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# Increased Sporulation Rate of Epidemic *Clostridium difficile* Type 027/NAP1▽†

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***Clostridium difficile* PCR ribotype 027 comprised 0.2% of a collection of Swedish isolates in 1997–2001 (3 of 1,325 isolates). These isolates had lower moxifloxacin MICs than the epidemic type 027 isolates, but they had the same *tcdC* sequence and toxin yield. Type 027 produced 3- to 13-fold more toxin than did major Swedish types. One epidemic strain (027/NAP1a) sporulated more than did other type 027 isolates, a feature that should contribute to its survival and spread.**

A strain of *Clostridium difficile* (PCR ribotype 027 and pulsed-field gel electrophoresis type NAP1 [027-NAP1]) producing high levels of toxin has in recent years been associated with increased infection rates, outbreaks, and severe disease (10, 12, 15, 16, 17, 18, 19, 22, 25, 26, 31, 34). Its high toxin yield may, in part, be caused by a frameshift mutation in *tcdC* (5), encoding a negative regulator of TcdR and part of the *C. difficile* pathogenicity locus (20, 21). Type 027 has been associated with the use of fluoroquinolones, especially moxifloxacin and gatifloxacin (3, 4, 8, 27, 32), that promote *C. difficile* growth and toxin production in an animal model (1). The role of these factors for the worldwide expansion of type 027/NAP1 is obscure. We compared historical and epidemic isolates of PCR ribotype 027 with respect to antibiotic susceptibility, *tcdC* sequence, toxin yield, S-layer, and sporulation.

*C. difficile* isolates were obtained from the following: a collection of 1,325 strains isolated in 1997–2001 in central and south Sweden (23, 29, 30, 35, 36, 37); the recent U.S.-Canadian epidemic (US1067 and US1165, representing PCR ribotype 027/pulsed-field gel electrophoresis types NAP1a and NAP1b, respectively); and the Culture Collection, University of Göteborg (CCUG), Göteborg, Sweden (CCUG 19125 [VPI 10463], CCUG 37783, and CCUG 20309 [8864]). For PCR ribotyping, see reference 30. Antibiotic susceptibility was determined by Etest (AB Biodisk, Solna, Sweden) using IsoSensitest agar (Oxoid Ltd, Basingstoke, United Kingdom) supplemented with 5% defibrinated horse blood and 20 μg/ml of β-NAD (Swedish Reference Group for Antibiotics [www.srga.org]). The isolation of bacterial DNA, PCR, and sequencing were performed as described previously (24) by using primers for *tcdC* (28). For growth experiments, overnight cultures were diluted $10^6$-fold into triplicate tubes containing peptone-yeast without cysteine or glucose that were further grown for 48 h (13, 14). Sampling, separation of intra- and extracellular fractions, sonication, and toxin measurements (by enzyme immunoassay) were performed as described previously (13, 14). Vegetative and sporulated cells were scored by microscopy by using a Bürker chamber; 10 squares containing 5 to 15 cells were counted per

TABLE 1. Clinical data for Swedish patients infected with *C. difficile* PCR ribotype 027

| Strain | Year | Age (yrs) | No. of stools per day | Temp (°C) | No. of leukocytes ($1{,}000/mm^3$) | Underlying disease[a] | Antibiotic treatment[b] |
|---|---|---|---|---|---|---|---|
| T-378 | 1997 | 53 | >10 | >39 | 17.9 | None | Penicillin V |
| Ö99-1670 | 1999 | 85 | 5–10 | 38 | 31.9 | Lung cancer | Amoxicillin |
| A177:1 | 2001 | 92 | 5–10 | >38 | 31.6 | None | Piperacillin-tazobactam metronidazole/ciprofloxacin |

[a] Indicates severe underlying disease. The patient infected with T-378 recovered, while the others died within 3 months after CDAD diagnosis.
[b] Used within the 2 months prior to CDAD onset; piperacillin-tazobactam treatment was followed directly by oral metronidazole/ciprofloxacin. After CDAD diagnosis, all patients were treated with metronidazole.

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TABLE 2. MICs (μg/ml) of antibiotics for *C. difficile* isolates by using Etest

| Antibiotic | MIC for strain | | | | | |
|---|---|---|---|---|---|---|
| | T-378[a] | Ö99-1670[a] | A177:1[a] | US1067[b] | US1165[b] | 8864[c] |
| Ciprofloxacin | >32 | >32 | >32 | >32 | >32 | >32 |
| Levofloxacin | >32 | >32 | >32 | >32 | >32 | >32 |
| Moxifloxacin | 0.5 | 2 | 1.0 | >32 | >32 | 1.0 |
| Clindamycin | 4 | 8 | 4 | 4 | 2 | 4 |
| Metronidazole[d] | 0.053 (3) | 0.125 (3) | 0.084 (3) | 0.33 (3) | 0.25 (3) | 0.19 (2) |
| Vancomycin | 0.5 | 0.25 | 0.5 | 0.5 | 0.5 | 1.0 |

