels.

therefore a necessary step in removing antibodies recognising high-M_r proteins common to MRSA and MSSA strains before it can be used to detect PBP 2′ specifically.

At a dilution of 1:100 the antibody could still detect the small amount of PBP 2′ present in membranes of MRSA strains grown under conditions unfavourable for the expression of resistance. It is technically difficult to detect such small amounts of PBP 2′ by conventional PBP assays without using high concentrations of expensive radiolabelled penicillin and improved separation techniques [6]. This suggests that the

immunological detection method described here has a greater sensitivity and specificity compared with the conventional method of detection of PBP 2′. Furthermore, the method is also more reliable in the identification of MRSA strains than the techniques currently used in clinical microbiological laboratories.

The use of nitrocellulose permits the detection of radiolabelled PBPs after immunological identification of PBP 2′, therefore allowing direct comparisons of bands seen on the immunoblot with those on the fluorogram (fig.2). This permits the definitive identification of the 78 kDa band reacting with the antibody as PBP 2′, the PBP found uniquely in methicillin-resistant staphylococci.

As the 78 kDa protein that was not PBP 2′ could be immunoprecipitated with membranes of a sensitive strain it was considered that the antibody might react with PBP 2′ in its native state (i.e. in membranes from the resistant strain) even though it was raised against denatured PBP 2′. However, although electroeluted PBP 2′ partially retained its ability to bind radiolabelled benzylpenicillin and therefore presumably retained its native conformation, the antibody raised against it was not immunoprecipitated by membranes from resistant strains (fig.1(III)–(IV)).

The antibody preparation has also been used to demonstrate the presence of PBP 2′ in methicillin-resistant strains of *S. epidermidis*, though the strains examined did not produce large amounts of the protein even when grown in the presence of β-lactam antibiotics.

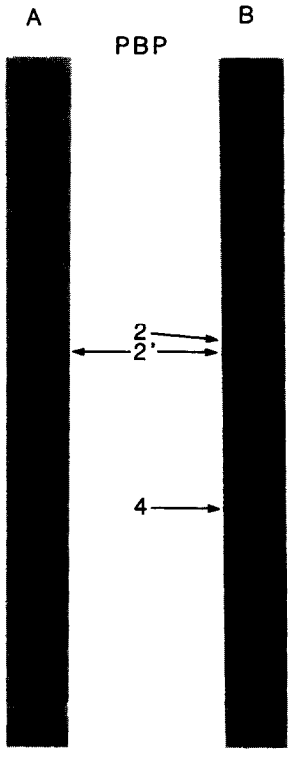

Fig.2. Membrane proteins from 13136 p^-m^+ were radiolabelled, separated by SDS-PAGE and transferred onto a nitrocellulose membrane as described in section 2. Antisera preadsorbed to membranes from 13136 p^-m^- were used at a dilution of 1:100 (A) and the PBPs were revealed by fluorography (B).

ACKNOWLEDGEMENTS

The authors are grateful to Dr P.J. Cassidy, Merck Sharp and Dohme Ltd, for generous gifts of [^{3}H]benzylpenicillin. The research was supported in part by an SERC research studentship to D.M.O'H.

REFERENCES

[1] Jevons, M.P. (1961) Br. Med. J. 1, 124–125.
[2] Parker, M.T. and Jevons, M.P. (1964) Postgrad. Med. J. Suppl. 40, 170–178.
[3] Brown, D.F.J. and Reynolds, P.E. (1980) FEBS Lett. 122, 275–278.
[4] Reynolds, P.E. (1984) Br. Med. Bull. 40, 3–10.
[5] Reynolds, P.E. (1985) Symp. Soc. Gen. Microbiol. 38, 13–40.
[6] Reynolds, P.E. and Brown, D.F.J. (1985) FEBS Lett. 192, 28–32.
[7] Ubukata, K., Yamashita, N. and Konno, M. (1985) Antimicrob. Agents Chemother. 27, 851–857.
[8] Hartman, B.J. and Tomasz, A. (1984) J. Bacteriol. 158, 513–516.
[9] Reynolds, P.E. and Fuller, C. (1986) FEMS Microbiol. Lett. 33, 251–254.
[10] Hakenbeck, R., Briese, T. and Ellerbrok, H. (1986) Eur. J. Biochem. 157, 101–106.
[11] Schryvers, A.B., Wong, S.S. and Bryan, L.E. (1986) Antimicrob. Agents Chemother. 30, 559–564.
[12] Hakenbeck, R., Ellerbrok, H., Briese, T., Handwerger, S. and Tomasz, A. (1986) Antimicrob. Agents Chemother. 30, 553–558.
[13] Lacey, R.W. (1972) J. Med. Microbiol. 5, 497–508.

[14] Chase, H.A., Shepherd, S.T. and Reynolds, P.E. (1977) FEBS Lett. 76, 199–203.
[15] Walker, J.E., Auffret, A.D., Carne, A., Gurnett, A., Hanisch, P., Hill, D. and Saraste, M. (1982) Eur. J. Biochem. 123, 253–260.
[16] Laemmli, U.K. and Favre, M. (1973) J. Mol. Biol. 80, 575–599.
[17] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[18] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[19] Wyke, A.W. (1984) FEMS Microbiol. Lett. 22, 133–138.