Aposymbiosis of a Burkholderiaceae-Related Endobacterium Impacts on Sexual Reproduction of Its Fungal Host

YUSUKE TAKASHIMA1,2, YOUSUKE DEGAWA3, TOMOYASU NISHIZAWA1,2, HIROYUKI OHTA1,2, and KAZUHIKO NARISAWA1,2*

1 United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, 3–5–8 Saiwai-cho, Fuchu-shi, Tokyo 183–8509, Japan; 2Haraki University College of Agriculture, 3–21–1 Chuo, Ami-machi, Ibaraki, 300–0393, Japan; and
3Sugadaira Research Station Mountain Science Center, University of Tsukuba, 1278–294, Sugadaira, Nagano 386–2204, Japan

(Received December 18, 2019—Accepted March 12, 2020—Published online April 15, 2020)

Bacterial endosymbionts inhabit diverse fungal lineages. Although the number of studies on bacteria is increasing, the mechanisms by which bacteria affect their fungal hosts remain unclear. We herein examined the homothallic isolate, Mortierella sugadairana YTM39, harboring a Burkholderiaceae-related endobacterium, which did not produce sexual spores. We successfully eliminated the bacterium from fungal isolates using ciprofloxacin treatment and asexual spore isolation for germinated asexual spores. Sexual spore formation by the fungus was restored by eliminating the bacterium from isolates. These results indicate that sexual reproduction by the fungus was inhibited by the bacterium. This is the first study on the sexual spore infertility of fungal hosts by endofungal bacteria.

Key words: Burkholderiaceae-related endobacteria, homothallism, Mortierella, zygospore

Recent studies using molecular techniques revealed that bacterial endosymbionts inhabit early diverging fungal lineages, such as Mucoromycota (Spatafora et al., 2016; Bonfante and Desirò, 2017). These bacterial endosymbionts were known as endofungal or endohyphal bacteria and mostly related to the family Burkholderiaceae, and, thus, were termed Burkholderia-related endobacteria (Bonfante and Desirò, 2017) or Burkholderiaceae-related endobacteria (BRE) (Takashima et al., 2018b). BRE were generally found in hyphae and asexual spores (Lumini et al., 2007; Sato et al., 2010; Ohshima et al., 2016; Mondo et al., 2017; Takashima et al., 2018a) as well as in sexual spores (Mondo et al., 2017). The host nutrient dependence of BRE due to genome reductions was suggested by genomic studies (Ghignone et al., 2012; Fujimura et al., 2014; Uehling et al., 2017; Sharmin et al., 2018). However, limited information is currently available on the effects of BRE on their fungal hosts.

The elimination of BRE from their fungal hosts previously revealed several effects on their hosts. BRE were found to alter gene expression (Lastovetsky et al., 2016; Salvioli et al., 2016; Uehling et al., 2017), metabolism (Salvioli, et al., 2010; Lastovetsky et al., 2016; Li et al., 2017), oxidative stress responses (Salvioli et al., 2016; Vannini et al., 2016; Venice et al., 2017), and chemotaxis (Lumini et al., 2007) in hosts. In contrast, only a few studies described the morphological alternations induced by BRE. The wall thickness of the chlamydospores of Gigaspora margarita was greater (Lumini et al., 2007), the sporangiospores of Rhizopus microsporus were not produced (Partida-Martinez et al., 2007; Lackner et al., 2011), and the aerial mycelia of Mortierella elongata were well developed (Uehling et al., 2017) in the absence of each BRE. In zygospore formation by R. microsporus, the number of zygospores produced in mating was lower for Burkholderia-free strains than for Burkholderia-harboring strains (Mondo et al., 2017). BRE are currently considered to be beneficial and non-harmful endosymbionts for their hosts.

