Numerical Taxonomy of Saccharolytic Clostridia, Particularly *Clostridium perfringens*-Like Strains: Descriptions of *Clostridium absonum* sp. n. and *Clostridium paraperfringens*

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Clostridium perfringens-like strains whose taxonomic position is uncertain were examined in detail. The lecithinases of these strains exhibited less avidity to the alpha-antitoxin of *C. perfringens* than did the *C. perfringens* lecithinase. On the basis of a computer analysis, the *C. perfringens*-like strains were grouped into two phenons, I and III, both of which are distinctly separable from *C. perfringens* (phenon II). Strains previously identified by us as belonging to *Clostridium paraperfringens* Nakamura et al. 1970, including strain G (= ATCC 27639), here designated as the type strain of *C. paraperfringens*, were found to belong to phenon I, and the strains of phenon III are regarded as constituting a new species, for which we propose the name *Clostridium absonum*. The type strain of *C. absonum* is HA-7103 (= ATCC 27555). The main characters differentiating *C. absonum* from *C. perfringens* are as follows: *C. absonum* produces a lecithinase which exhibits extremely low avidity to *C. perfringens* alpha-antitoxin, rapidly ferments salicin, does not ferment raffinose, and does not liquefy 10% gelatin; the main characters of *C. absonum* which differentiate it from phenon I strains are: larger cell width, a distinctly stronger lecithinase reaction, liquefaction of 2% gelatin, production of butanol, and weak toxicity for mice. Deoxyribonucleic acid (DNA)-DNA homology studies of phenons I, II, and III confirmed the validity of the above-mentioned groupings. A computer analysis of other saccharolytic clostridia revealed that *C. butyricum*, *C. acetobutyricum*, and *C. multifementans* are associated by similarity values higher than 90% and that *C. septicum* and *C. chauvoei* constitute separate, but closely related, taxa.

In a previous communication, Nakamura et al. (15) proposed a new species, *Clostridium paraperfringens*, for strains isolated from a case of gas gangrene, from normal human intestines, and from soil samples. A type strain was not designated for this new species. The lecithinases produced by most of these *C. perfringens*-like strains on half-antitoxin Nagler agar (26) were completely suppressed by an excessive amount of *C. perfringens* antitoxin although they exhibited less avidity to *C. perfringens* alpha-antitoxin than did the lecithinase of *C. perfringens*. These strains were nontoxic. Later, we encountered another group of *C. perfringens*-like strains which were toxigenic. The present work was carried out to elucidate the taxonomic relationship of the above-mentioned groups of *C. perfringens*-like strains to *C.*

perfringens as well as to other saccharolytic clostridia by the use of numerical taxonomy, to detect their genetic relatedness by deoxyribonucleic acid (DNA)-DNA homology analysis, and to designate the type strain of *C. paraperfringens*. Because no computer analysis has been performed on clostridia to date, we also considered it worthwhile to examine a number of saccharolytic strains by this method, particularly those belonging to *C. septicum* and *C. chauvoei*.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. The isolation of *C. perfringens*-like strains was performed by the same method as that used for *C. perfringens* (28).

Lecithinase. Lecithinase production was detected on an egg-yolk plate according to the method of Willis and Hobbs (26), and the activity of the lecithinase in culture filtrate was estimated by two methods, egg units (EU)/ml according to van Heyningen's method (25), and alpha-antitoxin equivalent (AE)/ml according to Evans' method (6). The medium used was previously described by Nishida et al. (16). To make a half-antitoxin egg-yolk plate, a 0.1-ml portion of *C.*

perfringens type A antitoxin (600 International Units/ml; Chiba Serum Institute, Chiba, Japan) was spread over one-half of an egg-yolk plate.

DNA-DNA homology. Cells for DNA isolation were grown in a medium containing 2% (wt/vol) proteose peptone (Difco), 0.5% yeast extract, 0.5% NaCl, 1% (wt/vol) glucose, and 0.1% sodium thioglycolate (pH 7.2). For isolation of ^{32}P -labeled DNA, 0.1 mCi of carrier-free ^{32}P was added to 100 ml of the

