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Penicillin-Binding Proteins of *Haemophilus ducreyi*

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The penicillin-binding protein (PBP) profile of *Haemophilus ducreyi* was determined by a whole-cell-labeling assay. Only two major PBPs, of molecular weights 90,000 (PBP 1) and 38,500 (PBP 2), were detected in six of eight strains studied. Competition binding experiments and the attendant morphological effects suggested that PBP 1 was either a functional amalgamation or a lack of resolution of two proteins equivalent to PBPs 1 and 3 of *Escherichia coli*.

Haemophilus ducreyi, a fastidious gram-negative coccobacillus, is the causative agent for chancroid, one of the six classical sexually transmitted diseases (16). Chancroid is endemic throughout the tropics and represents the most commonly diagnosed cause of genital ulcer disease in Nairobi, Kenya, ranking second to gonorrhea as the most frequently presenting clinical complaint (13). In contrast, the mean number of cases reported annually in the United States between 1971 and 1980 has not exceeded 900, although recent surveillance data portend its reemergence as a significant sexually transmitted disease (16). However, the perception that chancroid is a minor curiosity in the public health field has been radically altered with the advent of the acquired immunodeficiency syndrome epidemic. Prostitutes play a central role in the transmission of both diseases. This group is recognized as a major reservoir of both human immunodeficiency virus (9) and genital ulcer disease (13) and has been epidemiologically implicated in a number of localized outbreaks of chancroid that have occurred in Winnipeg, Canada (7), Orange County, Calif. (2), Massachusetts (5), and Dallas, Texas (M. C. Heard, B. J. Nobles, and G. Cartwright, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 822, 1987). The recent demonstration that genital ulcer disease independently facilitates human immunodeficiency virus transmission (D. W. Cameron, L. J. D'Costa, P. Karasira, J. O. Ndinya-Achola, P. Piot, A. R. Ronald, and F. Plummer, 27th ICAAC, abstr. no. 683, 1987) further underscores both the importance of chancroid as a risk factor in human immunodeficiency virus acquisition and the necessity of instituting appropriate therapeutic measures to control its spread.

Although β-lactam antibiotic resistance mediated by β-lactamase-specifying plasmids (3) is widely prevalent among *H. ducreyi* strains, nothing is known regarding the target sites, the penicillin-binding proteins (PBPs), of the β-lactam antibiotics. This study was conducted to identify the PBPs in *H. ducreyi* and to provide an initial understanding of the functional relationships of these PBPs to the PBPs in a more extensively studied gram-negative bacillus, *Escherichia coli*.

H. ducreyi strains which were maintained in stock cultures at −70°C were kindly provided by Robert Brunham (University of Manitoba, Winnipeg, Canada) (Table 1). *Haemophilus influenzae* Rd is a β-lactamase-negative, nonencapsulated isolate (12). MICs were determined on chocolate agar by using standard agar dilution techniques (21).

PBPs were determined in a modification of the whole-cell assay of Barbour (1). Colonies grown overnight on chocolate agar at 35°C in a moist CO_2 environment were labeled at either 30 or 37°C for 30 min (this period varied from 30 to 75 min in the time-course experiment) with [^{35}S]benzylpenicillin (3 μg/ml; Dupont, NEN Research Products, Boston, Mass. [specific activity ranged from 1.4 to 8.0 Ci/mmol]). *H. ducreyi* 35000 was used in the competition experiments (1). The PBPs were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli and Faure (10). After electrophoresis, the gels were prepared for fluorography by impregnation with 2,5-diphenyloxazole, dried, and then exposed to prefogged film (X-Omat R; Eastman Kodak Co., Rochester, N.Y.) for 14 days at −70°C. PBP quantitation and penicillin affinity determination were done by scanning densitometry of the fluorograms by using a video densitometer (model 620; Bio-Rad Laboratories, Richmond, Calif.) interfaced with an IBM PC computer incorporating the Bio-Rad 1-D Analyst Software Package. Molecular weights were determined by using known proteins as standards.

