Lack of Deoxyribonucleic Acid Relatedness Between *Haemophilus ducreyi* and Other *Haemophilus* Species

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Deoxyribonucleic acid relatedness studies (S1 nuclease method) showed that 25 strains of *Haemophilus ducreyi* from diverse geographic origins form a homogeneous deoxyribonucleic acid hybridization group. A total of 17 type or reference strains of established or proposed *Haemophilus* species were 0 to 6% related to *H. ducreyi* at the optimal temperature. These findings do not support the assignment of *H. ducreyi* to the genus *Haemophilus*.

The classification of the causative agent of chancroid, *Haemophilus ducreyi* (Neveu-Lemaire 1921) Bergey, Harrison, Breed, Hammer and Huntoon 1923 (1, 13), in the genus *Haemophilus* is based mainly on the demonstration of a requirement for hemin, a nitrate reductase activity, and a guanine-plus-cytosine content of the deoxyribonucleic acid (DNA) (38 mol%) similar to that of *Haemophilus influenzae* (11). However, *H. ducreyi*, a saliently unreactive bacterium in conventional biochemical tests, is phenotypically different from all other *Haemophilus* species (11).

The purpose of the present study was to determine the extent of genetic variation among chancroid isolates of *H. ducreyi* and the levels of relatedness between *H. ducreyi* and other *Haemophilus* species.

MATERIALS AND METHODS

Bacterial strains. The origins of the 25 *H. ducreyi* strains studied are listed in Table 1. The type or reference strains of the other *Haemophilus* species studied are listed in Table 2.

The *H. ducreyi* isolates from Hôpital Saint-Louis, Paris, France, were identified as described elsewhere (5). Stock cultures of *H. ducreyi* were maintained at -70°C in PPLO broth (Difco Laboratories, Detroit, Mich.) supplemented with 40% (vol/vol) Eagle minimal essential medium (Institut Pasteur Production, Marnes-la-Coquette, France), 20% (vol/vol) fetal bovine serum (Industrie Biologique Française, Villeneuve-la-garenne, France), and 15% (vol/vol) glycerol. Working cultures were maintained under 5% CO_2 at 36°C by inoculation every 3 days onto PPLO agar containing 40% (vol/vol) Eagle minimal essential medium and 20% (vol/vol) fetal bovine serum.

DNA preparation. After 2 to 3 days of incubation at 37°C in 5% CO_2 , the growth from 10 plastic flasks (Nunc, Nunc, Copenhagen, Denmark) containing 75 ml of PPLO agar containing 40% (vol/vol) Eagle minimal essential medium and 20% (vol/vol) fetal bovine serum was harvested in 200 ml of a solution containing 0.05 M tris(hydroxymethyl)aminomethane, 0.05 M ethylenediaminetetraacetate, and 0.1 M NaCl (pH 8.0). Then, 4 ml of a 25% (wt/vol) aqueous solution of sodium dodecyl sulfate (Sigma Chemical Co., St. Louis, Mo.) and 0.6 ml of a 2% (wt/vol) pronase (Calbiochem-Behring, La Jolla, Calif.) solution were added. The mixture was vigorously shaken and incubated at 37°C overnight to achieve cell lysis.

DNA was extracted and purified from the cell lysate by using previously described procedures (4). Some of the DNA preparation was sheared by sonication (4). Sheared DNAs were dialyzed overnight against 0.042 M NaCl and stored at 4°C over a layer of chloroform.

DNA-DNA hybridization. Native DNA was labeled in vitro by nick translation (10). The S1 nuclease method (8) used (S1 nuclease-trichloroacetic acid procedure) has been described elsewhere (10), except that all volumes were reduced by one-half.

The temperature (T_m) at which 50% of the reassociated DNA became hydrolyzable by the S1 enzyme was determined (8). The difference (ΔT_m) between the T_m of a homologous reaction and the T_m of a heterologous reaction is an estimate of divergence between two DNAs (3).