TABLE 1. *Strains used for computer analysis*^a

Strain no.	Name	Strain no.	Name
1	<i>Clostridium paraperfringens</i> FIS-1W(KZ)	33	<i>C. septicum</i> 8(NIHJ)
2	<i>C. paraperfringens</i> FIS-5(KZ)	34	<i>C. septicum</i> 94(NIHJ)
3	<i>C. paraperfringens</i> 3-3(KZ)	35	<i>C. septicum</i> C-61 (Gifu Univ., Gifu, Japan)
4	<i>C. paraperfringens</i> 9(KZ)	36	<i>C. septicum</i> 7281(KZ)
5	<i>C. paraperfringens</i> 3-1(KZ)	37	<i>C. septicum</i> 616(KZ)
6	<i>C. paraperfringens</i> G(KZ)	38	<i>C. chauvoei</i> Holland(SVSL)
7	<i>C. paraperfringens</i> H(KZ)	39	<i>C. chauvoei</i> Frankring(SVSL)
8	<i>C. paraperfringens</i> 2227 originally labeled <i>Inflabilis barati</i> 2227, Pasteur Institute, Paris	40	<i>C. chauvoei</i> Mørkov(SVSL)
9	<i>C. paraperfringens</i> 7606 originally labeled <i>I. barati</i> 7606, Pasteur Institute, Paris	41	<i>C. chauvoei</i> AA-11(SVSL)
10	<i>C. paraperfringens</i> 12/16B originally labeled <i>I. lacustris</i> 12/16B, Pasteur In- stitute, Paris	42	<i>C. chauvoei</i> C-59 (Gifu Univ., Gifu, Japan)
11	<i>C. perfringens</i> type A PB6K(NIHJ)	43	<i>C. chauvoei</i> CH6 (Leeds Univ., Leeds, England)
12	<i>C. perfringens</i> type A S002-3(KZ)	44	<i>C. chauvoei</i> 11(NIHJ)
13	<i>C. perfringens</i> type A S16550(KZ)	45	<i>C. parapatrificum</i> ATCC 17864
14	<i>C. perfringens</i> type A 1610(KZ)	46	<i>C. innocuum</i> ATCC 14501
15	<i>C. perfringens</i> type A S16102(KZ)	47	<i>C. botulinum</i> type E 59 (Institute of Health of Akita, Akita, Japan)
16	<i>C. perfringens</i> type A 1278A originally labeled <i>I. sanguicole</i> 1278A, Pasteur In- stitute, Paris	48	<i>C. sphenoides</i> ATCC 3560
17	<i>C. perfringens</i> type B NCTC 4964	49	<i>C. bifermentans</i> 315(KZ)
18	<i>C. perfringens</i> type C NCTC 3182	50	<i>C. sordellii</i> 4708 (Wellcome Research Laboratory Col- lection, England)
19	<i>C. perfringens</i> type D L9 (Leeds Univ., Leeds, England)	51	<i>C. sordellii</i> 12/16C originally labeled <i>I. lacustris</i> 12/16C, Pasteur Institute, Paris
20	<i>C. perfringens</i> type E 411(TSCI)	52	<i>C. sporogenes</i> 80011(KZ)
21	<i>C. perfringens</i> type F 398(TSCI)	53	<i>C. difficile</i> ATCC 17857
22	<i>C. perfringens</i> type F 410(TSCI)	54	<i>C. difficile</i> ATCC 9689
23	<i>C. tertium</i> 2C1-1(KZ)	55	<i>C. difficile</i> 2866 originally labeled <i>I. setiensis</i> 2866, Pas- teur Institute, Paris
24	<i>C. tertium</i> 4C-2(KZ)	56	<i>C. fallax</i> A33H Pasteur Institute, Paris
25	<i>C. tertium</i> 965 originally labeled <i>Plectridium pseudo-tetanicum</i> , Pasteur Institute, Paris	57	<i>C. tetani</i> PO5(KZ)
26	<i>C. multifementans</i> ATCC 17795	58	<i>C. tetanomorphum</i> NCTC 288
27	<i>C. multifementans</i> 2C-2(KZ)	59	Unidentified HA-7103(KZ)
28	<i>C. butyricum</i> IFO 3847	60	Unidentified HA-7107(KZ)
29	<i>C. butyricum</i> IFO 3858	61	Unidentified HA-9103(KZ)
30	<i>C. acetobutyricum</i> IFO 3346	62	Unidentified SO6-4(KZ)
31	<i>C. acetobutyricum</i> IFO 3859	63	Unidentified TFI(KZ)
32	<i>C. septicum</i> 5(NIHJ)		

^a Abbreviations: ATCC, American Type Culture Collection, Rockville, Md., U.S.A.; IFO, Institute for Fermentation, Osaka, Japan; KZ, strain isolated in Kanazawa, Japan; NIHJ, National Institute of Health, Tokyo, Japan; TSCI, Tarrasevich State Control Institute, Moscow, U.S.S.R.; SVSL, State Veterinary Serum Laboratory, Copenhagen, Denmark; NCTC, National Collection of Type Cultures, London, England.

