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# Differentiation of *cfiA*-Negative and *cfiA*-Positive *Bacteroides fragilis* Isolates by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry▽

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**Carbapenem resistance in *Bacteroides fragilis* is associated with *cfiA*-encoded class B metallo-beta-lactamase. *cfiA*-negative and *cfiA*-positive isolates belong to genotypically distinct groups. Of a total of 248 *B. fragilis* isolates included in this study, 214 were susceptible, 10 were intermediate, and 24 were resistant to meropenem. We show that matrix-assisted laser desorption ionization–time of flight mass spectrometry is able to differentiate between *cfiA*-negative and *cfiA*-positive isolates and predict carbapenem resistance in a routine laboratory setting.**

*Bacteroides fragilis* is a strictly anaerobic Gram-negative bacillus present in the human gut. It is recovered from various infection sites and frequently associated with abscess formation and sepsis. Carbapenem resistance in *B. fragilis* is emerging and associated with *cfiA*-encoded class B metallo-beta-lactamase, which is activated by insertion sequence (IS) elements. However, elevated MICs or resistance was also observed in strains that did not have activating IS elements in the upstream region of *cfiA* (17). In the 2003-2005 Belgian multicenter survey (18), the prevalence of the *cfiA* resistance gene was 7.4% (10/135) (19), which is high compared with the prevalence of *cfiA* positivity (2 to 7%) in other countries (5, 6, 13, 15, 20). In the survey, 4% (6/135) of *B. fragilis* isolates were intermediate or resistant to meropenem according to CLSI breakpoints (18).

By using various molecular typing methods, such as arbitrarily primed PCR, ribotyping, multilocus enzyme electrophoresis, and sequencing of *recA* and *glnA* genes, *cfiA*-negative and *cfiA*-positive strains were shown to belong to two genotypically distinct groups (9, 10, 13). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has the capacity to discriminate closely related species based on the spectrum of constantly expressed highly abundant proteins, such as ribosomal proteins. This technique was recently introduced as a rapid and accurate method for the identification of bacteria (2, 7, 14) and can also be applied in the identification of *Bacteroides* isolates (12). The aim of the present study was to examine the discriminatory power of MALDI-TOF MS to differentiate *cfiA*-positive from *cfiA*-negative *B. fragilis* strains in order to predict carbapenem susceptibility.

The 135 *B. fragilis* clinical isolates from the survey mentioned above (18, 19), originating from nine Belgian hospitals, as well as 113 *B. fragilis* isolates from routine cultures collected at our laboratory since 2005 were analyzed. Identification was performed by appropriate biochemical and enzymatic tests (11) and confirmed by MALDI-TOF MS. Meropenem susceptibility was determined by Etest methodology (bioMérieux, Marcy l'Etoile, France). The CLSI breakpoints for susceptible and resistant strains are ≤4 mg/liter and ≥16 mg/liter, respectively (4). PCR analysis was performed to detect the presence of the *cfiA* gene. The annealing temperature of the *cfiA* gene detection method described by Sóki et al. (16) was increased to 62°C to avoid aspecific reactions. In *cfiA*-positive strains, the *cfiA* promoter region was amplified. A PCR product of more than 0.4 kb was considered indicative of the presence of an IS element and was identified by sequencing (3, 20).

Susceptibility to meropenem and genotypic characteristics of the studied isolates are represented in Table 1. Out of 248 *B. fragilis* isolates included in the study, 214 were susceptible, 10 were intermediate, and 24 were resistant to meropenem. Although previous studies reported that the *cfiA* gene is not always activated, our 41 *cfiA*-positive isolates had high MICs of meropenem, ranging from 2 to >32 mg/liter ($MIC_{90}$, ≥32 mg/liter), while the MICs for *cfiA*-negative isolates ranged from 0.064 to 4 mg/liter ($MIC_{90}$, 0.5 mg/liter). Using CLSI breakpoints, only 7 of 41 *cfiA*-positive isolates were classified as susceptible, while all *cfiA*-negative isolates were susceptible. IS elements were detected only in 5 isolates, all with a MIC of ≥32 mg/liter, while 28 of 34 meropenem-intermediate or -resistant isolates lacked IS elements in the *cfiA* promoter region. In another study, this was the case for 19 of 25 isolates, and alternative mechanisms of activation, such as *cfiA* gene activation by its own promoter, were suggested (17).

