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Comparative phenotypic analysis of "Clostridium neonatale" and *Clostridium butyricum* isolates from neonates

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ABSTRACT

"Clostridium neonatale" was recently described as a new species within the Cluster I of the *Clostridium* genus *sensu stricto*. In this study, we characterized "C. neonatale" isolates (n = 42) and compared their phenotypic properties with those of *Clostridium butyricum* (n = 26), a close related species. Strains isolated from fecal samples of healthy neonates were tested for different phenotypic characteristics. Compared to *C. butyricum*, "C. neonatale" showed a significant higher surface hydrophobicity ($p = 0.0047$), exopolysaccharide production ($p = 0.0069$), aero-tolerance ($p = 0.0222$) and viability at 30 °C ($p = 0.0006$). A lower swimming ability ($p = 0.0146$) and tolerance against bile (0.3%) ($p = 0.0494$), acid (pH 4.5) ($p < 0.0001$), osmolarity (NaCl 5%, $p = 0.0188$) and temperature at 50 °C ($p = 0.0013$) characterized "C. neonatale" strains. Our results showed that "C. neonatale" behaves very differently from *C. butyricum* and suggests specific responses to environmental changes. Besides it is the first study on clinical isolates for these two anaerobic members of the newborns' gut microbiota and broadens our knowledge about their phenotypic traits.

1. Introduction

The genus *Clostridium* was proposed by Prazmowski in 1880 [1] and recently emended by Lawson and Rainey [2]. The *Clostridium* genus contains 220 species and 5 sub-species [3]. Clostridia as Gram-stain-positive obligate anaerobes are found in the environment but are also common inhabitants of the intestinal microbiota of humans and animals. In humans, some species include pathogens responsible for several diseases in adults and children, such as *Clostridium difficile* antibiotic-associated colitis and *Clostridium perfringens* enteric diseases [1], while others are present in a subdominant status in the gut microbiota [4,5]. In preterm neonates clostridia are the most common anaerobes found in fecal samples during the first 2 months of life at the expense of other anaerobes such as *Bacteroides* and *Bifidobacterium* species [6–10].

In 2002, an outbreak of necrotising enterocolitis (NEC) occurred in six neonates over a two-month period in a Canadian neonatal intensive care unit [11]. Blood cultures from three premature neonates grew the same strain proposed to belong to a novel species of *Clostridium*, "Clostridium neonatale" [12]. Although the "C. neonatale" ATCC BAA-265 reference strain was deposited, there was no clear characterization in order to either allow its formal classification as a new species or validate its name. The confusing status of this species explains the absence of data about its isolation, identification, or clinical significance. Besides, misidentification or/and underrepresentation of "C. neonatale" populations during previous studies may have occurred. Recently, combining a polyphasic analysis, we clarified "C. neonatale" status by demonstrating that it is a new species belonging to the Cluster I of the *Clostridium* genus *sensu stricto* [13]. In 2016, a draft genome of the "Clostridium neonatale" ATCC BAA-265 reference strains was announced [14]. Within this cluster, "C. neonatale" closest related species is *Clostridium butyricum*, the type species of the genus [15]. *C. butyricum* is part of the most frequently recovered species from fecal samples of neonates' gut microbiota [16] but has also been proposed to participate to NEC onset [17,18], as well as other clostridia species [19]. In the absence of data concerning "C. neonatale", we isolated "C. neonatale" strains from fecal samples of neonates and compared their phenotypic characteristics with those of

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C. butyricum. This study shows that "C. neonatale" behaves very differently from *C. butyricum,* in agreement with its status as a new species within the *Clostridium* genus. Besides we report for the first time data about clinical isolates of these two anaerobic members of the newborns' gut microbiota.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The 68 strains (*C. butyricum,* n = 26; "C. neonatale", n = 42) included in this study are part of our laboratory collection (EA4065, Laboratory of Microbiology) and were isolated from fecal samples obtained during the first month of life of unrelated healthy neonates. We included the *C. butyricum* ATCC 19398^T and "C. neonatale" ATCC BAA-265 reference strains. Species identification was performed by the PCR amplification of the 16S rRNA gene and a partial sequencing (Genewiz, UK) of the PCR product [20]. All strains were conserved at −80 °C. Unless otherwise stated, strains were grown on Columbia agar medium (Oxoid, France) supplemented with 5% v/v sheep blood at 37 °C in an anaerobic chamber (CO_2:H_2:N_2, 10:10:80) (MAC500, bioMérieux, France) for 24 h. For all tests, strains were cultured in TGY broth (Bacto Tryptone 30 g L^{-1}, Glucose 5 g L^{-1}, Yeast extract 20 g L^{-1}, L-cysteine 0.5 g L^{-1}) during 18 h at 37 °C under anaerobic conditions.

2.2. Motility assays

Motility assays were performed as previously described [21]. Briefly, swimming, swarming and twitching assays were performed on freshly poured TGY agar plates containing respectively 0.3%, 0.5%, and 1% w/v Bacto Agar (Difco, France) and incubated for 24 h at 37 °C in anaerobic condition. Bacterial motility was assessed by measuring the migration distance from the point of inoculation (9 cm corresponding to the petri dish diameter).

