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Published in:
National Academy of Sciences. Proceedings

Published: 23/04/2019

Document Version:
Final Published version, also known as Publisher’s PDF, Publisher’s Final version or Version of Record

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Publication record in CityU Scholars:
Go to record

Published version (DOI):
10.1073/pnas.1819254116

Publication details:

Citing this paper
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Download date: 08/08/2020
Complete biosynthetic pathways of ascofuranone and ascochlorin in Acremonium egyptiacum

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Edited by Craig A. Townsend, Johns Hopkins University, Baltimore, MD, and accepted by Editorial Board Member Stephen J. Benkovic March 12, 2019 (received for review November 8, 2018)

Ascofuranone (AF) and ascochlorin (AC) are meroterpenoids produced by various filamentous fungi, including Acremonium egyptiacum (synonym: Acremonium sclerotigenum), and exhibit diverse physiological activities. In particular, AF is a promising drug candidate against African trypanosomiasis and a potential anticancer lead compound. These compounds are supposedly biosynthesized from farnesylated of orsellinic acid, but the details have not been established. In this study, we present all of the reactions and responsible genes for AF and AC biosyntheses in A. egyptiacum, identified by heterologous expression, in vitro reconstruction, and gene deletion experiments with the aid of a genome-wide differential expression analysis. Both pathways share the common precursor, illicicolin A epoxide, which is processed by the membrane-bound terpene cyclase (TPC) AscF in AC biosynthesis. AF biosynthesis branches from the precursor by hydroxylation at C-16 by the P450 ascHIJ and a transcriptional factor (ascR) form a functional gene cluster, whereas those involved in the late steps of AF biosynthesis (ascHII) are present in another distantly located cluster. AF is therefore a rare example of fungal secondary metabolites requiring multi-locus biosynthetic clusters, which are likely to be controlled by the single regulator, AscR. Finally, we achieved the selective production of AF in A. egyptiacum by genetically blocking the AC biosynthetic pathway; further manipulation of the strain will lead to the cost-effective mass production required for the clinical use of AF.

Ascofuranone (AF) and ascochlorin (AC) are fungal natural products with similar chemical structures, originally isolated from Acremonium egyptiacum. Both have many useful biological properties; in particular, AF is a promising drug candidate against the tropical disease, African trypanosomiasis. However, the difficulty of the synthetic method and the inaccessibility of bioengineering methods have inhibited industrial production. This study identified all of the genes required for the branched biosynthetic pathways of AF/AC, which are clustered at two separate loci in the genome. In addition, we established the A. egyptiacum strain selectively producing AF, by genetically blocking the AC biosynthetic pathway. This study benefits the field of combinatorial biosynthesis through presenting biocatalysts and paves the way to cost-effective AF production with bioengineering.

A portion of this manuscript was presented as the master’s degree thesis of R.C. at Graduate School of Medicine, the University of Tokyo, in February 2015.


This article is a PNAS Direct Submission. C.A.T. is a guest editor invited by the Editorial Board.

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Data deposition: The data reported in this paper have been deposited in the DNA Data Bank of Japan (accession nos. DRA008136, E-GEAD-282, LC046756, and LC046757).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1819254116/-/DCSupplemental.