Morphological changes that were produced by the various β-lactam antibiotics were assessed by scraping cells of *H. ducreyi* 35000 from MIC chocolate agar plates that had been incubated at 35°C in a humid CO_2 atmosphere for 24 to 36 h and suspending them in 200 μl of brain heart infusion broth. Samples were examined immediately by phase-contrast microscopy (model BH-2 microscope; Olympus Opticals Co., Tokyo, Japan).

The following antibiotics were the generous gifts of the manufacturers: potassium benzylpenicillin G (Wyeth Laboratories, West Chester, Pa.), ampicillin sodium (Ayerst Laboratories, Montreal, Quebec, Canada), aztreonam (E. R. Squibb & Sons, Inc., Princeton, N.J.), cephaloridine and cephalothin (Eli Lilly & Co., Scarborough, Ontario, Canada), amdinocillin (Leo Laboratories, Toronto, Ontario, Canada), and piperacillin (Cyanamid of Canada Ltd., Montreal, Quebec, Canada).

The PBP profiles of *H. influenzae* Rd and of the five penicillin-susceptible and three penicillin-resistant β-lactamase-producing *H. ducreyi* strains are displayed in Fig. 1. The four high-molecular-weight PBPs of *H. influenzae* (M_r of PBP 1, 85,000; M_r of PBP 2, 72,000; M_r of PBP 3, 65,000; and M_r of PBP 4, 52,000) are clearly shown in Fig. 1, and two additional PBPs, PBP 5 and PBP 6, are also shown in Fig. 2. The pattern for these proteins conforms to that previously reported (12, 14). In contrast to *H. influenzae*, only two

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TABLE 1. *H. ducreyi* strains

Strain	Source	β-Lactamase
HD1	Sweden	−
CH28	Thailand	+
35000	Winnipeg, Manitoba, Canada	−
CIP542	Institut Pasteur, Paris, France	−
V1157	Seattle, Wash.	+
BG411	Nairobi, Kenya	+
36-F-2	Paris, France	−
E1673	Sweden	−

PBPs with apparent molecular weights of 90,000 (PBP 1) and 38,500 (PBP 2) were visualized in all the *H. ducreyi* strains. These two PBPs were the only PBPs seen in six of the eight *H. ducreyi* strains. In the remaining two strains (E1673 and 36-F-2), two additional PBPs of M_rs 70,000 and 65,000 were present, but they were never seen in the other strains examined (Fig. 1 and 2). Other bands that were present (Fig. 1, lanes c, g, i, j, and k) most likely represented nonspecific binding of [^{35}S]benzylpenicillin, because they were not consistently seen and because their appearance could not be eliminated by prebinding with unlabeled penicillin. No quantitative or qualitative alterations in the PBP profiles were observed when binding was performed at a reduced temperature (30 compared with 37°C), when the incubation time was extended from 30 to 75 min, or when the time of fluorography was lengthened from 2 to 4 weeks (data not shown). Additionally, no change in PBP number was discernible when whole-cell labeling was performed with [^{35}S] benzylpenicillin of higher specific activity (8.0 Ci/mmol) (data not shown). Collectively, these results would seem to exclude the presence of a slow-reacting, low-affinity PBP.

The PBP profile of membrane preparations derived from *H. ducreyi* 35000 demonstrated a pattern identical to that observed in the whole-cell assay.