above-mentioned medium. All organisms were incubated at 37 C. Bacteria grown to the logarithmic phase were harvested by centrifugation and washed once with $1 \times \text{SSC}$ (0.15 M NaCl and 0.015 M sodium citrate). Highly polymerized DNA was prepared by the method of Marmur (12). The cells thus obtained were suspended in 0.1 M ethylenediamine tetraacetic acid $\text{SSC} (\times 1)$ solution, and treated at 37 C for 20 min with 1 mg of lysozyme per ml (Boehringer Mannheim GmbH, Mannheim, Germany). Sodium lauryl sulfate was added to a final concentration of 3% (wt/vol), and the cell lysate was kept at 60 C for 10 min. The suspension was cooled to room temperature and deproteinized two or more times with chloroform-octyl alcohol. Ribonucleic acid (RNA) was removed by treatment with ribonuclease (RNase) (Worthington Biochemical Corp., Illinois). The labeled DNA was sheared by passing it through a French pressure cell (12,000 to 15,000 lb/in²) (Otake, Tokyo, Japan). DNA, either native or sheared, was denatured by heating for 5 min at 100 C. The procedure for preparation of DNA-agar was carried out according to the method described by McCarthy and Hoyer (11). DNA was trapped in a 3% (wt/vol) agar (Oxoid Ionagar no. 2) suspension. Hybridization was carried out by incubating at 60 C for 14 h the mixture of 0.1 g of DNA-agar containing 30 to 60 μg of DNA and ³²P-labeled DNA fragments of one-tenth the DNA-agar in $1 \times \text{SSC}$ according to the method of McCarthy and Bolton (10) as modified by Hanaoka et al. (8). Unbound DNA fragments were eluted with $2 \times \text{SSC}$ at 60 C, and bound DNA was eluted with $0.01 \times \text{SSC}$ at 70 C. Each fraction was collected and then counted in a liquid scintillation system, Unilex I-A (Nuclear-Chicago Co., Des Plaines, Ill.). Experiments were performed in duplicate and, when further confirmation was necessary, were repeated.

Determination of similarity values. Strains examined were clustered by the single-linkage method described by Sneath (22). The data were coded according to procedure III of Beers et al. (2), and the similarity values were calculated as described by these same authors. The computer used was the NEAC 2230 (Nippon Electric Co., Tokyo, Japan). The properties used in the characterization of the organisms studied are listed in Table 2, and the number of features used was 152. Due to the morphological variability of clostridia, we did not use cell length as a character. The features of each strain were determined as follows. For morphological examinations, organisms were streaked on 1% glucose blood agar, and the plates were incubated for 36 h at 37 C. These cultures were used for the observation of colony shape and Gram stain. For detection of endospores, the organisms were cultured in Roberts cooked meat broth for 5 days (21) and stained with Wirtz stain (27). Estimation of the growth response to sugars was made as follows. Each sugar tested (Table 2) was added to a 3.5-ml portion of PY medium in a small test tube (10 by 106 mm) to give a 1% (wt/vol) concentration of the sugar. (PY medium consists of 2% [wt/vol] polypeptone [Daigo, Osaka, Japan], 0.5% yeast extract, and 0.5% NaCl.) Each tube was inoculated with a 0.15-ml portion of a liver broth culture. The growth response to each sugar

