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Enzyme Profile of *Haemophilus ducreyi* Strains Isolated on Different Continents

E. Van Dyck, P. Piot*

Two hundred strains of *Haemophilus ducreyi*, isolated in different parts of the world, were investigated using the API-ZYM system, which included 95 different substrates. All strains produced aminopeptidase against beta-naphthylamide derivates of L-lysine, glycine, L-arginine, L-alanine, D-L-methionine, glycyl-glycine, glycyl-L-alanine and L-leucine. All strains also produced alkaline and acid phosphatase and phosphohydrolase. Nearly all strains showed esterase activity against butyrate, valerate, caproate and caprylate, but the reactions were very weak. No glycosidase activity could be detected. Of the 47 aminopeptidase tests showing variable reactions, only the results for S-benzyl-L-cystine, L-ornithine, L-alanyl-L-phenyl-alanyl-L-proline, L-hystidyl-L-leucyl-L-histidine and L-histidyl-L-serine arylamidase obtained on strains from Asia, Africa and Europe were significantly different ($p < 0.05$). On the basis of test results for L-ornithine arylamidase and L-alanyl-L-phenyl-alanyl-L-proline arylamidase, the distribution of three biovars found among the isolates of the different continents was significantly different ($p < 0.0001$), whereas African strains isolated in Kenya and South Africa yielded the same enzymatic pattern. Thus, these enzymes may constitute a marker system for the epidemiological study of *Haemophilus ducreyi*.

Haemophilus ducreyi, the causative agent of chancroid, is a fastidious organism with very limited biochemical activity. Since the development of new selective and enriched media, *Haemophilus ducreyi* can now be isolated from 60 to 70 % of clinical cases of chancroid, and large numbers of strains are available for microbiological studies (1, 2). Our knowledge of the epidemiology of chancroid suffers from the lack of a typing system for *Haemophilus ducreyi*. Some authors have found variable antimicrobial susceptibilities and antigenic differences, but antimicrobial susceptibility patterns or serotyping have not been used as yet for epidemiologic purposes (3, 4).

The classic biochemical activity of *Haemophilus ducreyi* is limited to the reduction of nitrate and the production of phosphatase (5). The results of an enzymatic profile study of 30 clinical isolates done by Casin et al. provided new information on the biochemical activity of *Haemophilus ducreyi* (6). In this study, we determined the enzymatic activity on 95 different substrates for 200 strains of *Haemophilus ducreyi* isolated in different parts of the world.

Department of Microbiology, Institute of Tropical Medicine, B-2000 Antwerp, Belgium.

Materials and Methods

Strains. Two hundred *Haemophilus ducreyi* strains were isolated between 1978 and 1985 from genital lesions of chancroid patients in the following places: Bangkok, Thailand (93 strains); Johannesburg, South Africa (32 strains); Nairobi, Kenya (24 strains); Amsterdam, The Netherlands (24 strains); Paris, France (8 strains); Copenhagen, Denmark (6 strains); Winnipeg, Canada (4 strains); Göteborg, Sweden (3 strains); Seattle, USA (4 strains); Manchester, UK and Antwerp, Belgium (1 strain each). All strains were identified on the basis of morphological and cultural characteristics, reduction of nitrate and production of alkaline phosphatase. All had a negative porphyrin test. Suspensions of the strains in skim milk were kept frozen at – 70 °C.

Enzyme Tests. The enzyme assays were performed using nine different API strips (API System SA, France) as follows: API-ZYM (19 tests), API-ZYM esterases (10 tests), API-ZYM aminopeptidases (59 tests), API-ZYM osidases (20 tests). Thirteen tests were included twice: the 95 different substrates consisted of naphthyl derivates of fatty acids (esterases), beta-naphthyl derivates of peptides and amino acids (aminopeptidases) and nitrophenol derivates of carbohydrates (osidases).

Test Performance. Bacteria were cultured on Mueller-Hinton agar (BBL, UK), enriched with 5 % chocolated horse blood (Gibco, USA), 5 % calf serum (Gibco) and 1 % Iso-Vitalex (BBL). After incubation for 48 h at 35 °C in a moist CO_2 atmosphere, bacterial colonies were scraped off with a cotton swab and suspended in distilled water or phosphate buffered saline to a turbidity standard No. 5 on the McFarland scale; 0.04 ml of this dense suspension was dropped into each cupule of the API-strips, which were fitted into moistened plastic chambers and incubated aerobically in darkness at 37 °C

for 4 h. After incubation, one drop (0.02 ml) of API-ZYM reagent A (tris-(hydroxymethyl)-aminomethane, 250 g; HCl 37 %, 110 ml; lauryl-sulfate, 100 g; aqd. to 1000 ml) and one drop of API-ZYM reagent B (fast blue BB, 3.5 g in 1000 ml 2-methoxy-ethanol) were added to each cupule of the aminopeptidase and esterase tests. To the cupules of the glycosidase strip one drop of 0.1 NaOH was added. The strips were left for 10 min, after which they were exposed to the light of a 1000 W lamp for 10 s to eliminate the yellow base caused by an excess of fast blue BB which had not reacted, leaving the negative reactions colourless. The results were classified according to the API colour-reading-scale (0 = negative, 1–5 = positive). To ensure reproducibility of the results, five strains were retested four times on different days using different subcultures. Some tests with very weak reactions (1+) sometimes gave negative results (0), and were thus disregarded for data analysis; no other discrepancies were seen.