For MALDI-TOF MS analysis, all strains were cultured on fastidious anaerobic agar (Lab M, Bury, United Kingdom) at 35°C in an anaerobic chamber for 24 to 48 h. As a first step, four *cfiA*-positive (isolates 1 to 4) and four *cfiA*-negative (iso-

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TABLE 1. Meropenem susceptibility and genotypic characteristics of the 248 isolates studied[a]

| MIC (mg/liter) | No. of isolates that were *cfiA* PCR | | Length of *cfiA* promoter (kb) | Insertion sequence identification |
|---|---|---|---|---|
| | Negative ($n$ = 207) | Positive ($n$ = 41) | | |
| ≤0.125 | 146 | 0 | ND | |
| 0.25 | 30 | 0 | ND | |
| 0.5 | 21 | 0 | ND | |
| 1 | 3 | 0 | ND | |
| 2 | 4 | 2 | For *cfiA*-negative isolates, ND; for *cfiA*-positive isolates, 0.4 kb | |
| 4 | 3 | 5 | For *cfiA*-negative isolates, ND; for *cfiA*-positive isolates, 0.4 kb | |
| 8 | 0 | 10 | 0.4 kb in all isolates | |
| 16 | 0 | 9 | 0.4 kb in all isolates | |
| ≥32 | 0 | 15 | 0.4 kb in 9 isolates | |
| | | | 1.5-2 kb in 5 isolates | IS*612B* like (1 isolate) |
| | | | | IS*1187* like (2 isolates) |
| | | | | IS*1169* like (2 isolates) |
| | | | ND (we failed to amplify a *cfiA* promoter of 1 isolate) | ID |

[a] MICs of *cfiA*-negative isolates ranged from 0.064 to 4 mg/liter ($MIC_{90}$, 0.5 mg/liter), and MICs of *cfiA*-positive isolates ranged from 2 to ≥32 mg/liter ($MIC_{90}$, ≥32 mg/liter). The expected length of a *cfiA* promoter amplification product is 0.4 kb; longer fragments indicate the presence of an insertion element. ND, not determined; ID, indeterminate.

lates 5 to 8) isolates were spotted on the target plate after ethanol-formic acid extraction (7) to obtain high-quality spectra. All spots were overlaid with 1 μl alfa-cyano-4-hydroxycinnamic acid matrix in 50% acetonitrile and 2.5% trifluoroacetic acid. Spectra were obtained with a Microflex LT mass spectrometer in the linear positive mode within a mass range of 2,000 to 20,000 Da and analyzed with MALDI Biotyper 2.0 software and reference library 3.1.1.0 (Bruker Daltonik GmbH, Bremen, Germany). Spectra were internally calibrated each week and controlled every day using *Escherichia coli* ribosomal proteins (bacterial test standard; Bruker Daltonik GmbH). On each plate, a blank spot overlaid with 1 μl matrix was used as a negative control. Visual inspection of the mass spectra revealed about 10 peak differences between the spectra of the two groups. Since determining qualitative differences between spectra is subject to personal bias, the relatedness between spectra was determined using the composite correlation index (CCI) tool of MALDI Biotyper. It is a modification of the mathematical algorithm to compare and distinguish MALDI mass spectra of whole bacterial cells described by Arnold and Reilly (1). CCI values around 1 represent a high relationship between spectra. A CCI matrix was created using 48 raw spectra of isolates 1 to 8 (Fig. 1). Means of CCI values between *cfiA*-positive (1 to 4) and *cfiA*-negative (5 to 8) isolates were 0.98 and 0.93, respectively, and were higher than the mean (0.63) of CCI values if *cfiA*-positive isolates were compared with *cfiA*-negative isolates (unpaired $t$ test; $P < 0.001$) (MedCalc software, version 11.4.4.0; MedCalc Software bvba, Mariakerke, Belgium).