Mortierella spp. produce sporangiospores and zygospores as asexual and sexual spores, respectively, in their life cycle for dispersal, dormancy, and recombination at the sexual stage. We recently obtained three fungal isolates, YTM39, YTM128, and SUT-174, from cool regions in Hokkaido and Nagano, Japan (Table 1). These isolates were described as Mortierella sugadairana, which form zygospores in a homothallic manner and are related to the first heterothallic species described in the genus (Takashima et al., 2018a). Among them, one isolate, M. sugadairana YTM39, did not form zygospores under zygospore-inducing conditions, whereas the other two did. The isolate, M. sugadairana YTM39, was different from the other two isolates because it harbored a BRE, which was phylogenetically clustered within MorBRE group C assigned by Takashima et al. (2018b). Based on the differences observed in zygospore formation by the isolate, M. sugadairana YTM39 and the two other isolates as well as the presence of the bacterium, we hypothesized that the bacterium affects zygospore formation by this isolate. In order to prove this hypothesis, we attempted to eliminate the bacterium from M. sugadairana YTM39 and observed zygospore formation by BRE-harboring and BRE-free clonal lines obtained from the original line. The results obtained showed that zygospore formation was restored in the BRE-free clonal lines of the isolate. The present study is the first to show sexual spore infertility by a fungal host in the presence of BRE.

* Corresponding author. E-mail: kazuhiko.narisawa.kkm@vc.ibaraki.ac.jp; Tel: +81–29–888–8667; Fax: +81–29–888–8667.
Materials and Methods

Elimination of BRE from the fungal host

In order to eliminate BRE from the fungal host, we employed antibiotic treatments, such as ciprofloxacin, which was previously shown to be effective against BRE associated with R. microsporum (Partida-Martínez et al., 2007). To assess the ciprofloxacin treatment, sporangiospores of the original isolate M. sugadairana YTM39 (wild-type; BRE-harboring [B+]) and two clonal lines (clonal line names including “s” and “mc” hereafter indicate clonal lines obtained by single-sporangiospore isolation and the ciprofloxacin treatment, respectively) generated by the following single-sporangiospore isolation, M. sugadairana YTM39s3 (B+) and YTM39s4 (B+), were used. Small agar pieces containing several germinated sporangiospores were added to separate 1.5-mL tubes for each clonal line. Four, ten, and ten small agar pieces were obtained, respectively. After every single-sporangiospore isolation were used and ten, ten, seven, and sixteen clonal lines were obtained, respectively. Four, ten, and ten small agar pieces were added to separate 1.5-mL tubes for each clonal line. Four, ten, and ten small agar pieces were obtained, respectively. After every single-sporangiospore isolation were used and ten, ten, seven, and sixteen clonal lines were obtained, respectively. After every single-sporangiospore isolation were used and ten, ten, seven, and sixteen clonal lines were obtained, respectively. After every single-sporangiospore isolation were used and ten, ten, seven, and sixteen clonal lines were obtained, respectively. After every single-sporangiospore isolation were used and ten, ten, seven, and sixteen clonal lines were obtained, respectively. After every single-sporangiospore isolation were used and ten, ten, seven, and sixteen clonal lines were obtained, respectively.

Single-sporangiospore isolation

Although ciprofloxacin is an antibacterial agent, a previous study reported that it functioning as an abiotic stress factor in some fungi (Yuan et al., 2011). To avoid placing any abiotic stress on the fungal host, in single-sporangiospore isolation, sporangiospores formed by the representative clonal lines, M. sugadairana YTM39s3 (B+) and YTM39s4 (B+), were used. Sporangiospores of each clonal line immobilized by 2% low melting point agarose were obtained, respectively. To eliminate endofungal bacteria, 200 µL of 50 µg mL⁻¹ ciprofloxacin hydrochloride (Wako Pure Chemical) solution was added to the tubes, which were then incubated at room temperature (ca. 23°C) for 24 h. After the incubation, agar pieces were disrupted in the solution by pipetting, and all of the solution was inoculated onto fresh LCA medium, and incubated at 18°C in the dark for three weeks, which are the conditions used in the previous study (Takashima et al., 2018a).

Diagnostic PCR

The presence of BRE in each clonal line was checked by diagnostic PCR. Template DNA was extracted from the six- to twelve-day-old mycelia of each clonal line incubated on a sterilized cellophane sheet placed on 1/2 CMMY agar (8.5 g corn meal agar [Difco], 10 g malt extract [Difco], 1 g yeast extract [Difco], and 7.5 g Bacto agar [Difco] in 1 L distilled water) using Prepman™ Ultra sample reagent (Applied Biosystems) in accordance with Sato et al. (2010). Diagnostic PCR was performed using the bacterial universal primers 10F and 1541R in accordance with Takushima et al. (2018b). After PCR, amplification of the 16S rRNA gene was checked by agarose gel electrophoresis.