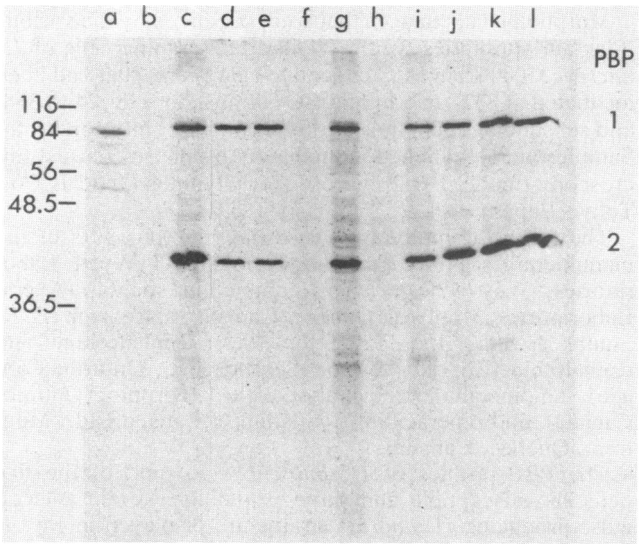

FIG. 1. PBP profiles of *H. influenzae* Rd (lane a) and eight *H. ducreyi* strains (lanes b through l). Lanes b through l: b, CH28; c, CH28; d, 35000; e, CIP542; f, V1157; g, V1157; h, BG411; i, BG411; j, 36-F-2; k, E1673; l, HD1. Samples in lanes c, g, and i were preincubated with 1 μg of clavulanic acid per ml. Molecular weight markers (10^3) are displayed on the left.

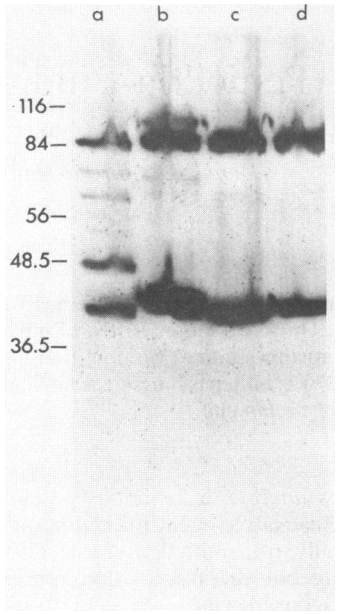

FIG. 2. PBP profiles of *H. influenzae* Rd (lane a) and three *H. ducreyi* strains (lanes b through d). Lanes b through d: b, 36-F-2; c, E1673; d, 35000. Molecular weight markers (10^3) are displayed on the left.

Several antimicrobial agents were chosen for use in competition assays because of their well-known preferences for either PBP 1, 2, or 3 of *E. coli* (17–19, 22). PBP 1 of *H. ducreyi* preferentially bound compounds with highest affinity for both PBP 1 (cephaloridine, Fig. 3) and PBP 3 (piperacillin, Fig. 3; cephalothin 50% inhibitory doses, 1.5 μg/ml for PBP 1 and >32 μg/ml for PBP 2, data not shown) of *E. coli*. A similar preference of PBPs 3a and 3b of *H. influenzae* for piperacillin has also been previously shown (14). Ampicillin, with a relatively less-pronounced preference for PBP 3 of *E. coli*, also bound with highest affinity to PBP 1 of *H. ducreyi* (Fig. 3). Amdinocillin demonstrated preferential affinity for PBP 2, a characteristic shared by PBP 2 of *E. coli*. The morphological effects seen at the MIC corresponded to those seen in *E. coli*. Ampicillin and piperacillin caused filamentation, cephaloridine produced early lysis, and amdinocillin resulted in the formation of ovoid cells (data not shown).

Two intriguing observations emerge from this study. First, in contrast to all the eubacteria examined to date, most strains of *H. ducreyi* demonstrated only two detectable PBPs instead of the usual complement of three to eight (6, 19, 22). This unusual PBP pattern, which was present in six of eight strains examined, is consistent with the taxonomic distinctiveness of *H. ducreyi* compared with other members of the genus *Haemophilus* (4, 11). However, we cannot exclude non-PBP involvement in peptidoglycan synthesis or a pool of "senescent" PBPs acting as a binding sink (20). In addition, other PBPs possessing either very low β-lactam affinities or rapid deacylation rates may exist but could not be detected by the conventional PBP assay that was used.