[a] Historical Swedish PCR ribotype 027 isolates.
[b] Recent epidemic PCR ribotype 027–pulsed-field gel electrophoresis type NAP1 isolates.
[c] Reference strain (toxin $A^-$ $B^+$) obtained from CCUG, Göteborg, Sweden.
[d] Averages of independent experiments (the number of experiments is indicated in parentheses). Means were 0.088 and 0.31 μg/ml for the historical and epidemic isolates, respectively ($P = 0.015$ [$t$ test using unequal variance]).

isolate, and the values were averaged. Two-dimensional gel electrophoresis was performed as described in reference 13; duplicate 24-h intracellular protein samples of US1067, US1165, and T-378 and a single sample of Ö99-1670 were focused on 180-mm linear immobilized pH gradient strips (pH 4 to 7; Amersham Biosciences, Uppsala, Sweden). For identification, protein spots were excised from Coomassie-stained gels and processed using the Montage in-gel digestion kit (Millipore, Billerica, MA), together with a vacuum manifold unit. Two microliters of eluted peptides was loaded on an anchor chip plate (Bruker Daltonics, Inc., Bremen, Germany) and covered by 1 μl of α-cyano-4-hydoxycinnamic acid. Peptide mass mapping of tryptic peptides was performed by a Bruker Daltonics Reflex IV matrix-assisted laser desorption ionization–time of flight apparatus equipped with a nitrogen laser (337.1 nm) and operated in a reflective positive mode. Spectrum calibration was performed by internal use of trypsin fragments of 842.510, 1,045.564, and 2,211.105 Da and the external use of a 1,000- to 4,000-Da peptide calibration standard (Bruker Daltonics, Inc.). Identified peptide masses were analyzed in the Mascot search engine at www.matrixscience.com.

By using two *C. difficile* PCR ribotype 027 reference strains, three type 027 isolates were identified in the Swedish national database; these isolates represented 0.2% of strains collected in 1997-2001. (PCR ribotype 027 corresponded to type SE10 according to the Swedish nomenclature.) The type 027-infected patients had developed moderate to severe *Clostridium difficile*-associated diarrhea (CDAD) (Table 1), and like other historical 027 isolates (22), the Swedish ones had lower MICs for moxifloxacin compared to the MICs for the recent epidemic type 027/NAP1 (Table 2). In addition, the MICs for metronidazole were about threefold lower in the Swedish isolates. The *tcdC* sequence was identical in the type 027 isolates Ö99-1670, US1067, and US1165, including the characteristic 18-bp deletion and the frameshift mutation at position 117, while isolates representing the major Swedish types SE20 and SE30 had a wild-type *tcdC* allele (see Fig. S1 in the supplemental material). The toxin yield was similar in historical and epidemic type 027 isolates but 3- to 13-fold higher than the yield in isolates representing the common types SE20, SE30, and SE21 (Fig. 1).

Three of the type 027 isolates had sporulation frequencies of about 25% at 48 h (Fig. 2). T-378 was morphologically different from the other 027 isolates, showing elongated doublet cells usually containing a single spore (not shown). Thus, the actual sporulation frequency per unit cell was about twofold lower than the scored one, i.e., closer to 25% than to 45% (Fig. 2). Strain US1067 (NAP1a) sporulated more (60%; $P < 0.001$) (Fig. 2) and had a smaller cell and colony size compared to the case for the other type 027 isolates. Excluding the low-sporulating strains VPI 10463 and 8864, US1067 also had a 20% higher optical density at 24 h than those of all other isolates ($P < 0.001$) (data not shown), i.e., consistent with characteristics of the epidemic 027 type (34). Despite the differences in