was compared with that in a control tube with no sugar added and was read positive when the optical density of each culture was more than two times as much as that of the control tube. A 0.2-ml portion of pH indicator consisting of methyl red (0.1 g), bromthymol blue (0.3 g), 95% (vol/vol) ethyl alcohol (300 ml), and distilled water (300 ml) was added after the estimation of optical density. A change in the pH indicator from blue to yellow and then to red indicated acid production. To detect anaerobiosis, organisms streaked on 1% glucose blood (human) agar were incubated aerobically and anaerobically. Hemolysis was determined on the same medium. For detection of capsules, observations were made on an overnight culture (10% [vol/vol] serum broth containing 1% [wt/vol] glucose [9]) stained with India ink. Indole production, nitrate reduction, and liquefaction of 2 and 10% (wt/vol) gelatin were determined according to the method of Sterne and van Heyningen (23). To test for H₂S production, SIM medium (Eiken Chemical Co., Tokyo, Japan) was used. The digestion of casein was determined by Grob's method (7). The production of urease was determined by the method of Brooks and Epps (4). For the digestion of tributyrin, a brain-heart infusion agar (Eiken Chemical Co., Tokyo, Japan) plate containing 1% (vol/vol) tributyrin (Wako Co., Osaka, Japan) was used. RNase and deoxyribonuclease (DNase) were detected as follows: RNA was added to a basal medium (2.5% [vol/vol] heart infusion [Nissui Co., Tokyo, Japan], 0.045% CaCl₂, 0.5% yeast extract, and 1.5% [wt/vol] agar) to a final concentration of 0.2%; after 48 h of incubation at 37 C, 1.5 N HCl was added to detect the transparent zone around the RNase-positive colonies; to test for DNase, the Eiken DNA medium (Eiken Chem., Tokyo, Japan) containing DNA in a concentration of 0.25% was used; the result was read by the same procedure as used in the RNase test. To test for phosphatase, phenolphthalein-diphosphate (Wako Co., Tokyo, Japan) was added, to a final concentration of 0.15%, to a basal medium (pH 7.2) consisting of 2% (wt/vol) proteose peptone (Difco), 0.5% K₂HPO₄, 0.5% glucose, and 1.5% (wt/vol) agar; colonies which developed on the plates were exposed to NH₃ gas. To test for collagenase, holes were punched out on plates containing 2% (wt/vol) agar and 0.4% fragmented hide powder (BDH), and 0.5 ml of a 24-h chopped meat broth culture was pipetted with a drop of chloroform into the holes; a positive test was indicated by the appearance of a clear zone around a hole after 24 h of incubation at 37 C. To test for gas production from glucose, PY medium containing 1% (wt/vol) glucose and 0.5% agar was used. Toxicogenicity for mice was determined by the use of 6-h-old cultures grown in the medium of Nishida et al. (16). Alcohol and fatty acid production was detected by gas chromatography according to the method of Moore et al. (13) but using a Shimadzu gas chromatography model GC-4A (Shimadzu Co., Kyoto, Japan). A stainless steel column (4 mm inside diameter, 2 m long) was packed with Resoflex Lac-1-R-296 (Nishio Co., Tokyo, Japan). The chromatograph was operated at 120 C and with a N₂ flow of 60 ml/min. The product with the greatest peak on the chromatogram was marked as a main product.

(This does not mean that the greatest peak represented the major product in the culture, because not all acids are extracted to the same degree by the ether, and the detector is not equally sensitive to all kinds of acids.) To test for deamination, 1 ml of bacterial suspension (1

mg of N/ml), 0.4 ml of 0.1 M substrate, and 1.0 ml of 0.1 M phosphate buffer (pH 7.2) were mixed and shaken for 4 h under anaerobic conditions at 37 °C, and the evolved NH₃ was detected by Nessler reagent. The sensitivity of each strain to antibiotics was tested

TABLE 2. *Characters determined*

Test	Features scored
Colonies on 10% glucose blood agar	
Growth	Swarming or localized
Edge	Entire, uneven, or irregular
Surface	Smooth or rough
Color	Not colored, white, or green
Mucosity	Mucous or not
Morphology of cell	
Gram stain	Negative or positive
Shape of cell	Straight rod or curved
Arrangement	Occurring singly or clustered, filamentous or not
Width	Slender (< 0.5 µm) or stout (> 0.5 µm)
Spore	Terminal or not terminal, oval or spherical, swelling the rods or not
Capsule	Capsulated or not
Anaerobiosis	Anaerobic or microaerophilic
Hemolysis	Negative, incomplete, or complete haemolysis
Growth in PYG broth	
At 18 °C	Negative or positive
At 50 °C	Negative or positive
Growth stimulation by sugars	Negative or positive
Glucose, maltose, lactose, sucrose, galactose, fructose, xylose, mannitol, arabinose, salicin, mannose, raffinose, inulin, sorbitol, adonitol, dulcitol, glycerin	
Biological tests	
Indole production, nitrate reduction	Negative or positive
Gelatin liquefaction	Negative, weakly positive, or strongly positive
Casein digestion	Negative, incomplete, or complete
Hydrogen sulfide production	Negative or positive
Enzymatic activity	Negative or positive
Lecithinase, urease, RNase, DNase, phosphatase, collagenase, lipase	
Sensitivity to antibiotics	Sensitive or resistant
Chloramphenicol, tetracycline, penicillin, erythromycin, streptomycin, colistin, kanamycin, fradiomycin	
Toxigenicity to mouse	Toxic or nontoxic
Alcohol and fatty acid production in PYG medium	Negative or positive
Butanol, isobutanol, isoamyl alcohol, acetic acid, propionic acid, isobutyric acid, <i>n</i> -butyric acid, isovaleric acid, <i>n</i> -valeric acid, isocaproic acid, <i>n</i> -caproic acid	
A main product	Isoamyl alcohol, acetic acid, <i>n</i> -butyric acid, iso-valeric acid, <i>n</i> -valeric acid, or isocaproic acid
Utilization of nitrogen compounds as sole nitrogen and carbon source	
L-Glutamic acid	Negative or positive
L-Histidine	
Deamination of	Negative or positive
L-Aspartic acid, L-glutamic acid, L-Histidine, L-methionine, DL-serine	
Gas from glucose	Negative or positive

