J Ind Microbiol Biotechnol (2014) 41:1175–1179
DOI 10.1007/s10295-014-1461-0

SHORT COMMUNICATION

SIMB
Society for Industrial Microbiology and Biotechnology

Efficient strategy for maintaining and enhancing the huperzine A production of *Shiraia* sp. Slf14 through inducer elicitation

Riming Yan · Zhibin Zhang · Ya Wang · Huilin Yang · Qinggui Zeng · Du Zhu

Received: 14 February 2014 / Accepted: 8 May 2014 / Published online: 28 May 2014

Abstract Huperzine A (HupA), a naturally occurring lycopodium alkaloid, is a potent, highly specific and reversible inhibitor of acetylcholinesterase and is a potential treatment for Alzheimer's disease. However, isolating HupA from Huperziaceae plants is inefficient; thus, extracting this compound from endophytic fungi may be more controllable and sustainable. However, the large-scale production of this chemical from endophytes is limited by the innate instability of endophytic fungi. In this study, we maintained the stability and viability of the HupA-producing endophytic fungus *Shiraia* sp. Slf14 and enhanced the HupA titers during fermentation by adding *Huperzia serrata* extracts (HSE), L-lysine, and acetic acid into the culture as inducers. Adding trace amounts of HupA clearly improved the HupA production of *Shiraia* sp. Slf14, reaching a maximum content of approximately 40 μg g^{-1}. Moreover, the addition of HSE and L-lysine promoted HupA production in the flask fermentation. The aforementioned bioprocessing strategy may be potentially applied to other endophytic fungal culture systems for the efficient production of plant secondary metabolites.

R. Yan · Z. Zhang · H. Yang · Q. Zeng · D. Zhu (✉)
Key Laboratory of Protection and Utilization of Subtropic Plant Resources of Jiangxi Province, Jiangxi Normal University, Nanchang 330022, China
e-mail: zhudu12@163.com

R. Yan
Key Laboratory for Research on Active Ingredients in Natural Medicine of Jiangxi Province, Yichun University, Yichun 336000, China

Y. Wang · D. Zhu
Jiangxi Science and Technology Normal University, Nanchang 330013, China

Keywords Huperzine A production · *Shiraia* sp. Slf14 · Maintain stability · Passage · Inducer

Introduction

Huperzine A (HupA) is a pharmaceutical *Lycopodium* alkaloid derived from the Chinese herb *Huperzia serrata* that that has symptomatic efficacy comparable to cholinesterase inhibitors that are currently used to treat Alzheimer's disease (AD). Phase II trials suggest that 400 μg of HupA BID has a demonstrable cognitive effect in patients with mild to moderate AD [18]. In China, HupA has been approved by the China Food and Drug Administration as therapy for benign senescent forgetfulness and AD.

Most HupA currently used in herbal supplements and medicines is extracted from *H. serrata* and other Huperziaceae species. However, the HupA content of *H. serrata* is only approximately 70 μg g^{-1} DCW, making this compound difficult to harvest [27]. Moreover, *H. serrata* is slow growing and requires at least 15 years for spore germination from the gametophyte stage to the mature sporophyte stage, during which plant tissues can be harvested for medicinal use. No applicable commercial cultivation has been reported for *H. serrata* or other Huperziaceae species. Therefore, HupA isolation from Huperziaceae plants is inefficient. Furthermore, wild plant populations are being threatened and may soon become extinct if the current over-harvesting continues [14]. Increasing efforts have been exerted to develop alternative means of HupA production, such as Huperziaceae plant cell culture and complete chemical synthesis [6, 17]. Despite these efforts, *H. serrata* cell culture is rarely successful [13]. Although HupA can be chemically synthesized, the resulting racemic mixture is considerably less potent for AChE inhibition than the natural HupA derived from plant extracts.