Data Analysis. One hundred and seventy-three strains of *Haemophilus ducreyi* isolated in four centres on three different continents (Bangkok 93 strains, Johannesburg 32, Nairobi 24, Amsterdam 24) were compared using the r × c Chi-square test.

Results

All 200 strains showed aminopeptidase activity against beta-naphthyl derivates of L-lysine, L-arginine, L-alanine, D-L-methionine, L-glycine, glycyl-glycine, glycyl-L-alanine and L-leucine. They were also positive for alkaline phosphatase, acid phosphatase and phosphohydrolase. None of the strains showed aminopeptidase activity against gamma-L-glutamine, N-benzoyl-L-leucine, N-CBZ-4-methoxy-L-arginine, beta-alanine, N-benzoyl-4-methoxy-L-alanine, N-acetyl-glycyl-L-lysine and N-benzoyl-D-L-arginine. No esterase activity was found against laurate, palmitate and stearate. None of the strains demonstrated glycosidase activity against paranitrophenol derivates of alpha-D-galactopyranoside, beta-D-galactopyranoside, beta-D-galactopyranoside-6-phosphate, alpha-L-arabinofuranoside, alpha-D-glucopyranoside, beta-D-glucopyranoside, beta-D-galacturonide, beta-D-glucoronide, alpha-maltoside, beta-maltoside, N-acetyl-alpha-D-glucosaminide, N-acetyl-beta-D-glucosaminide, alpha-L-fucopyranoside, beta-D-fucopyranoside, beta-L-fucopyranoside, beta-D-lactoside, alpha-D-mannopyranoside, beta-D-mannopyranoside, alpha-D-xylopyranoside and beta-D-xylopyranoside.

The results for 47 aminopeptidases and seven esterases showing variable reactions are given in Table 1. The esterase results were either weakly positive or negative. However, the reproducibility for C4, C8 and C14, as determined on two different test strips, was too poor to use these test results as identification markers.

In a comparison of 173 *Haemophilus ducreyi* isolates from four geographic areas only five of the 47 aminopeptidase tests showed significantly variable reactions

Table 1: Enzyme activity of 200 *Haemophilus ducreyi* strains, with 47 aminopeptidases and seven esterases showing variable results.

Substrate (beta-naphthylamide derivate)	No. of positive strains (%)	
L-tyrosine	197	(98)
L-pyrrolidone	1	(0.5)
L-phenylalamine	198	(99)
L-hydroxyproline	45	(42)
L-histidine	67	(33)
L-aspartate	171	(85)
S-benzyl-L-cysteine[a]	65	(32)
Glycyl-L-phenylalanine	160	(80)
Glycyl-L-proline	151	(75)
L-leucyl-glycine	122	(61)
L-seryl-L-tyrosine	77	(38)
L-glutamine	195	(97)
Alpha-L-glutamate	12	(6)
L-isoleucine	11	(5)
L-ornithine[a]	121	(60)
L-proline	52	(26)
L-serine	195	(97)
L-threonine	66	(33)
L-tryptophane	79	(39)
N-CBZ-glycyl-glycyl-L-arginine	2	(1)
L-alanyl-L-arginine	189	(94)
L-alanyl-L-phenylalanyl-L-proline[a]	40	(20)
L-alanyl-L-phenylalanyl-L-prolyl-L-alanine	34	(17)
L-arginyl-L-arginine	98	(49)
Alpha-L-aspartyl-L-alanine	68	(34)
Alpha-L-aspartyl-L-arginine	45	(22)
Alpha-L-glutamyl-L-alpha-L-glutamine	9	(4)
Alpha-L-glutamyl-L-histidine	29	(14)
Glycyl-L-arginine	198	(99)
Glycyl-L-tryptophane	19	(9)
L-histidyl-L-leucyl-L-histidine[a]	29	(14)
L-histidyl-L-serine[a]	27	(13)
L-leucyl-L-alanine	134	(67)
L-leucyl-L-leucyl-L-valyl-L-tyrosyl-L-serine	78	(39)
L-lysyl-L-alanine	177	(88)
l–lysyl-L-lysine	175	(87)
L-phenylalanyl-L-arginine	59	(29)
L-phenylalanyl-L-proline	95	(47)
L-phenylalanyl-L-prolyl-L-alanine	37	(18)
L-prolyl-L-arginine	35	(17)
L-seryl-L-methionine	140	(70)
L-valyl-L-tyrosyl-L-serine	15	(7)
L-histidyl-L-phenylalanine	5	(2)
4-Methoxy-L-lysyl-L-serine	4	(2)
L-valine	58	(29)
L-cystine	16	(8)
N-glutaryl-phenylalanine	3	(1)
Butyrate (C4)	198/196[b]	(99–98)
Valerate (C5)	199	(99)
Caproate (C6)	199	(99)
Caprylate (C8)	199/181[b]	(99–90)
Nonanoate (C9)	15	(7)
Caprate (C10)	1	(0.5)
Myristate (C14)	0/30[b]	(0–15)