Guanine-plus-cytosine content. The melting temperatures of 50- $\mu\text{g}/\text{ml}$ DNA solutions in $0.1\times$ SSC buffer ($1\times$ SSC is 0.15 M NaCl plus 0.015 M trisodium citrate) were measured with a Gilford spectrophotometer. The guanine-plus-cytosine contents of DNAs were determined twice from melting temperatures by the equation of Owen et al. (14). *Escherichia coli* K-12 DNA taken to have a guanine-plus-cytosine content of 50.6 mol% was included as a standard.

RESULTS

Guanine-plus-cytosine content. The guanine-plus cytosine content of the DNA of *H. ducreyi* type strain CIP 542 was 39 mol%.

DNA relatedness. The DNA relatedness results obtained with labeled reference DNA from strain CIP 542^T (T = type strain) and unlabeled DNAs from 24 strains of *H. ducreyi* are shown in Table 1. The S1 nuclease-resistant core (in the incubated control tubes containing only denatured labeled DNA) was calculated to be $3 \pm 1\%$ (mean $\pm$ standard deviation). At 60°C , the 24 *H. ducreyi* strains were $94 \pm 6\%$ related to strain CIP 542^T. The ΔT_m values between *H. ducreyi* strains and strain CIP 542^T were less than 1°C .

The DNA relatedness results obtained with the same labeled DNA from strain CIP 542^T and DNAs from 17 other representative strains of the genus *Haemophilus* are shown in Table 2. All *Haemophilus* species studied (other than *H. ducreyi*) were 0 to 6% related to *H. ducreyi* CIP 542^T.

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DISCUSSION

The guanine-plus-cytosine content of the DNA of *H. ducreyi* found in this work (39 mol%) is within 1 mol% of the value (38 mol%) published by Kilian (11).

The DNA relatedness results provide evidence that strains identified as *H. ducreyi* constitute a homogeneous genomic species. Since the level of DNA hybridization between *H. ducreyi* and the other *Haemophilus* species was very low, our results provide no support for assignment of *H. ducreyi* to the genus *Haemophilus*.

Most species in the family *Pasteurellaceae* (i.e., members of the genera *Pasteurella*, *Haemophilus*, and *Actinobacillus*) were at least 30% related when the spectrophotometric method was used to assess DNA relatedness (16). However, DNA reassociation values determined with the hydroxyapatite method (6, 18), the nitrocellulose filter method (6, 7), or the S1 nuclease method (9) showed the *Pasteurellaceae* to be genomically heterogeneous, with some groups showing little or no DNA relatedness (0 to 10% similarity). No *H. ducreyi* strain had been included in published DNA relatedness studies prior to the present work.

The etiological agent of chancroid was first isolated on a medium supplemented with 30% (vol/vol) fresh rabbit blood (2), and this apparent requirement for blood led to the inclusion of "*Coccobacillus ducreyi*" (13) in the genus *Haemophilus* (1). Lwoff and Pirowsky (12) showed that *H. ducreyi* requires X factor (protoporphyrin IX or protoheme). A key characteristic of the genus *Haemophilus* is the requirement for X factor or V factor (nicotinamide adenine dinucleotide or nicotinamide adenine dinucleotide phosphate) or

TABLE 2. Reassociation of DNA from *H. ducreyi* CIP 542^T with DNAs from other *Haemophilus* species

