

Identification of a Group of *Haemophilus influenzae* Penicillin-Binding Proteins That May Have Complementary Physiological Roles

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[³⁵S]penicillin bound to different *Haemophilus influenzae* proteins in assays performed at 20, 37, or 42°C. Penicillin-binding proteins 3a, 3b, 4, and 4' formed a group characterized by their affinity for moxalactam, cefotaxime, and piperacillin. Penicillin-binding protein 4' showed specific properties that may reflect its complementary role in septation.

To exert their inhibitory effects, antibiotics of the β -lactam family must bind to specific targets (penicillin-binding proteins [PBPs]) which have essential enzymatic functions in cell wall peptidoglycan synthesis (7, 8). We have recently provided evidence concerning the temperature-modulated distinctive character of *Haemophilus influenzae* PBPs 1a and 3; the penicillin-binding activity of PBP 1a was activated at 42°C, and PBP 3 activity was suppressed (3). Here we show that penicillin-binding assays performed at three different temperatures resulted in other distinct PBP profiles for *H. influenzae* Rd. We also show that a specific group of four PBPs (3a, 3b, 4, and 4') that have different penicillin-binding properties is involved in septation.

The medium used for growth of *H. influenzae* Rd was supplemented brain heart infusion agar or broth (GIBCO Diagnostics, Madison, Wis.) (5). The procedure for binding radiolabeled penicillin to whole bacterial cells was previously described (3). Cells were labeled at 20, 37, or 42°C for 45 min with a specific concentration (0.1 to 5 μ g/ml) of [³⁵S]benzylpenicillin at 5.3 Ci/mmol (Dupont NEN Canada, Lachine, Quebec, Canada). The labeled cells were washed and loaded for electrophoresis on discontinuous sodium dodecyl sulfate–12% polyacrylamide gels (1). Gels were stained with Coomassie brilliant blue R and destained before they were soaked for 20 min in Amplify (Amersham Corp., Oakville, Ontario, Canada) and thereafter subjected to 7 days of fluorography at –70°C with prefogged film (X-Omat AR; Eastman Kodak Co., Rochester, N.Y.). In some experiments, bacterial cells were prelabeled with various concentrations of a competing β -lactam for 20 min at 37°C before the penicillin-binding assay. This allowed identification of a PBP(s) targeted preferentially by β -lactams other than penicillin. The concentration of the competing β -lactam needed to block at least 50% of the subsequent binding of the radiolabeled penicillin to a particular PBP was determined by scanning the PBP profiles obtained on the X-ray film with a 2202 Ultrascan laser densitometer (LKB, Bromma, Sweden) with a 3390A integrator (Hewlett-Packard Canada, Ltd., Mississauga, Ontario, Canada). To observe the morphological effects of β -lactams on *H. influenzae* cells, bac-

terial cultures were inoculated and adjusted to an A_{600} of 0.1. The nonshaking cultures were then incubated at 20, 37, or 42°C for 1 h before addition of β -lactams at the MIC. Heat-fixed culture fractions were microscopically examined after 0 and 18 h of incubation with the antibiotic. MICs of β -lactams were determined by a broth dilution technique as described previously (5).

PBP expression as a function of labeling temperature. The PBP profiles of *H. influenzae* Rd are shown in Fig. 1. With bacterial cells grown at 37°C, [³⁵S]penicillin G bound covalently to seven resolved PBPs in whole-cell labeling assays performed at 20°C. PBPs 1a and 1b were not resolved in these experiments but were previously identified in other gel systems (3, 5). When cells were labeled at 37°C, there was a decrease in PBP 3a, 3b, 4, and 6 labeling and a new 45-kilodalton PBP, PBP 4', was observed. The reductions in the labeling of PBPs 3a, 3b, 4, and 6 at 37°C were 100, 48, 62, and 67%, respectively, as measured by densitometry. Only PBPs 1, 2, 3b, and 5 were labeled at 42°C, and the same temperature modulation of PBP labeling was seen in the lag, log, and stationary phases of growth. When less radiolabeled penicillin was used in assays performed at 42°C (Fig. 2), PBP 1 showed increased binding of [³⁵S]penicillin while PBP 3b showed less binding, as previously reported (3).