TABLE 3. *Lecithinase activities of Clostridium perfringens*-like strains

Strains	α -Antitoxin equivalents/ml	Egg units/ml	Lethal toxicity ^a	Lecithinase reaction ^b on half- α -antitoxin agar spread with 0.1 ml of α -antitoxin of	
				60 U/ml	600 U/ml
<i>C. paraperfringens</i>					
2227	<0.1	<1.0	—	+	—
7606	<0.1	<1.0	—	+	—
12/16 B	<0.1	<1.0	—	+	—
G	<0.1	<1.0	—	+	—
H	<0.1	<1.0	—	+	—
3-3	<0.1	<1.0	—	+	—
9	<0.1	<1.0	—	+	—
FIS-5	1.0	<1.0	—	+	—
FIS-1W	2.0	<1.0	—	+	—
3-1	2.0	<1.0	—	+	—
Unidentified strains					
HA-7103	>5.0	4.9	+ or —	+	±
HA-7107	>5.0	5.2	+ or —	+	±
HA-9103	>5.0	2.2	+ or —	+	±
CPP-8	>5.0	6.7	+	+	±

^a Symbols: +, toxic; —, nontoxic; + or —, inconsistently toxic.

^b Symbols: +, lecithinase reaction not suppressed; ±, lecithinase reaction slightly suppressed; —, lecithinase reaction suppressed; + or —, inconsistently toxic.

by the disk method. In scoring sensitivity to antibiotics, the absence of a zone of inhibition was interpreted as resistance, and an inhibition zone of any size was interpreted as sensitivity to an antibiotic. The utilization of L-glutamic acid and L-histidine as sole nitrogen and carbon sources was determined as follows: to a medium consisting of 0.03% yeast extract, 0.02% KH_2PO_4 , and 0.01% MgSO_4 in a volume of 3.5 ml in a small test tube (10 by 106 mm), each amino acid was added to give a final concentration of 0.75%; the medium was inoculated with 0.15 ml of a liver broth culture; after anaerobic incubation for 48 h at 37 C, the turbidity was estimated by optical density readings. To test for growth at 18 and 50 C, a PY medium containing 1% glucose was used.

RESULTS

Lecithinase reaction. The zones of the lecithinase reactions around the colonies of *C. paraperfringens* on Nagler agar were definitely thinner than those produced by *C. perfringens*. The lecithinase reaction on half-antitoxin Nagler agar spread with 600 U of alpha-antitoxin was completely suppressed in most cases and was slightly suppressed by 60 U of the antitoxin, although the stronger lecithinase reactions of the *C. perfringens* strains were completely or almost completely suppressed by the 60 U of alpha-antitoxin. Further examination of the lecithinase produced by *C. paraperfringens* strains in chopped meat-broth culture filtrates confirmed the low avidity between the lecithinase of these strains and *C. perfringens*

alpha-antitoxin (Table 3). The lecithinase activity, when examined according to the method of van Heyningen, was very slight, less than 1 EU/ml. However, when the lecithinases of some of the strains were re-examined for combining ability with the alpha-antitoxin, they exhibited AE titers extraordinarily higher than the titer calculated from the ratio of AE/EU of *C. perfringens* alpha-toxin. The lecithinase activities of the remaining strains were so weak that they could not be measured by this method. The new *C. perfringens*-like isolates, HA-7103 (ATCC 27555), HA-7107 (ATCC 27637), HA-9103 (ATCC 27635), and CPP-8 (ATCC 27636), were compared with the *C. paraperfringens* and *Inflabilis barati* strains. The colonial and morphological characteristics of these strains were in agreement with those of *C. perfringens*. The zones of the lecithinase reaction around the colonies of the new isolates grown on Nagler agar were as wide as those of *C. perfringens*, but the lecithinase reaction on half-antitoxin Nagler agar spread with 600 U of alpha-antitoxin was only slightly suppressed by the antitoxin (Fig. 1). The lecithinase produced by the new isolates in chopped meat-broth cultures exhibited activities as weak as 10 EU/ml, but the lecithinase reaction could not be neutralized by an excessive amount of the alpha-antitoxin. One strain, CPP-8, was consistently toxigenic to mice, and the remaining strains, HA-7103, HA-7107, and HA-9103, were inconsistently toxigenic.