Recently, endophytic fungi from plants have been widely accepted as an important source of drugs. Many compounds with new structures and various bioactivities are continually isolated. Many bioactive agents produced by plants could also be produced by endophytic fungi. Some of these agents include taxol [19], vincristine [7], and podophyllotoxin [23]. Therefore, finding a microbial source of HupA has attracted considerable interest in recent years. Several HupA-producing endophytes, such as *Acremonium* sp. 2F09P03B [9], *Blastomyces* sp., and *Botrytis* sp. [5], have been isolated from different Huperziaceae plants. In our previous study, we evaluated *H. serrata* collected from natural populations at the Lushan Botanical Garden (Jiangxi Province, China) and acquired 69 isolates of endophytic fungi from *H. serrata*. Among these collections, several fungi such as *Shiraia* sp. Slf14 [27], *Cladosporium cladosporioides* LF70 [25], and *Aspergillus flavus* LF40 [22] have high acetylcholinesterase (AChE) inhibitory activity and produce HupA.

Endophytic fungi offer a great opportunity to produce bioactive chemicals without limiting plant resources, especially the rare, slow-growing plants. However, many critical obstacles hinder microbial production. The stability of endophytic fungi is currently one of the most formidable challenges in the field [26]. Many laboratories have difficulty with the progressive degeneration of strain vitality and the consistency of its bioactive chemical productivity. The large-scale production of active chemicals from endophytic fungi has rarely been reported. In our previous study, we found that the endophytic fungus *Shiraia* sp. Slf14 produces 142.6 μg g^{-1} DCW [27]. However, the strain degenerates sharply and only produces approximately 30 μg g^{-1} DCW of HupA. In this study, we investigated several factors that possibly promote the HupA production of endophytic fungi during fermentation.

Materials and methods

Materials

All solvents used for spectroscopic analyses were HPLC grade, whereas the remaining chemicals were of analytical grade. All chemicals and culture medium components were obtained from Sinopharm Chemical Reagent Co., Ltd., China. The HupA standard was supplied by the National Institutes for Food and Drug Control of China.

Endophytic fungus/culture microorganism

Shiraia sp. Slf14, which produced the highest amounts of HupA in our initial screening, was used in the experiment. The seeds were stored at −70 °C in 15 % glycerol for long-term storage.

Strain passage in PDA slant

The *Shiraia* sp. Slf14 was cultured on potato dextrose agar (PDA) medium supplemented with 200 μg L^{-1} HupA at 28 °C and then subcultured every 7 days for seven passages. All passaged strains were stored in PDA at 4 °C for subsequent submerged cultivation.

Preparation of *H. serrata* extracts (HSE)

Huperzia serrata Leaves and stems were washed with water, dried overnight at 60 °C, and powdered. Each sample of dried raw material (20.0 g) was extracted with 1.0 L of 60 % ethanol for 3 days at 25 °C. The extract was filtered through four layers of cheesecloth and then concentrated through evaporation under a vacuum at 40 °C to 0.10 g mL^{-1}, which was designated as HSE for subsequent experiments.

Culture conditions

Shiraia sp. Slf14 spores were collected from PDA stock slants with 0.05 M phosphate-buffered saline (PBS) at pH 6.8. The spores were washed, suspended in sterile water, and then used as liquid inoculant. The liquid growth medium for the spores contained 200 g of potatoes (soup extract) and 20 g of glucose in 1,000 mL of deionized water was autoclaved at 121 °C for 20 min. Every 100 mL of culture medium was inoculated with approximately 1×10^6 spores per milliliter in a 500 mL flask and then cultured for 3 days at 28 °C with rotation at 120 rpm. During the fermentation stage, 180 mL of sterilized liquid medium in 500 mL flask was inoculated with 5 mL of the liquid inoculant. The medium was prepared and inoculated as described in 200 g of potato (soup extract) and 10 g of sucrose in 1,000 mL liquid medium. For the elicitation study, different concentrations of HSE, L-lysine, and acetic acid were added into the fermentation culture. Each treatment was fermented for 14 days at 28 °C with rotation at 120 rpm and was prepared in triplicate.

Preparation of fungal extracts

The fermented *Shiraia* sp. Slf14 mycelia were harvested by centrifugation at 12,000×*g* for 10 min and then dried at 45 °C overnight. The dried mycelia were then powdered. Each sample of raw material (1.0 g) was extracted thrice with ethanol (50 mL) for 30 min with ultrasonication in a water bath at 40 °C. The extracts were filtered. The pooled filtrates were evaporated under reduced pressure. Then, the dry residue was dissolved in 50 mL of 2.5 % hydrochloric acid and purified by shaking twice with chloroform and then with ethylic ether. The water phase was rendered