Binding of β -lactams to PBPs. The degree of saturability of each *H. influenzae* Rd PBP obtained with various concentrations of ³⁵S-labeled penicillin G was plotted as shown in Fig. 3. PBPs 3b, 4, and 6 were saturated by 0.5 μ g of radiolabeled penicillin, while other PBPs (in particular, PBPs 4' and 5) showed greater binding with increasing [³⁵S]penicillin concentrations when labeled at 37°C. Figure 4 shows the major target PBPs of moxalactam, cefotaxime, and piperacillin. All three β -lactams bound preferentially to PBPs 3b, 4, and 4', although different PBPs served as secondary targets: PBPs 5 and 6 for moxalactam, PBP 6 for cefotaxime, and PBP 2 for piperacillin. For all three β -lactams, the MIC (0.024, 0.001, and 0.006 μ g/ml for moxalactam, cefotaxime, and piperacillin, respectively) was similar to the concentration needed to block at least 50% of radiolabeled penicillin binding to the three targets. The concentrations needed to block 50% of moxalactam, cefotaxime, and piperacillin binding were 0.009, 0.001, and 0.001 μ g/ml, respectively, for all three PBPs (3b, 4, and 4'). Any one of these three β -lactams at the MIC caused cell filamentation at 20 or 37°C, and filamentous cell growth was also

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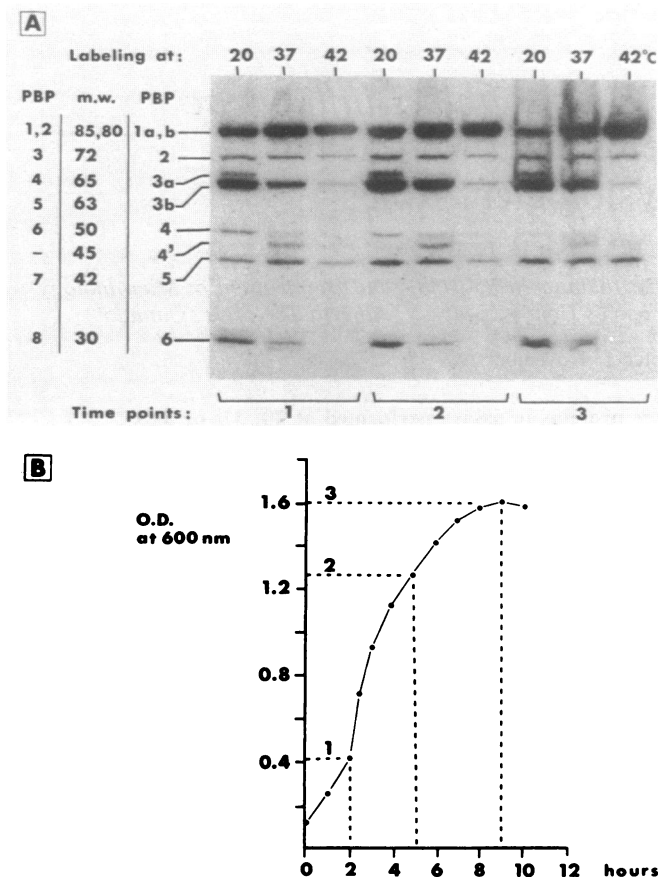


FIG. 1. Fluorograph (A) of *H. influenzae* Rd cell samples collected at various times (B) during growth. Whole cells were grown at 37°C and collected by centrifugation at time points 1 to 3. The cells were then labeled with 1.5 μg of ^{35}S -labeled penicillin G (5.3 Ci/mmol) per ml at 20, 37, or 42°C. The cells were electrophoresed in a sodium dodecyl sulfate-polyacrylamide (12%) gel system. In panel A, the PBPs of *H. influenzae* Rd and their apparent molecular weights (m.w.; 10^3) are indicated on the basis of the numbering system of Parr and Bryan (5) on the left or that of Makover et al. (2) on the farther left. O.D., Optical density.

observed when the cells were grown at 42°C with no antibiotic. Penicillin G (MIC, 0.2 $\mu\text{g}/\text{ml}$) caused cell lysis at all three growth temperatures (data not shown).

In conclusion, binding of radiolabeled penicillin G to

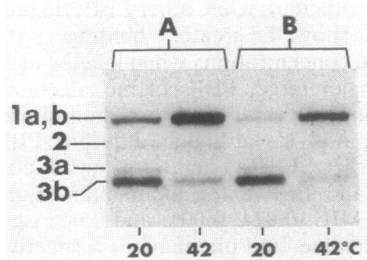


FIG. 2. Fluorograph of *H. influenzae* Rd showing temperature activation of PBP 1. Whole cells were grown at 37°C and then labeled with 0.5 (A) or 0.1 (B) μg of ^{35}S -labeled penicillin G (5.3 Ci/mmol) per ml at 20 or 42°C. The cells were electrophoresed in a sodium dodecyl sulfate-polyacrylamide (12%) gel system. The PBPs of *H. influenzae* Rd are indicated on the left on the basis of the numbering system of Parr and Bryan (5).