Source of unlabeled DNA ^a	% Relative binding at 60°C
<i>H. influenzae</i> NCTC 8143 ^T	4
<i>Haemophilus aegyptius</i> CIP 52.129 ^T (- ATCC 11116 ^T)...	2
<i>Haemophilus aphrophilus</i> CIP 7073 ^T (- NCTC 5906 ^T)...	1
<i>H. avium</i> ATCC 29546 ^T	1
<i>Haemophilus haemoglobinophilus</i> NCTC 1659 ^T	3
<i>Haemophilus haemolyticus</i> NCTC 10659 ^T	1
<i>Haemophilus paragallinarum</i> ATCC 29545 ^T	3
<i>Haemophilus parahaemolyticus</i> NCTC 8479 ^T (- ATCC 10014 ^T).....	4
<i>Haemophilus parainfluenzae</i> NCTC 7857 ^T	4
<i>Haemophilus paraphrohaemolyticus</i> NCTC 10670 ^T	1
<i>Haemophilus paraphrophilus</i> CIP 70.72 ^T (- NCTC 10557 ^T)	1
<i>Haemophilus parasuis</i> CIP 58.8 ^T (- ATCC 19417 ^T).....	1
<i>H. pleuropneumoniae</i> ATCC 27088 ^T	6
" <i>Haemophilus agni</i> " CIP 58.63.....	0
" <i>Haemophilus somnus</i> " WK 724-75 ^b	0
<i>Haemophilus equigenitalis</i> NCTC 11184 ^T	0
<i>Haemophilus piscium</i> ATCC 10801 ^{Tc}	0

^a ATCC, American Type Culture Collection, Rockville, Md.; CIP, Collection de l'Institut Pasteur, Paris, France; NCTC, National Collection of Type Cultures, London, England.

^b Received from Institut Mérieux, Lyon, France.

^c Now considered an atypical strain of *Aeromonas salmonicida* (15).

TABLE 1. Reassociation of DNA from *H. ducreyi* CIP 542^T with DNAs from other *H. ducreyi* strains

Source of unlabeled DNA		% Relative binding at 60°C
Strain ^a	Geographic origin	
CIP 542 ^T	Vietnam (Hanoi)	100 (0.0) ^b
HSL 82013	Portugal (Lisbon)	95
HSL 83002	France (Paris)	85
HSL 82010	Senegal	97
HSL 79001	France (Paris)	100
HSL 83004	India	93
HSL 76005	Singapore	92 (0.4)
HSL 82006	Singapore	82
HSL 80002	India	98 (0.7)
HSL 82009	Portugal (Porto)	94
HSL 83013	France (Paris)	88
HSL 82011	Antilles	80 (0.1)
HSL 83003	France (Paris)	96
HSL 82014	France (Paris)	90 (0.3)
HSL 83014	France (Paris)	99
CCUG 7309	Canada (Winnipeg)	99
CCUG 7310	Canada (Winnipeg)	93 (0.2)
CCUG 7312	Canada (Winnipeg)	98
CCUG 7781	Denmark (Copenhagen)	100
CCUG 8157	Thailand	95
CCUG 8224	Spain (Mallorca)	98
CCUG 8759	Portugal	96
CCUG 9276	East Africa	94
CCUG 10058	Sweden (Karlstad) or Spain	93
Slootmans 3141	Kenya	95

^a CIP, Collection de l'Institut Pasteur, Paris, France; HSL, Laboratoire Central de Microbiologie, Hôpital Saint-Louis, Paris, France; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden (E. Falsen); Slootmans, H. Slootmans, Instituut voor Tropische Geneeskunde Prins Leopold, Antwerp, Belgium.

^b The values in parentheses are ΔT_m values (in degrees Celsius).

both. However, this key characteristic for circumscribing the genus *Haemophilus* has been challenged by DNA homology studies. Pohl et al. (17) have shown that *Haemophilus pleuropneumoniae* is genetically closer to *Actinobacillus lignieresii* than to *H. influenzae* and that V-factor-requiring and V-factor-nonrequiring strains could constitute a single genomic species. Accordingly, *H. pleuropneumoniae* was transferred to the genus *Actinobacillus* (17). Similarly, *Haemophilus avium* belongs to the same genomic group as *Pasteurella gallinarum* irrespective of different growth factor requirements (17).

The family *Pasteurellaceae* is undergoing complete revision. At this time, we refrain from proposing nomenclatural changes until DNA relatedness studies of the genus *Haemophilus* and the family *Pasteurellaceae* provide more comprehensive results.

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