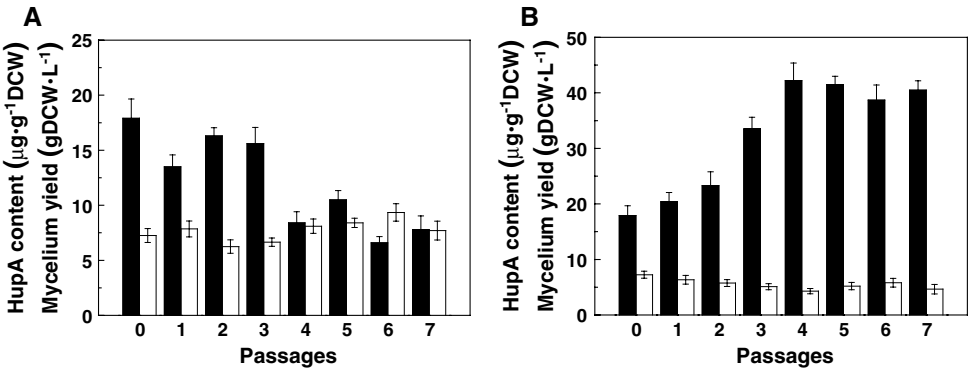

Fig. 1 HupA production (*filled square*) and mycelial yield (*open square*) of *Shiraia* sp. Slf14 in different passages of **a** without and **b** with adding 2.0 mg L^{-1} HupA, respectively

basic with 25 % ammonia solution (pH 9), salted out with sodium chloride, and then exhaustively extracted with chloroform. In the last stage, the combined chloroform extracts were evaporated to dryness. The dry residue was dissolved in 10 mL of methanol (HPLC grade). The methanolic extracts were filtered through a 0.45 μm filter prior to chromatographic separation [21].

HupA quantification by HPLC

HupA content was measured via HPLC using an ODS-C 18 reverse phase column (4 μm, 4.6 × 250 mm) (YMC Inc., Japan). The temperature of the column compartment was maintained at 30 °C. The injection volume was set to 20 mL and the flow rate was 0.5 mL min^{-1} using a methanol–water mobile phase (6:4, v/v). The effluent was monitored at 310 nm. The HupA content was quantified using a standard curve generated from a HupA standard at concentrations ranging from 0.50 to 9.0 mg L^{-1}, with the peak area and the height linearly correlated with absorbance ($r^2 = 0.9967$).

Statistical methods

Each experimental data point was the result of an average of three replicated flasks simultaneously conducted. The standard deviation of the mean is shown as error bars in the figures.

Results and discussion

Attenuation of HupA production in endophytic fungi

The fundamental obstacle in utilizing endophytic fungi is maintaining the vitality and stability of the cells during passages. In our previous study, various endophytic fungi were isolated from *H. serrata* and identified, and their HupA yields were measured. The strain with the highest HupA yield was *Shiraia* sp. Slf14, producing approximately 142.6 μg g^{-1} DCW of mycelia when cultured for 14 days in 100 mL of liquid PDA medium at 28 °C with shaking at 120 rpm. However, the HupA productivity of *Shiraia* sp. Slf14 sharply declined after nearly 3 years of repeated passages, yielding only 17.6 μg g^{-1} DCW of HupA. We examined the attenuation of HupA production through successive serial passages under the culture conditions. The HupA production of *Shiraia* sp. Slf14 significantly decreased from 17.6 to <10 μg g^{-1} DCW after four transfers (Fig. 1a). This result signifies that *Shiraia* sp. Slf14 is unstable and easily attenuated independent of the host plant. Interestingly, the mycelial yield significantly increased with decreasing HupA production during the strain transfers.

Maintaining the stability and viability of HupA-producing endophytic fungi

Maintaining the HupA productivity of seeds during transfer is so crucial that the possible mechanisms of the synthesis of plant metabolites by endophytic fungi need to be elucidated. However, the reasons for such declines remain poorly studied. We hypothesize that the lack of host stimulus in the culture media and silencing of genes in axenic cultures cause the decline in production [20]. Amending axenic cultures with host stimulus may be a practical and feasible way to reverse the decline. Similar to paclitaxel, podophyllotoxin, and camptothecin, HupA is also an important secondary metabolite produced occasionally by endophytic fungi from *H. serrata*. Plant endophytes produce host-specific metabolites as a survival strategy or resistance mechanism to tolerate toxic host metabolites [8]. Thus, we speculated that HupA in host plant is a key host stimulus for HupA production in endophytic fungi.