Computer analysis. Computer studies were

carried out on *C. paraperfringens*, *I. barati*, and the new *C. perfringens*-like isolates to determine their taxonomic position with relation to other saccharolytic clostridia and particularly to elucidate correlations, if any, between these strains and *C. perfringens*. The result of these studies, presented as a dendrogram (Fig. 2), show that 53 strains of saccharolytic clostridia are associated at 81% similarity (81-phenon). In this 81-phenon, seven phenons were separated at 90% similarity. The remaining strains outside the saccharolytic 81-phenon consist of reference strains of various species of *Clostridium*.

Phenon I. The mean similarity values (MSV) of each strain to each of the other strains in phenon I ranged between 86 and 93%. This phenon is linked to phenon II through *C. perfringens* 1278 A at the 89% level. However, none of the MSVs of each strain in phenon I to all of the strains in phenon II was more than 81%. This indicates that although both phenons are taxonomically close to each other, they should be sorted into different species. This phenon includes all of the strains of *C. paraperfringens* used in this study.

Phenon II. The MSVs of each strain to each of the other strains in this phenon ranged from 85 to 92%. When the MSVs were compared, that of *C. perfringens* 1278 A was the lowest. This strain was sent from the Pasteur Institute, Paris, as *Inflabilis sanguicole*. It was sorted into this phenon because it was linked to *C. perfringens* S16102 at an SV of 91%. When grown on Nagler agar, *C. perfringens* 1278 A

showed a distinct lecithinase reaction which was typically inhibited by the alpha-antitoxin of *C. perfringens*. This phenon includes all of the strains of *C. perfringens* studied here.

Phenon III. The three new *C. perfringens*-like strains included in the computer analysis belonged to this phenon, and each was linked with the other strains of this same phenon at MSVs ranging from 92 to 96%. Each strain in this phenon was linked to the strains in phenon I at MSVs ranging from 80 to 81% and to those in phenon II at MSVs ranging from 71 to 74%. Another strain, CPP-8, which is cited in Table 3 but was not subjected to computer analysis, was placed in this group when examined for its morphological and biological properties. Culture filtrates of strains belonging to this phenon killed mice when injected intravenously.

Phenon IV. The three strains in this phenon belong to *C. tertium*.

Phenon V. *C. multifementans*, *C. butyricum*, and *C. acetobutyricum* belong to this phenon.

Phenon VI. All strains sorted into this phenon belong to *C. septicum*. They were associated at high SVs. The MSVs of each strain to each of the other strains in this phenon ranged from 94 to 97%.

Phenon VII. This phenon consists only of *C. chauvoei* strains. The MSVs of each strain to each of the other strains of this phenon ranged from 94 to 97%. Phenons VI and VII were associated at fairly high similarities, indicating that *C. septicum* and *C. chauvoei* are taxonomically close to each other.

Phenon VIII. This phenon, which lies outside of the above-mentioned 81-phenon, consists of *C. difficile* strains. Each of these strains is associated at 95% similarity.

Remaining strains. The remaining strains constitute a heterogenous group consisting of reference strains associated at SVs of less than 86%. They belong to the following species: *C. sordellii*, *C. bifermentans*, *C. sporogenes*, *C. fallax*, *C. tetani*, and *C. tetanomorphum*.

The most useful criterion for differentiating *C. perfringens* (phenon II) and the two groups of *C. perfringens*-like strains (phenons I and III) from the other phenons in the saccharolytic 81-phenon is lecithinase production. Table 4 implies that phenons I and III are fairly similar to each other although both are distinctly different from *C. perfringens*. The most useful criteria for differentiating phenon I from phenon III strains are cell width, the inhibition of lecithinase by 600 Units of *C. perfringens* alpha-antitoxin, the liquefaction of 2% gelatin, the production of butanol, and the colonial morphology.

DNA-DNA homology. Further attempts to

FIG. 1. Lecithinase reaction of *Clostridium perfringens* and *C. perfringens*-like strains on half-alpha-antitoxin Nagler agar. 1, Strain HA-7103; 2, *C. perfringens* PB6K; 3, *C. paraperfringens* H.

FIG. 2. Dendrogram of strains of saccharolytic clostridia and of selected reference strains.