After these preliminary tests, main spectra (MSP) containing information on average peak masses, average peak intensities, and peak frequencies were created for one *cfiA*-negative (A1) and one *cfiA*-positive (05/0113) isolate by processing at least 25 mass spectra after ethanol-formic acid extraction with MALDI Biotyper according to the manufacturer's instructions. All isolates were analyzed by the direct-transfer method routinely used in our laboratory. A small amount of a single colony was smeared directly on the target plate in a thin film using an inoculation needle, and spots were overlaid with 1 μl matrix (7). They were identified as *B. fragilis* by MALDI-TOF MS using the Bruker reference spectra database, with a log(score) of ≥2. The spectra of all isolates were matched with the CCI matrix. These results classified unequivocally all strains in the expected group. In the dendrogram (Fig. 2), the spectra of these isolates clustered in *cfiA*-positive and *cfiA*-negative groups without overlap. The mean log(score) for *cfiA*-positive isolates against *cfiA*-positive MSP (05/0113) was 2.33 and

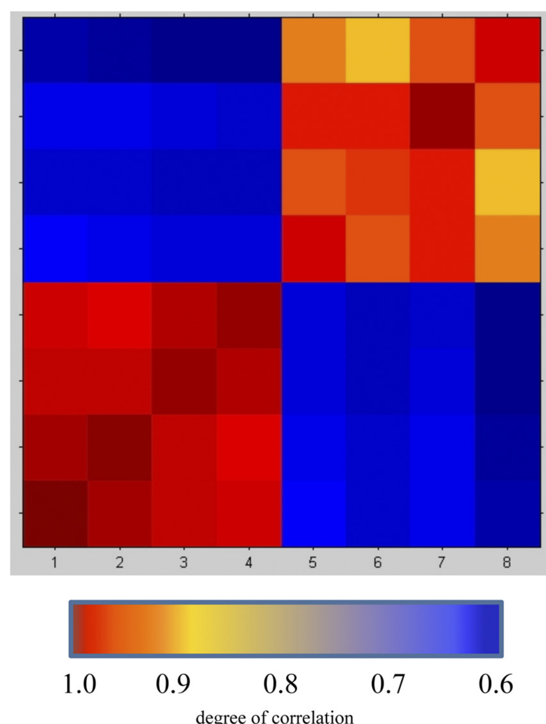


FIG. 1. The composite correlation index (CCI) values between *cfiA*-positive (1 to 4) and *cfiA*-negative (5 to 8) isolates were higher than CCI values if *cfiA*-positive isolates were compared with *cfiA*-negative isolates.