In the present study, we attempted to improve or maintain the stability and viability of the HupA-producing endophytic fungus by supplementing HupA into subculture generations. *Shiraia* sp. Slf14 supplemented with 200 μg L^{-1} HupA were continually transferred to determine the viability and stability of the fungus. The HupA production of *Shiraia* sp. Slf14 obviously increased from 17.9 to 40 μg g^{-1} DCW after four continuous transfers (Fig. 1b). The results indicated that it successfully increased the HupA yield of *Shiraia* sp. Slf14 by adding trace amounts of HupA into the slant culture. More interestingly, the color of the culture changed from reddish brown to having almost unpigmented after four transfers. We examined the culture and inferred that the fading of pigmentation was caused by a decline in hypocrellin formation (data not shown). Previous studies hypothesized that the initial biosynthesis of fungal perylenequinone pigments and HupA share the polyketide synthase pathway [24].

Activators (inducers) of HupA production

Certain specific compounds in higher plants influence the production of secondary metabolites by fungi. Therefore, we assessed the influence of compounds supplemented into the PDA liquid culture on the HupA yield and mycelial production of *Shiraia* sp. Slf14. We compared the effects of HSE, L-lysine, and acetic acid at different concentrations as inducers. The results showed that cultures supplemented with HSE and L-lysine produced significantly more mycelia and HupA than those supplanted with acetic acid (Fig. 2). Especially, culturing *Shiraia* sp. Slf14 with 0.2 g L^{-1} L-lysine increased the HupA yield to 296.7 μg L^{-1}, which about 40 % higher than that of the control. We are uncertain whether L-lysine acid is an activator or a precursor of HupA. Considering the pathway of HupA biosynthesis remains unclear, previous investigations speculated that L-lysine is a precursor in lycopodine biosynthesis [2]. However, Ma and Gang [12] proposed a biosynthetic pathway for the synthesis of HupA and related lycopodium alkaloids consisting of pelletierine coupled with 4PAA based on previous studies [1, 4, 15, 16]. Initially, L-lysine is decarboxylated by lysine decarboxylase to form cadaverine. The subsequent reactions on the intermediates include a series of oxidation, decarboxylation, and *N*-methylation reactions that lead to the production of HupA and related lycopodium alkaloids [3, 12]. Luo et al. [10, 11] also described a transcript of lysine decarboxylase in *H. serrata* leaves that may be involved in HupA biosynthesis. Hence, L-lysine could be a vital precursor for HupA production in *Shiraia* sp. Slf14.

Some studies improved secondary metabolite production in endophytes by optimizing the culture conditions. However, few studies have focused on the HupA production in endophytic fungi. The only report so far indicated that ethanol and methanol improve HupA production [26]. However, we have failed to increase HupA production using this approach. In the present study, we maintained the stability of the endophytic fungi and enhanced HupA production using inducers. These inducers possibly act as positive regulators of HupA production in *Shiraia* sp. Slf14. Future studies should determine the pathways involved in HupA synthesis and regulation. From a practical standpoint, these results strongly suggest that HupA production in some