TABLE 4. Differences between *Clostridium perfringens* (phenon II) and *C. perfringens*-like strains (phenons I and III)

Features	Phenon I <i>C. paraperfringens</i>	Phenon II <i>C. perfringens</i>	Phenon III <i>C. absonum</i> sp. n.
Width of cells	0.5-0.6 μ m	1.0-1.5 μ m	0.9-1.0 μ m
Colonial morphology	Serrate margin, rough, not green	Entire round, smooth, green	Entire, round, smooth, green (weak)
Hemolysis	— ($\pm$ or +)	+	+
Acid from raffinose	—	+	—
Salicin fermentation	Immediate	Delayed or none	Immediate
Capsule formation	Narrow	Wide	Narrow
Gelatin liquefaction			
2%	—	+	+
10%	—	+	—
Butanol production	—	+	+
Toxicogenicity to mouse	—	+	+ or —
Lecithinase inhibition on half-antitoxin Nagler agar	Complete	Complete	Slight

TABLE 5. DNA-DNA hybridization

Labeled DNA	Homology (%)		
	Phenon I <i>Clostridium para-</i> <i>perfringens</i> (2227)	Phenon II <i>C. perfringens</i> (PB6K)	Phenon III <i>C. absonum</i> sp.n. (HA-7103)
Phenon I			
<i>Clostridium paraperfringens</i> 2227	100	17	28
<i>C. paraperfringens</i> 3-3	67	12	29
<i>C. paraperfringens</i> 9	75	14	42
<i>C. paraperfringens</i> G	95	14	43
Phenon II			
<i>C. perfringens</i> type A PB6K	13	100	14
<i>C. perfringens</i> type A S002-3	16	76	17
<i>C. perfringens</i> type B 4964		65	
<i>C. perfringens</i> type C 3182		74	
<i>C. perfringens</i> type E 411	13	92	15
<i>C. perfringens</i> type F 410	12	66	11
<i>C. perfringens</i> type A 1278 A	12	71	17
Phenon III			
<i>C. absonum</i> sp.n. HA-7103	37	10	100
<i>C. absonum</i> sp.n. HA-7107	38	7	100
<i>C. absonum</i> sp.n. HA-9103	34	12	95
Reference strains			
<i>C. tertium</i> 4C-2	14	10	14
<i>C. butyricum</i> 3858	11	9	13
<i>C. septicum</i> 7281	17	12	17

elucidate the taxonomic relatedness of the strains of phenons I, II, and III were made by studying their DNA-DNA homologies. The hybridization results (Table 5) and the above-mentioned computer analysis results agree in showing that the strains of phenons I, II, and III constitute three distinct groups and that phenon III is more closely related to phenon I than to phenon II (Table 5).

DISCUSSION

Due to differences in the morphological, biological, and lecithinase properties, as well as to the DNA-DNA homologies of phenons I, II, and III, we regard these three phenons as constituting separate but closely related species, and the *C. perfringens*-like strains of phenon III we consider to belong to a new species, for which we propose the name *Clostridium absonum*. A description of this species follows.

Clostridium absonum sp. nov. (L. adj. *absonus* deviating [intended to mean "deviating from *C. perfringens*"].)

Gram positive, straight rods, 0.9 to 1.3 by 4.0 to 12.0 μ m. Not motile. Spores oval, eccentric to subterminal, not swelling the cells.

Surface colonies are circular, 1 to 3 mm in diameter, raised, slightly green.

Profuse and rapid growth in broth with fermentable carbohydrates.

Ferments fructose, galactose, glucose, lactose, maltose, mannose, salicin, and sucrose; does not ferment adonitol, arabinose, dulcitol, inulin, mannitol, raffinose, or sorbitol.

Fermentation products include acetic and butyric acids and butanol.

Gelatin is slowly liquefied. Milk is digested; frequently there is a storming of the milk. Urease is not produced. Nitrate is reduced.

Blood agar (human blood) is completely hemolyzed. Lecithinase is produced on egg-yolk agar. Lipase is not produced.

Anaerobic.

Toxin is weakly produced by most strains.

Habitat: widely distributed in soil.

Type strain. The type strain is HA-7103. It has been deposited in the American Type Culture Collection (ATCC), Rockville, Md., as ATCC 27555.

Phenon I includes three named strains which were sent from the Pasteur Institute, Paris. Two of them, 2227 and 7606, were labeled *Inflabilis barati*. Prévot (17, 20) stated that *I. barati* was the same as "le Bacille de Barat." The two

strains of *I. barati*, however, exhibited cultural properties distinctively different from those given in the original description of "le Bacille de Barat" by Tissier (24). Although Tissier stated that his strains formed round spores, produced the characteristic "limande" form, contained granules stainable by iodine solution, were motile, could grow rapidly and abundantly in the absence of sugars as well as in the presence of them, caused stormy fermentation of milk as *C. perfringens* did, produced no volatile fatty acid except acetic acid, fermented mannitol, and attacked fats, the two strains of the Pasteur Institute differed from "le Bacille de Barat" in all of the above-mentioned characters. The two strains, however, were demonstrated to be in agreement with *C. paraperfringens* in their cultural and biological properties and, therefore, were renamed as *C. paraperfringens*. A detailed description of *Clostridium paraperfringens* follows.