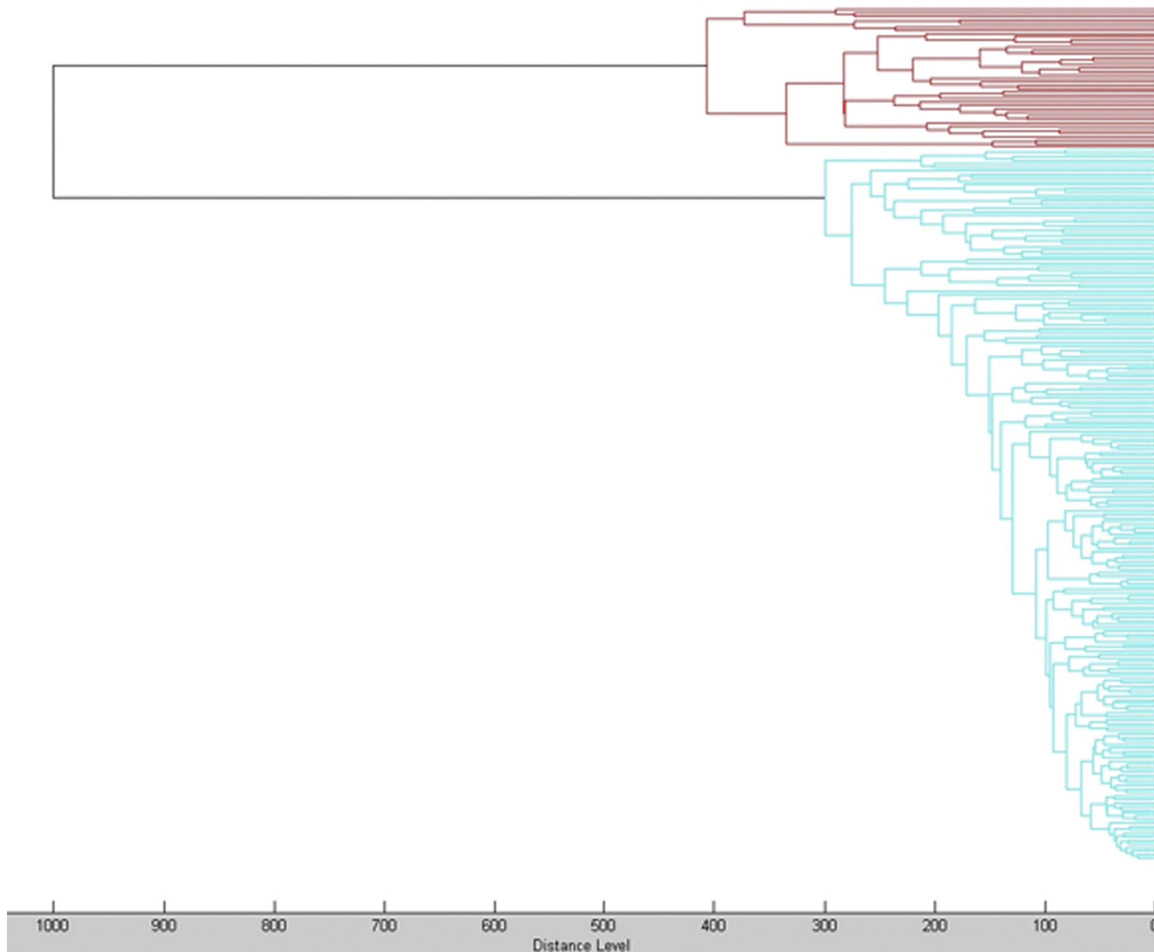


FIG. 2. Dendrogram of all tested isolates. *cfiA*-positive *B. fragilis* isolates are represented in red, and *cfiA*-negative isolates are in blue.

against *cfiA*-negative MSP (A1) was 1.76, with a mean difference of 0.56. Inversely, the mean log(score) for *cfiA*-negative isolates against *cfiA*-positive MSP (05/0113) was 1.81 and against *cfiA*-negative MSP (A1) was 2.45, with a mean difference of 0.63.

Our data suggest that it is possible to differentiate *cfiA*-positive from *cfiA*-negative isolates in a routine laboratory setting and so pinpoint *B. fragilis* strains potentially resistant to carbapenems. This discrimination is not based on the absence or occurrence of one specific peak. The protein profiles of these two genotypically distinct groups differ at approximately 10 peaks. Since the two separate genetic divisions of *B. fragilis* are not clustered geographically (13), the rapid detection of carbapenem resistance can probably be applied universally.

As the *cfiA* gene is not always activated, the positive predictive value for the presence of this gene in the isolates of the Belgian 2003-2005 multicenter survey for the detection of meropenem-intermediate or -resistant isolates was 60% and the negative predictive value was 100% according to CLSI breakpoints. EUCAST breakpoints differed only in that the lower breakpoint was 1 dilution lower (≤2 mg/liter) than the CLSI breakpoint (8). Using EUCAST breakpoints, positive and negative predictive values for the presence of the *cfiA* gene are 90 and 99.2%, respectively, for this collection of isolates.

Although the possibility of obtaining false positives exists because of the silent presence of the *cfiA* gene, it would be interesting to add *cfiA*-positive isolates to the MALDI-TOF MS databases used for bacterial identification and use them as surrogate markers for the detection of carbapenem resistance. Because MALDI-TOF MS analysis can be performed from the primary plate and is available in less than 10 min, this information can be used to guide empirical treatment of *B. fragilis* infections, as susceptibility testing takes another 48 h.

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