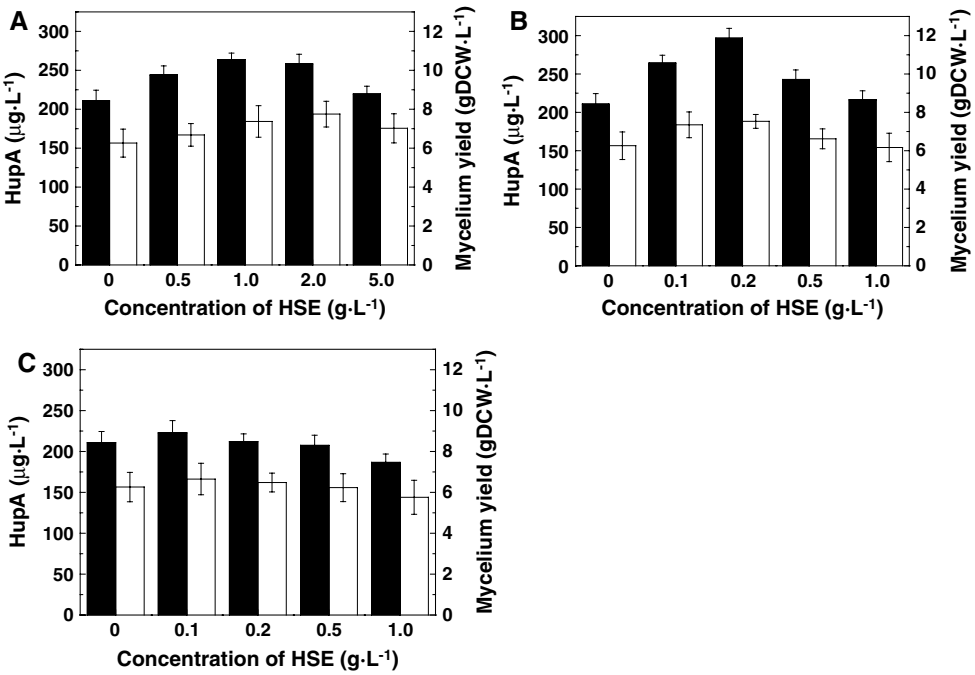

Fig. 2 Effect of different inducers on mycelial and HupA yields of *Shiraia* sp. Slf14 cultures. The fungi were cultured in liquid PDA medium by adding the indicated amounts of: **a** HSE, **b** L-lysine, and **c** acetic acid, respectively. HupA production (*filled square*) and mycelial yield (*open square*) were quantified after 14 days of cultivation

fungi is regulated by plant products or other small molecular compounds and may contribute to commercial HupA production via industrial fermentation. In addition, we are now sequencing the expression profile of *Shiraia* sp. Slf14 to elucidate the pathways involved in HupA biosynthesis and regulation through comparison with known unique host transcripts. We also plan to examine the molecular cascades of HupA production and the regulatory mechanism of L-lysine.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Grant Nos. 31300051, 21066014 and 81260617), China Postdoctoral Science Foundation (2012M511454), Jiangxi Natural Science Foundation of China (20114BAB204020), and Major Science and Technology Subject of Jiangxi Province (2010AZD00307). We appreciate the help of Prof. Lehua Zhang from the Lushan Botanical Garden of China for providing the plant materials. We are also grateful for the constructive comments of anonymous reviewers.