***Clostridium paraperfringens* Nakamura et al.**

Straight rods, 0.5 to 0.6 by 1.3 to 6.7 μ m. Gram positive. Not motile. Spores oval, eccentric to subterminal, swelling the cells.

Surface colonies are circular, 0.5 to 1.0 mm in diameter, slightly raised, and translucent with a dull, smooth surface, but colonies are frequently rhizoid, 2 to 4 mm in diameter, flat, and slightly grayish with a matt surface.

Growth is moderately good in liquid media with fermentable sugars but is poor in sugar-free media; diffuse turbidity.

Ferments cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, salicin, starch, and sucrose. Variable on ribose, melibiose, and trehalose. Does not ferment adonitol, arabinose, dulcitol, esculin, glycerol, inositol, inulin, mannitol, melezitose, raffinose, rhamnose, sorbitol, or xylose. Fermentation products include acetic and butyric acids without butanol.

Casein and gelatin are not hydrolyzed. Milk becomes acid but is otherwise unchanged.

Hydrogen sulfide is produced; indole is not formed. Urease is not formed; nitrate is reduced by most strains. Blood agar (human blood) is incompletely hemolyzed by some strains. Lecithinase is produced on egg-yolk agar; lipase is not produced. Anaerobic. Toxin is not formed; nonpathogenic for mice. Optimal temperature for growth, 35 to 45 C. Isolated from the normal human intestine and from wounds.

Type strain. Because a type strain was not designated for *C. paraperfringens* when this organism was originally named and described (15), strain G, one of the strains on which the original description was based, is here designated as the type strain. It has been deposited

in the American Type Culture Collection, Rockville, Md., as ATCC 27639. Two additional strains, 3-3 and 2227, have been deposited in the American Type Culture Collection as ATCC 27640 and ATCC 27638, respectively.

Also included in phenon I was another named strain, *Inflabilis lacustris* 12/16 B. We received two strains of *I. lacustris*, 12/16 B and 12/16 C, from the Pasteur Institute. However, when subjected to a computer analysis, the two were linked to each other at a low SV (70%). In checking the authenticity of these strains, we found that 12/16 B differed from the original description of *I. lacustris* (19) in many important respects: strain 12/16 B was urease-negative, did not produce a foul odor, did not produce indole, and produced capsules. The incorrectly labeled *I. lacustris* 12/16 B was re-identified as *C. paraperfringens*. On the other hand, the morphological, cultural, and biological properties of strain 12/16 C were in agreement with those of *C. sordellii*. The genus *Inflabilis*, which is not recognized in the present edition of *Bergey's Manual* (3), is clearly inappropriate for the three strains mentioned above. Although Prévot (17) stated that strains of the genus *Inflabilis* were not encapsulated, we could demonstrate the presence of capsules in the three strains received from the Pasteur Institute as well as in our *C. paraperfringens* isolates by the use of 10% serum broth containing 1% glucose, although the capsule was definitely thinner than that of *C. perfringens*.

Inflabilis setiensis (18), described in Prévot's *Manual* (17), is designated *Clostridium setiense* (Prévot and Raynaud) by McClung and McCoy in *Bergey's Manual* (3). The two strains of *I. setiensis* (2866 and 3131) examined by us, however, exhibited biological properties distinctly different from those reported by Prévot himself. *I. setiensis* 2866 was associated with *C. difficile* ATCC 17857 and ATCC 9689 at SVs as high as 95 and 96%, respectively. *I. setiensis* 3131 did not attack any sugar examined, and we did not include this strain in the computer analysis. We have received a few other strains of the genus *Inflabilis* from the Pasteur Institute. Most of the strains identified as belonging to this genus seemed to necessitate further investigation before being established as distinct species. Our results demonstrated that *C. multifementans*, *C. butyricum*, and *C. acetobutyricum* belonged to the same phenon. A recent investigation (5) revealed that strains designated as *Clostridium butyricum* constituted two distinct groups on the basis of

cell-wall sugar patterns, nucleotide-sequence similarities of DNA preparations, and nutritional requirements.

Also, DNA-DNA homology tests revealed that phenon I strains belong to a species distinctly different from *C. perfringens* and that *C. absonum* is not related to *C. perfringens* and is related only at a low level of homology to phenon I strains.

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