[a] Substrates yielding significantly different results among the four geographic centres.
[b] Tests done in duplicate on two different strips.

(2 X 4 Chi-square test, $p < 0.05$) (Table 2). Further data analysis comparing the results of these five tests for each center in relation to each other showed significant differences between the isolates from Asia, Africa and Europe but not between the strains isolated in the two African cities, Nairobi and Johannesburg.

The best discrimination of strains of different geographic origin was obtained by combining the test results of L-ornithine and L-alanyl-L-phenylalanyl-L-proline. As shown in Table 3, the distribution of these three biovars was significantly different when comparing strains from Bangkok, Nairobi, Johannesburg and Amsterdam. Considering Nairobi and Johannesburg as a single African group, the statistical difference obtained between the strains from Asia, Africa and Europe was even stronger ($X^2 = 33.61$, $p = 0.00001$, 3 X 3 test).

Discussion

The purpose of this study was to compare isolates from different geographic origins. In a comparison of the combined results of all five significantly different tests to each other, the best type distribution showing the highest discrimination among the different groups of strains was found by the combination of L-alanyl-L-phenylalanyl-L-proline and L-ornithine. These two enzymes and perhaps also S-benzyl-L-cysteine, L-histidyl-L-leucyl-histidine and L-histidyl-L-serine may provide a marker system which could be used for the epidemiological study of *Haemophilus ducreyi*.

It may be difficult to compare the results obtained in our study with those of Casin on 30 isolates of *Haemophilus ducreyi* (6). Different growth conditions (media, incubation time, atmosphere, tempera-

Table 2: Arylamidase activity of 173 *Haemophilus ducreyi* strains isolated in different geographical areas.

Substrate	No. strains giving a positive reaction: Bangkok n = 93	Nairobi n = 24	Johannesburg n = 32	Amsterdam n = 24	X^2 [a]
S-benzyl-L-cysteine	26	6	10	14	8.83 $p < 0.05$
L-ornithine	69	8	14	19	21.96 $p < 0.001$
L-alanyl-L-phenylalanyl-L-proline	23	5	10	0	8.80 $p < 0.05$
L-histidyl-L-leucyl-L-histidine	18	7	3	0	9.40 $p = 0.02$
L-histidyl-L-serine	13	2	3	8	8.02 $p < 0.05$

[a] 2 X 4 test comparing results for each substrate among the four centres.

Table 3: Distribution of *Haemophilus ducreyi* strains from different geographic areas combining the test results of L-ornithine- and L-alanyl-L-phenylalanyl-L-proline arylamidase.

Substrate	Bangkok n = 93	Nairobi n = 21	Johannesburg n = 32	Amsterdam n = 24
L-alanyl-L-phenylalanyl-L-proline positive (l-ornithine negative or positive)	23	5	10	0
L-alanyl-L-phenylalanyl-L-proline negative and l-ornithine positive	47	5	5	19
L-alanyl-L-phenylalanyl-L-proline negative and L-ornithine negative	23	14	17	5

$X^2 = 34.48$, $p = 0.00004$; 3 X 4 test combining the three result combinations among the four centres.

ture, pH) of the bacterial strains may influence the production of several enzymes. We observed such a variation in reaction patterns during a multicenter evaluation of API-ZYM tests for the identification of *Neisseria* spp. (unpublished observations) and during a taxonomic study on *Gardnerella vaginalis* (7). Different geographic origin of the strains tested may also cause different test results as compared with the French isolates (6). In general, a higher proportion of our strains exhibited positive aminopeptides reactions. However, isolates from Amsterdam resembled closely those isolated in Paris, and were strikingly more enzymatically inert than Asian and African strains. In contrast, reaction patterns for esterases and glycosidases and for most aminopeptidases were in agreement with the results of Casin et al., confirming the relatively poor biochemical activity of *Haemophilus ducreyi*.

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