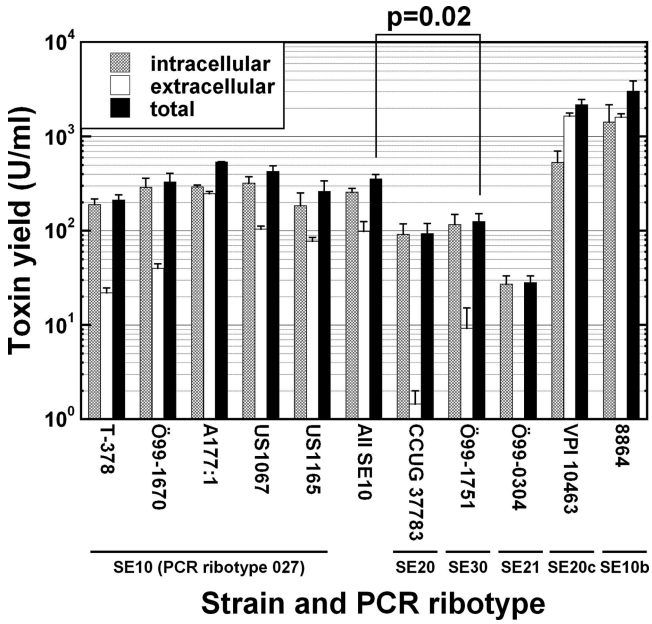


FIG. 1. Toxin yields of 24-h *C. difficile* cultures. Isolates T-378, Ö99-1670, A177:1, US1067, and US1165 are type SE10, i.e., PCR ribotype 027. Isolates representing major Swedish PCR ribotypes, CCUG 37783 (SE20, i.e., PCR ribotype 001), Ö99-1751 (SE30), Ö99-0304 (SE21), and two high-level toxin producing reference strains, VPI 10463 (SE20c) and 8864 (SE10b), were included for comparison. Values are averages of duplicate cultures, and bars indicate standard errors. Statistics were calculated using logarithmic average values of total toxin for "All SE10," CCUG 37783, Ö99-1751, Ö99-0304, VPI 10463, and 8864 by using analysis of variance and Bonferroni post hoc compensation for multiple comparisons (the $P$ value for the comparison of "All SE10" and strain Ö99-1751 is shown).

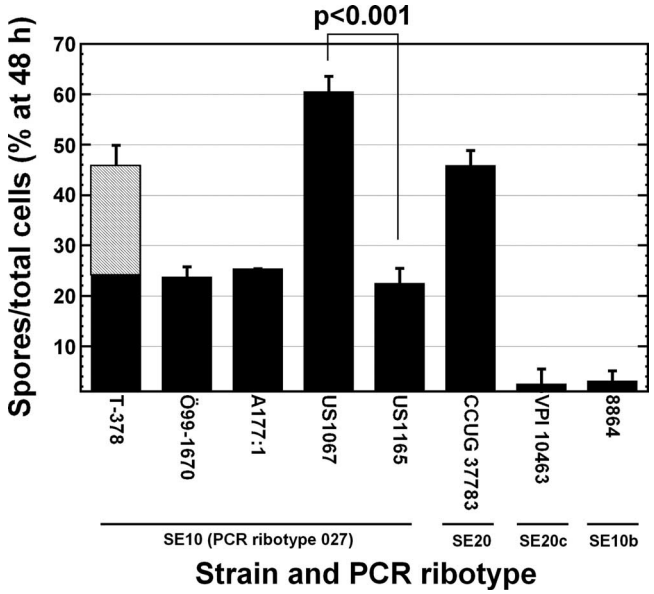


FIG. 2. Sporulation frequencies of *C. difficile* cultures. For further explanation of strains, see the legend for Fig. 1. Bars indicate standard errors. Statistics were calculated by using analysis of variance and Bonferroni post hoc compensation for multiple comparisons. The *P* value for the comparison of US1067 and US1165 is indicated. As T-378 formed doublet cells, the total frequency scored (filled and hatched areas) and an estimate of the true sporulation frequency per unit cell (filled area) are shown. For details, see the text.

sporulation frequency and morphology, the expressed protein patterns of US1067, US1165, and Ö99-1670 were similar (see Fig S2 in the supplemental material). However, T-378 expressed an additional S-layer (see Fig. S2 and Table S1 in the supplemental material), possibly causing its unique morphology.

The "hypervirulence" of *C. difficile* 027/NAP1 (16, 25, 26) has been ascribed to its higher (about 20-fold) toxin yield in vitro compared to those of toxinotype 0 strains (34), caused by loss of TcdC function (5, 21). However, the moderate threefold-higher toxin yield of type 027 (defective *tcdC*) compared to those of types SE30/SE20 (wild-type *tcdC*) indicates that other factors may also affect toxin production. For example, the nutritional sensor CodY may further affect the range of toxin levels (6). The fact that strains VPI 10463 and 8864 yielded few spores but superior amounts of toxin during stationary phase was in accord with the inverse relation between spore and toxin yield generally found among *C. difficile* isolates (2). No clinical isolate of the same PCR ribotype as that of strain 8864 and only two isolates sharing type and characteristics with VPI 10463 were found in our national database, suggesting that high-level toxin producers with low sporulation capacities have poor transmission rates. Although antibiotics that promote growth and toxin production by resistant *C. difficile* in vivo are major risk factors for developing CDAD (1, 7, 9, 11, 33), our data showed that certain PCR ribotype 027 strains have different morphologies and growth characteristics as well as high capacities for both toxin and spore production, features that may contribute to disease severity, therapy failure, relapse, and spread.

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