Fluorescence in situ hybridization (FISH)

In order to confirm the elimination of the bacterium by single-sporangiospore isolation, sporangiospores formed by the representative clonal lines, M. sugadairana YTM39s3 (B+) and YTM39s14 (BRE-free [B–]), generated by single-sporangiospore isolation were subjected to FISH. Young germlings of the sporangiospores of each clonal line immobilized by 2% low melting point agar were transferred to a 1.5-mL tube. FISH was performed using agar pieces containing sporangiospores in accordance with Takushima et al. (2018b).

Mycelial growth of BRE-harboring and BRE-free clonal lines

To examine the effects of the presence/absence of BRE and the ciprofloxacin treatment on mycelial growth by the fungal host, mycelial growth by representative BRE-harboring and BRE-free clonal lines of the fungus was investigated in accordance with Takushima et al. (2018a). Differences among the means of mycelial growth (mm d⁻¹) of each clonal line were statistically evaluated with Tukey’s honestly significant difference (HSD) test (Significance level defined as P<0.01) using the R package “multcomp” in R version 3.3.1 (https://www.r-project.org/).

Zygospore induction

To induce zygospores, six and sixteen clonal lines of M. sugadairana YTM39 obtained by the ciprofloxacin treatment and single-sporangiospore isolation, respectively, were used (Table 2). Among them, 10 out of the 16 clonal lines generated by single-sporangiospore isolation harbored BRE (B+) and the other twelve clonal lines were BRE-free (B–) (Table 2). Regarding the zygospore induction of isolates of M. sugadairana YTM128 and SUT-174, single-sporangiospore isolation was also conducted in advance. These clonal lines of M. sugadairana YTM39, YTM128, and SUT-174 were incubated on lCA medium at 18°C for five to seven days prior to zygospore induction. Three inoculation discs were cut from incubated mycelia using an autoclave-sterilized plastic straw (8 mm in diameter) instead of a cork borer, and were then placed onto lCA medium and hemp seed agar (HSA) (100 mL of hemp seed extract prepared by autoclaving 10 g of hemp seeds in 100 mL of distilled water and 15 g Bacto agar [Difco] in 1 L of distilled water), as shown in Fig. 1, with duplicates for each medium, and incubated at 18°C in the dark for three weeks, which are the conditions used in the previous study (Takashima et al., 2018a).

Table 1. Isolates of Mortierella sugadairana used in the present study

| Isolate name | Culture collection No. | Endofungal bacterium | Collection site | Isolation source |
|--------------|------------------------|----------------------|----------------|------------------|
| YTM39        | NBRC 112366            | +                    | Rakuno Gakuen University, Ebetsu-shi, Hokkaido, Japan | Root, tomato seedling |
| YTM128       | NBRC 112976            | -                    | Midorigaoka Park, Obihiro-shi, Hokkaido, Japan | Soil under Abies veitchii |
| SUT-174      | NBRC 104553            | -                    | Mountain Science Center Sugadaira Research Station, University of Tsukuba, Ueda-shi, Nagano, Japan | Decayed twig, Fagus crenata |
Zygospore Infertility by an Endobacterium

Table 2. Zygospore induction of clonal lines originating from Mortierella sugadairana YTM39, YTM128, and SUT-174 obtained by single-sporangiospore isolation and the ciprofloxacin treatment.

| Name of clonal linesa | PCR amplificationb | Homothallic zygospore formationc |
|-----------------------|--------------------|--------------------------------|
| YTM39s3               | P                  | –                              |
| YTM39s4               | P                  | –                              |
| YTM39s7               | P                  | –                              |
| YTM39s11              | P                  | –                              |
| YTM39s12              | P                  | trace                         |
| YTM39s13              | P                  | –                              |
| YTM39s3_s1            | P                  | –                              |
| YTM39s3_s2            | P                  | –                              |
| YTM39s4_s1            | P                  | –                              |
| YTM39s4_s2            | P                  | –                              |
| YTM39s1              | N                  | –                              |
| YTM39s2              | N                  | +                              |
| YTM39s6              | N                  | +                              |
| YTM39s9              | N                  | +                              |
| YTM39s10             | N                  | +                              |
| YTM39s14             | N                  | +                              |
| YTM39mc1             | N                  | +                              |
| YTM39mc2             | N                  | +                              |
| YTM39s3_mc1          | N                  | +                              |
| YTM39s3_mc2          | N                  | +                              |
| YTM39s4_mc1          | N                  | +                              |
| YTM39s4_mc2          | N                  | +                              |
| YTM128s1             | None              | +                              |
| YTM128s2             | None              | +                              |
| SUT-174s1            | None              | +                              |
| SUT-174s2            | None              | +                              |
| SUT-174s3            | None              | +                              |
| SUT-174s4            | None              | +                              |