Second, the results derived from the competition binding experiments, in which PBP 1 of *H. ducreyi* bound β-lactams showing a preference for either PBP 1 (cephaloridine) or PBP 3 (piperacillin and cephalothin) of *E. coli*, support the contention that PBP 1 of *H. ducreyi* possesses properties of both of these *E. coli* PBPs (18, 19, 22). The functional correlate of this duality in *H. ducreyi* was reflected in the

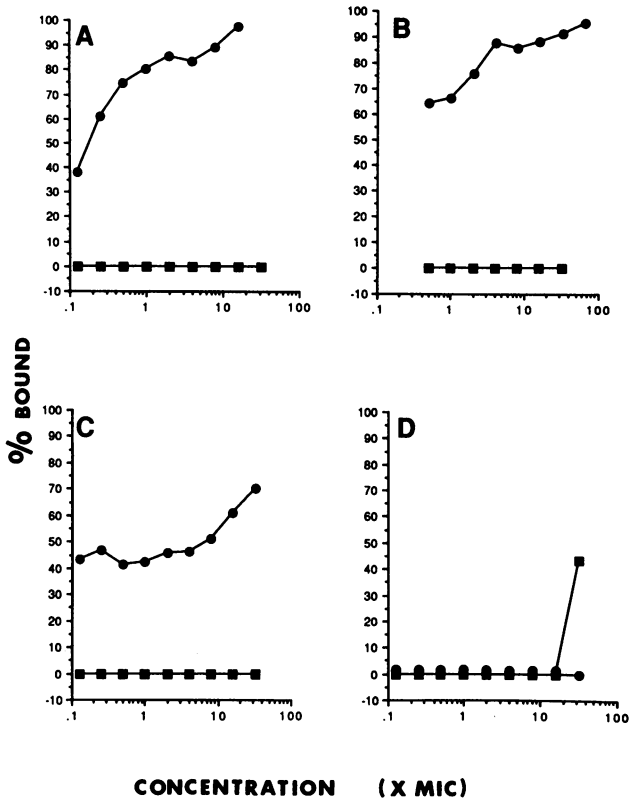

FIG. 3. Competition assays using various β-lactam antibiotics for PBPs of *H. ducreyi* 35000. (A) Ampicillin. (B) Cephaloridine. (C) Piperacillin. (D) Amdinocillin. ●, PBP 1; ■, PBP 2.

morphological outcome following exposure to β-lactam antibiotics with preferential affinities for PBP 1 (cephaloridine) and PBP 3 (piperacillin) of *E. coli*, in which cephaloridine produced spheroplast-like structures with subsequent lysis and piperacillin resulted in filamentation. Analogous effects are observed in *E. coli*, in which selective inhibition of PBP 1 (which catalyzes the synthesis of peptidoglycan involved in cell elongation) produces cell lysis, and inhibition of PBP 3 (which mediates septum formation) produces filamentation (8, 15, 18, 19, 22). It is possible that PBP 1 may represent more than one protein that has not been resolved. However, even if it comprises two proteins, their molecular weights would be more similar than the molecular weights of PBP 1 and PBP 3 of either *E. coli* or *H. influenzae*. Alternatively, the roles of PBP 1 and PBP 3 of *E. coli* may have been functionally consolidated into one PBP, PBP 1, in *H. ducreyi*.

These observations prompt the speculation that *H. ducreyi* may be a very ancient organism in terms of PBP evolution. It is possible that more recent evolutionary steps have led to the development of two PBPs performing the function of PBP 1 of *H. ducreyi* or to two unresolved PBPs with functions of PBP 1 and PBP 3 of *E. coli* but with very similar molecular weights. Alternatively, *H. ducreyi* may have pursued quite a separate evolution resulting in a single highly efficient high-molecular-weight PBP or two PBPs of similar molecular weights.

A similar distinct evolutionary route may exist for PBP 2. A function corresponding to PBP 2 in *E. coli* has apparently been preserved in *H. ducreyi*. However, the molecular weight of PBP 2 in *E. coli* is approximately twice that of its functional counterpart in *H. ducreyi*.

More extensive structural and genetic studies are warranted to corroborate these conjectures. Nevertheless, this study clearly demonstrates that *H. ducreyi* has followed a PBP evolutionary pathway that is unique in comparison with those of other *Haemophilus* species and, indeed, with those of other gram-negative rod bacteria.

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