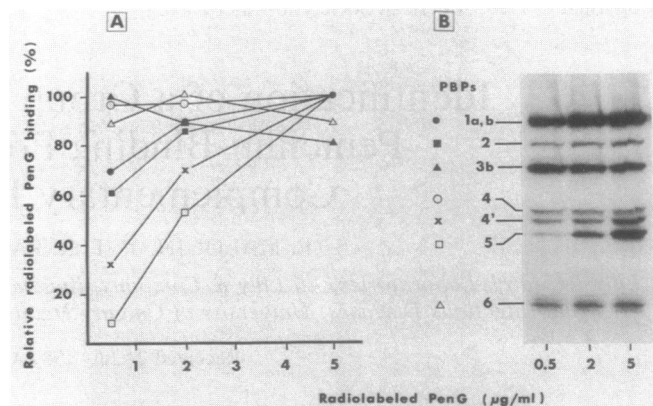


FIG. 3. Relative ^{35}S -labeled penicillin G saturation of *H. influenzae* Rd PBPs (A) calculated from laser densitometry data obtained from fluorographs (B). Whole cells grown at 37°C were labeled with 0.5 (lane 1), 2 (lane 2), or 5 (lane 3), μg of ^{35}S -labeled penicillin G (5.3 Ci/mmol) per ml at 37°C. The cells were electrophoresed in a sodium dodecyl sulfate-polyacrylamide (12%) gel system. For panel A, fluorographs were scanned by laser densitometry and the data are expressed as relative penicillin binding to individual PBPs. For panel B, the PBPs of *H. influenzae* Rd are indicated on the left on the basis of the numbering system of Parr and Bryan (5).

whole *H. influenzae* cells at three different temperatures resulted in distinct PBP profiles partially described previously (3). Use of [^{35}S]penicillin with high specific activity permitted identification of a new PBP, PBP 4', in labeling assays performed at 37°C. We believe that PBP 4' was present in previously shown PBP profiles but was not commented on by other investigators (4, 6). By labeling the PBPs at 37°C, the present study showed that PBPs 3b, 4, and 4' form a distinct group of PBPs which are the primary targets of three different β -lactams that cause inhibition of septation (moxalactam, cefotaxime, and piperacillin). We previously reported that PBPs 3a and 3b were the primary targets of piperacillin in labeling experiments performed at room temperature (5). Although PBPs 3a, 3b, 4, and 4' were

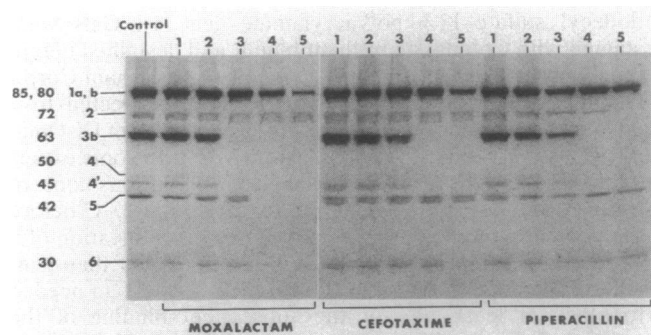


FIG. 4. Fluorographs of *H. influenzae* Rd in a competition experiment with various β -lactams. Whole cells were grown at 37°C, collected by centrifugation, and preincubated for 20 min at 37°C with 10-fold dilutions of the competing β -lactams. The concentrations used for the competing β -lactams in lanes 1 to 5 were 0.0001 to 1 μg of moxalactam per ml and 0.00001 to 0.1 μg of cefotaxime or piperacillin per ml. The cells were then labeled with 1.5 μg of ^{35}S -labeled penicillin G (5.3 Ci/mmol) per ml for 45 min at 37°C and electrophoresed in a sodium dodecyl sulfate-polyacrylamide (12%) gel system. The PBPs of *H. influenzae* Rd and their apparent molecular weights (10^3) are indicated on the left on the basis of the numbering system of Parr and Bryan (5).

the specific targets of the above-mentioned β -lactams, these proteins displayed different labeling properties. The maximal labeling concentration and optimal labeling temperature of PBP 4' were clearly distinct from those of the rest of the group. We reported previously the characteristics of a β -lactam-resistant strain of *H. influenzae* (isogenic to strain Rd) and showed that resistance was caused by alterations in PBPs 3a and 3b (5) and more recently that it was caused by alterations in PBP 4' at 37°C in addition to PBPs 3a and 3b (F. Malouin, T. R. Parr, Jr., and L. E. Bryan, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1127, 1989). This strain was particularly resistant to β -lactams that cause cell filamentation, and transformation experiments showed a correlation between resistance and the alterations in PBPs 3a, 3b, and 4'. Our data are consistent with the view that PBPs 3a, 3b, 4, and 4' are all involved in the septation process and that PBP 4' may complement reduced enzymatic activity of PBPs 3a, 3b, and 4 at 37°C. The cell filamentation that results at 42°C is coincidental to the altered binding activity of PBP 4' at that temperature and supports this conclusion. However, we still do not know whether further reduction of PBP 3b activity at 42°C also causes filamentation to occur. The importance of PBP 4' in *H. influenzae* cell physiology during human infections with fever remains unclear. It is possible that PBP 4' permits the initial transition to a higher growth temperature, and its function in septal peptidoglycan synthesis is of scientific and medical interest. At a higher growth temperature (42°C), the increased penicillin-binding activity of PBP 1a (3) certainly represents a target of choice for β -lactam antibiotics.

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