a: Names including “s” and “mc” indicate clonal lines obtained by single-sporangiospore isolation and the ciprofloxacin treatment, respectively. b: P and N indicate positive and negative PCR amplification in diagnostic PCR, respectively. “None” indicates BRE-free isolates since original isolates were obtained. c: +; zygospore formation in both or either of LCA and HSA media, –; zygospores were not formed in LCA medium. trace; trace zygospore formation was noted in LCA medium.

Results and Discussion

Elimination of BRE from M. sugadairana

Previous studies successfully eliminated BRE from the host using an antibiotic treatment within the host mycelia of R. microsporus (Partida-Martinez et al., 2007) and M. elongata (Uehling et al., 2017). In the present study, we established 24 clonal lines of M. sugadairana YTM39 by the ciprofloxacin treatment and PCR amplification of the 16S rRNA gene was not observed in these clonal lines (Table 3). These results demonstrated that the ciprofloxacin treatment was effective not only for eliminating BRE-harboring isolates of R. microsporus, but also those of M. sugadairana. On the other hand, 97 clonal lines of M. sugadairana YTM39 were established by single-sporangiospore isolation and PCR amplification of the 16S rRNA gene was observed in 83.5% (81 out of 97) of clonal lines (Table 4). Since the production of the sporangiospores of clonal lines was not related to the presence of BRE, the presence and absence of the bacterium were confirmed in the sporangiospores of the BRE-harboring clonal line (Fig. 2A, B, and C) and BRE-free clonal line (Fig. 2D, E, and F), respectively, by FISH. Therefore, we obtained 24 and 16 bacteria-free clonal lines by the ciprofloxacin treatment and single-sporangiospore isolation, respectively (Table 3 and 4). This result indicated that BRE associated with M. sugadairana YTM39 was eliminated from host fungal cells by the successive subculturing of single spores, similar to G. marginata (Bianciotto et al., 2004; Lumini et al., 2007), M. elongata (Sato et al., 2010), and R. microsporus (Mondo et al., 2017). Our mycelial growth assay showed that the presence/absence of BRE and the ciprofloxacin treatment had no harmful effects on mycelial growth by the fungus (Fig. S1). This result ensured that the observation of zygospore formation in the present study was not affected by mycelial growth differences among clonal lines.

The bacterial endosymbiont inhibits the sexual development of its fungal host

In the present study, six and sixteen clonal lines of M. sugadairana YTM39 were used for zygospore induction, which was achieved by the ciprofloxacin treatment and single-sporangiospore isolation, respectively (Table 2). Among them, 10 out of 16 clonal lines obtained by single-sporangiospore isolation harbored BRE (B+) and the other 12 clonal lines were BRE-free (B–) (Table 2). Two isolates of M. sugadairana, YTM128 and SUT-174, were used as representative bacteria-free isolates for zygospore induction. Regarding the results of zygospore induction, fungal colonies with hyphal masses were observed in all clonal lines of M. sugadairana YTM128 and SUT-174, and most of the BRE-free (B–) clonal lines of M. sugadairana YTM39 (Fig. 1A, B, D, and E). Zygospores were always produced in the hyphal masses (Fig. 1A’ and A”; magnified images of hyphal masses in clonal lines of M. sugadairana YTM39s9 [Fig. 1A], and other magnified images of M. sugadairana YTM39s3 mc1 [Fig. 1B] and YTM39s12 [Fig. 1C] were shown in Fig. S2). On the other hand, hyphal masses were not observed in the BRE-harboring clonal lines of M. sugadairana YTM39 (B+) (Fig. 1G and H), except for the clonal line M. sugadairana YTM39s12 (Fig. 1C). Therefore, zygospore formation by the fungus was inhibited by the presence of the bacterium (9 out of 10 clonal lines) (Table 2). These results showed the sexual spore infertility of M. sugadairana YTM39 by the presence of the bacterium. Even though an exceptional case was found in the BRE-harboring clonal line M. sugadairana YTM39s12 showing trace zygospore formation (Fig. 1C), this result is the first to show that BRE inhibited zygospore formation by its fungal host. It currently remains unclear why exceptional cases occurred in the clonal lines M. sugadairana YTM39s1 (B–) and YTM39s12 (B+). However, we presumed that zygospore inhibition was related to a population of the bacterium within the mycelia.