References

1. Castillo M, Gupta RN, Ho YK, MacLean DB, Spenser ID (1970) Biosynthesis of lycopodine. Incorporation of Δ1-piperideine and of pelletierine. Can J Chem 48(18):2911–2918
2. Castillo M, Gupta RN, MacLean DB, Spenser ID (1970) Biosynthesis of lycopodine from lysine and acetate. The pelletierine hypothesis. Can J Chem 48(12):1893–1903
3. Comins DL, Al-awar RS (1995) Model studies toward the synthesis of the lycopodium alkaloid, phlegmarine. J Org Chem 60(3):711–716
4. Hemscheidt T, Spenser ID (1993) Biosynthesis of lycopodine: incorporation of acetate via an intermediate with C2v symmetry. J Am Chem Soc 115(7):3020–3021
5. Ju Z, Wang J, Pan SL (2009) Isolation and preliminary identification of the endophytic fungi which produce Huperzine A from four species in Huperziaceae and determination of Huperzine A by HPLC. Fudan Univ J Med Sci 36(4):445–449
6. Koshiba T, Yokoshima S, Fukuyama T (2009) Total synthesis of (−)-huperzine A. Org Lett 11(22):5354–5356
7. Kumar A, Patil D, Rajamohanan PR, Ahmad A (2013) Isolation, purification and characterization of vinblastine and vincristine from endophytic fungus *Fusarium oxysporum* isolated from *Catharanthus roseus*. PLoS One 8(9):e71805
8. Kusari S, Košuth J, Čellárová E, Spiteller M (2011) Survival-strategies of endophytic *Fusarium solani* against indigenous camptothecin biosynthesis. Fungal Ecol 4(3):219–223
9. Li WK, Zhou JY, Lin ZW, Hu ZB (2007) Study on fermentation condition for production of huperzine A from endophytic fungus 2F09P03B of *Huperzia serrata*. Chin Med Biotechnol 2(4):254–259
10. Luo H, Li Y, Sun C, Wu Q, Song J, Sun Y, Steinmetz A, Chen S (2010) Comparison of 454-ESTs from *Huperzia serrata* and *Phlegmariurus carinatus* reveals putative genes involved in lycopodium alkaloid biosynthesis and developmental regulation. BMC Plant Biol 10(1):209–224
11. Luo H, Sun C, Li Y, Wu Q, Song J, Wang D, Jia X, Li R, Chen S (2010) Analysis of expressed sequence tags from the *Huperzia serrata* leaf for gene discovery in the areas of secondary metabolite biosynthesis and development regulation. Physiol Plantarum 139(1):1–12
12. Ma X, Gang DR (2004) The lycopodium alkaloids. Nat Prod Rep 21(6):752–772
13. Ma X, Gang DR (2008) In vitro production of huperzine A, a promising drug candidate for Alzheimer's disease. Phytochemistry 69(10):2022–2028
14. Ma X, Tan C, Zhu D, Gang DR, Xiao P (2007) Huperzine A from *Huperzia* species—an ethnopharmacolgical review. J Ethnopharmacol 113(1):15–34
15. Marshall WD, Nguyen TT, MacLean DB, Spenser ID (1975) Biosynthesis of lycopodine. The question of the intermediacy of piperidine-2-acetic acid. Can J Chem 53(1):41–50
16. Nyembo L, Goffin A, Hootele C, Braekman J-C (1978) Phlegmarine, a likely key intermediate in the biosynthesis of the Lycopodium alkaloids. Can J Chem 56(6):851–856
17. Qian L, Ji R (1989) A total synthesis of (±)-huperzine A. Tetrahedron Lett 30(16):2089–2090
18. Rafii MS, Walsh S, Little JT, Behan K, Reynolds B, Ward C, Jin S, Thomas R, Aisen PS (2011) A phase II trial of huperzine A in mild to moderate Alzheimer disease. Neurology 76(16):1389–1394
19. Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of *Pacific yew*. Science 260(5105):214–216
20. Sudhakar T, Dash SK, Rao RR, Srinivasan R, Zacharia S, Atmanand MA, Subramaniam BR, Nayak S (2013) Do endophytic fungi possess pathway genes for plant secondary metabolites? Curr Sci India 104(2):178–182
21. Szypuła W, Pietrosiuk A, Suchocki P, Olszowska O, Furmanowa M, Kazimierska O (2005) Somatic embryogenesis and in vitro culture of *Huperzia selago* shoots as a potential source of huperzine A. Plant Sci 168(6):1443–1452
22. Wang Y, Zeng QG, Zhang ZB, Yan RM, Wang LY, Zhu D (2011) Isolation and characterization of endophytic huperzine A-producing fungi from *Huperzia serrata*. J Ind Microbiol Biot 38(9):1267–1278. doi:10.1007/s10295-010-0905-4
23. Yang X, Guo S, Zhang L, Shao H (2003) Select of producing podophyllotoxin endophytic fungi from podophyllin plant. Nat Product Res Dev 15(5):419–422
24. Yi M-H, Kim J-A, Kim J-M, Park J-A, Kim B-T, Park S-M, Yang M-S, Hwang K-J, Kim D-H (2011) Characterization of a mutant strain of a filamentous fungus *Cladosporium phlei* for the mass production of the secondary metabolite phleichrome. J Microbiol 49(4):680–683
25. Zhang ZB, Zeng QG, Yan RM, Wang Y, Zou ZR, Zhu D (2011) Endophytic fungus *Cladosporium cladosporioides* LF70 from *Huperzia serrata* produces huperzine A. World J Microbiol Biotechnol 27(3):479–486
26. Zhao X-M, Wang Z-Q, Shu S-H, Wang W-J, Xu H-J, Ahn Y-J, Wang M, Hu X (2013) Ethanol and methanol can improve huperzine A production from endophytic *Colletotrichum gloeosporioides* ES026. PLoS One 8(4):e61777
27. Zhu D, Wang J, Zeng Q, Zhang Z, Yan R (2010) A novel endophytic huperzine A-producing fungus, *Shiraia* sp. Slf14, isolated from *Huperzia serrata*. J Appl Microbiol 109(4):1469–1478