2.3. Hydrophobicity assays

Hydrophobicity assay was performed according to Pan et al. [22]. Briefly, 18 h TGY bacterial culture was centrifuged, washed twice with 1X PBS buffer (Thermo Fisher, France), and resuspended in 1X PBS buffer to obtain an absorbance at 600 nm of 1.0 (A0). Then, 300 µl of xylene were added to 3 ml of the suspension, mixed and the aqueous phase absorbance (A) was measured (600 nm). Hydrophobicity percentage (H%) was calculated as follows: H % = [(A0-A)/A0] X 100]. The surface hydrophobicity levels were considered as follows: high, H>70%; intermediate 30% < H<70%; low, H<30%.

2.4. Exopolysaccharides (EPS) production

One milliliter of 18 h TGY bacterial culture or TGY broth (control) was loaded into 2 ml polypropylene microtubes (eppendorf, Germany). After incubation for 2 h at 37 °C in anaerobic conditions, the bacterial culture was removed, the microtubes were washed once with 1X PBS buffer, and 1 ml of a 0.5% w/v crystal violet solution was added and incubated for 5 min at room temperature. After washing 3 times with 1X PBS buffer, the adsorbed remaining stain was eluted with 1 ml of pure ethanol (96.2%) and the absorbance at 595 nm was measured.

2.5. H_2O_2 disk diffusion assay

To determine strains susceptibility to H_2O_2, a disk diffusion assay was performed as described [23]: an 18 h TGY bacterial culture was adjusted to 1 unit MacFarland turbidity and used to inoculate TGY agar plates. A 6-mm filter paper disc containing 15 µl of 1% v/v H_2O_2 was placed onto the agar plates. The inhibition zone was measured after 24 h of incubation at 37 °C in anaerobic condition.

2.6. Bile, aero-tolerance, temperature, acid, and NaCl tolerance assays

Tolerance to bile salt was evaluated using 0.1 ml of an 18 h TGY bacterial culture grown anaerobically mixed either with 1 ml of a 0.3% w/v porcine bile salt (Sigma Aldrich, France) solution (0.2 M NaHCO3, pH6.3) or 1 ml of TGY (control) and incubated at 37 °C in anaerobic conditions for 5 h.

Aero-tolerance was evaluated using a 18 h TGY bacterial culture adjusted to an absorbance at 600 nm of 0.5, and 3 ml of this suspension was homogenized and incubated either aerobically in ambient air or anaerobically (control) at 37 °C for 24 h.

Temperature tolerance was evaluated by incubating 1 ml of an 18 h TGY bacterial culture at 30 °C, 37 °C (control) and 50 °C in anaerobic conditions for 5 h.

Tolerance to acid was performed using 0.1 ml of an grown anaerobically mixed either with 1 ml of TGY adjusted at pH 4.5 or at pH 7.0 (control) and incubated at 37 °C in anaerobic conditions for 24 h.

NaCl tolerance was evaluated by incubating 0.1 ml of 18 h TGY cell suspension grown anaerobically mixed with 1 ml of TGY (control), TGY supplemented with 5% or 9% w/v NaCl at 37 °C in anaerobic conditions for 5 h.

For all assays, 5 µl of serial dilutions were spotted on TGY agar plates to count colonies forming units (CFU)/ml after incubation during 24 h at 37 °C under anaerobic conditions.

2.7. Statistical analysis

XLSTAT 2014 add-on software was used for statistical analysis. The Wilcoxon-Mann and Whitney *U* test for ranked data ($p < 0.05$ two-tailed) was used to compare the quantitative values between strains populations. The correlation matrix of Pearson coefficients ($p < 0.05$) was used to search for dependence among the tested parameters. A principal component analysis (PCA) was used to represent each strain variables by observed correlations.

3. Results and discussion

Recently, we demonstrated that "C. neonatale" is a new species within the Cluster I of the *Clostridium* genus *sensu stricto* [13]. Albeit some phenotypic differences exist, they were not sufficiently discriminant to allow a differential identification between "C. neonatale" and *C. butyricum* strains. In this study, 68 strains were tested for their phenotypic characteristics under different conditions. Data calculated from three independent experiments (Figs. 1 and 2; Tables 1–3) evidenced clear differences between "C. neonatale" and *C. butyricum* strains.

3.1. Bacterial motility

Motility was evaluated by monitoring the swimming, swarming and twitching capabilities of 26 *C. butyricum* and 42 "C. neonatale" strains. *C. butyricum* strains exhibited an average swimming diameter of 4.9 cm: 12 strains (46%) showed the maximal migration distance from the point of inoculation (Table 1, Fig. 1a). *C. butyricum* showed significantly higher means of swimming ($p = 0.0146$) (Fig. 1a) with an average of 2.4 times longer growth distances. Indeed, compared to *C. butyricum*, "C. neonatale" average