An evolutionary perspective of the symbiotic status of M. sugadairana and BRE

The sexual spore infertility of the fungal host by the presence of BRE in the present study raises a question about the relationship between BRE and their Mucoromycotan hosts.
Mondo et al. (2017) predicted that the current R. microsporus-BRE association originated from an antagonistic relationship. This is because the control of the expression of a ras2-1 gene, encoding a small GTP-protein responsible for reproductive development was hijacked by BRE in R. microsporus (Mondo et al., 2017). If the M. sugadairana-BRE association follows a similar evolutionary trajectory to that of the R. microsporus-BRE association introduced by Mondo et al. (2017), as shown in Supplementary Fig. 4, the M. sugadairana-BRE association may represent an “early symbiotic status” among Mucoromycota-BRE associations. Further comparative transcriptome analyses between BRE-harboring and BRE-free clonal lines of M. sugadairana YTM39 and among different BRE-harboring fungal species are needed to answer this open question.

Acknowledgements

We thank Dr. Y. Sato for providing information on the molecular techniques, Dr. K. Kai for advising on the method of FISH in Mortierella spp., and Dr. E. Usui for providing the isolate, M. sugadairana YTM39. This research was supported by a Grant-in-Aid from the Japan Society for the Promotion of Science (JSPS) Fellows (No. 16J08716), a JSPS Grant-in-Aid for Challenging Exploratory Research (No. 15K14902), and a Grant-in-Aid for Scientific Research (C) (No. 17K07695) from JSPS. This research was also supported by a grant from the Institute for Fermentation, Osaka (IFO).
Table 4. The number of PCR-positive clonal lines of Mortierella sugadairana YTM39 obtained by serial single-sporangiospore isolation and the frequency of vertical transmission by the endofungal bacterium through sporangiospores.

| Origin     | Incubation period for DNA extraction (day) | Series of single-sporangiospore isolation | No. of total clonal lines | No. of PCR-positive clonal lines | Frequency of vertical transmission |
|------------|-------------------------------------------|-------------------------------------------|---------------------------|---------------------------------|-----------------------------------|
| YTM39      | 6, 7, 12*                                 | Wild-type                                 | 12                        | 6                              | 50%                               |
| YTM39s3    | 7                                         | Wild-type                                 | 34                        | 34                             | 100%                              |
| YTM39s4    | 7                                         | 1st                                       | 10                        | 9                              | 90%                               |
| YTM39s3s1  | 8                                         | 2nd                                       | 7                         | 7                              | 100%                              |
| YTM39s3s1s1| 7                                         | 3rd                                       | 8                         | 7                              | 87.5%                             |
| YTM39s3s1s6| 7                                         | 3rd                                       | 8                         | 0                              | 0%                                |
| YTM39s3s1s6| 7                                         | 3rd                                       | 8                         | 8                              | 100%                              |
| **Total**  | **—**                                     | **—**                                     | **97**                    | **81**                         | **83.5%**                         |

*: The presence of BRE in twelve clonal lines was checked by diagnostic PCR three times with different incubation periods and the results obtained were identical.

Fig. 2. Fluorescence and bright field images of sporangiospores of clonal lines of Mortierella sugadairana YTM39 with PCR-positive (B+) and -negative (B−) amplification in diagnostic PCR of the endofungal bacterium. Columns: left; FISH with a Cy3-labeled EUB338 probe (Red), middle; DAPI (blue), right; bright field. Rows: A–C; YTM39s3, D–F; YTM39s14. Rod-shaped endofungal bacterial cells were detected within the sporangiospores of the clonal line YTM39s3 (B+: A, B) by FISH and DAPI staining (arrowheads), and were not detected within YTM39s14 (B−: D, E). Scale bars: 10 μm.

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