Published online April 5, 2019.
Interestingly, these compounds have been isolated from various fungi; for example, 2 from *Cylindrocladium ilicicola* (15, 16), *Cylindrocarpon* sp. (17), *Fusarium* sp. (18), *Microcera* sp. (19), and *Nectria coccinea* (20), and 1 from *Paecilomyces variotii* (21) and *Verticillium hemipterigenum* (22), indicating the broad distribution of the biosynthetic pathways. Compounds related to 1 and 2 are considered to be synthesized through the prenylation of orsellinic acid (3) and terminal cyclization via epoxidation (17, 18, 23) (Fig. 2A), but the details of their biosyntheses have not been established. Recently, we reported that *Stachybotrys bishyi* PYH05-7 encodes the 3-producing polyketide synthase (PKS) StbA, the UbiA-family prenyltransferase StbC, which produces ilicicolic acid B (4) (Fig. 2A), and the nonribosomal peptide synthetase (NRPS)-like reductase StbB for the synthesis of LL-Z1272β (ilicicolin B) (5), a putative precursor of 1 and 2 (24). By analogy to the biosynthetic pathways of other fungal meroterpenoids, such as pyripyropene, paclitaxel, and aflatoxin (25), the terminal olefin of the prenyl chain of 5 is thought to be epoxidized by a flavin monooxygenase (FMO), and then cyclized by a membrane-bound terpene cyclase (TPC). Considering the different cyclization patterns of 1 and 2, two distinct TPCs were assumed to exist in *A. egyptiacum*. However, it is difficult to predict the relevance of genes with novel functions and to find biosynthetic clusters separated at multiple loci. For plant metabolites, in contrast, coexpression analyses have been effective in identifying nonclustered biosynthetic genes (28, 29); naturally, such a transcriptome-based approach is also promising in the case of fungal multilocus clusters (30).

In this study, we aimed to find the biosynthetic genes for 1 by using a comparative transcription analysis of *A. egyptiacum* cultures exhibiting all-or-none production. Taking advantage of heterologous expression systems in *Aspergillus* spp., in vitro reconstruction assays, and a newly established gene disruption method for *A. egyptiacum*, we identified the complete biosynthetic enzymes of 2 encoded by the most prominently induced gene cluster. We then discovered another gene cluster at a discrete locus involved in the remaining steps of AF production, by virtue of a combination of motif-based and differential expression approaches. In addition, we analyzed the transcriptional factor regulating the biosynthetic genes of both compounds located in separate chromosomal regions.

**Results and Discussion**

**Discovery of the asc-1 Cluster.** First, we performed a genome-wide transcription analysis using the *A. egyptiacum* strain F-1392, to identify the differentially expressed gene clusters possibly responsible for the biosynthesis of 1 or 2. The production of 1 and 2 in *A. egyptiacum* varied dependent on the culture medium, and two media, designated as F1 and AF here, showed the virtual all-or-none difference. We thus prepared poly(A) selected RNAs from mycelia grown in F1 and AF media, which yielded 0.96 and 399 mg of 1 from mycelia grown in F1 and AF media, and obtained 611,740 and 846,811 RNA-seq reads, respectively. Although the experiment was preliminary, in terms of read numbers and lacking replicates, mapping the differential expression profiles to the 31-Mb draft genome (SI Appendix, Table S1) shed light on a 32-kb region on
scaffold 2 (named the asc-1 cluster; DNA Data Bank of Japan (DDBJ)/European Nucleotide Archive (ENA)/GenBank accession LC406756) (Fig. 3A and SI Appendix, Fig. S1). The transcription of eight genes (ascABCDEFGR) in the asc-1 cluster was more strongly induced (log20 values ≥ 2.5) in AF medium than in F1 medium, and some of them were predicted to encode characteristic enzymes required for the biosyntheses of 1 and 2; that is, PKS, prenyl transferase, and halogenase (SI Appendix, Table S2). Therefore, we named the eight genes ascR and ascA-G as candidates for the 1 or 2 biosynthetic genes (Fig. 3A).

The functions of AscR and AscA-G were deduced by bioinformatics analyses, as follows. AscR possessed a Zn2+ coordination site for DNA binding and was presumably a transcription regulator. AscA, AscB, and AscC exhibited more than 50% amino acid identities with the ilicicolin B (5) biosynthetic enzymes (StbABC) in S. bisbyi (24). A flavin-binding enzyme, AscD, was thought to be a halogenase, because it shares 68% amino acid identity with the halogenase that catalyzes the 5-chlorination of ilicicolinic acid B (4) (33). The Pfam motifs indicated that AscG is a membrane-bound P450 monoxygenase, and AscE is a P450 monoxygenase/P450 reductase fusion protein, such as the bacterial soluble P450 monoxygenase BM3 (34). AscF was predicted to be a membrane-bound TPc, sharing 29% amino acid identity to AndB, a TPC in antitoxin biosynthesis in Emericella varicelloides (35). The multiple alignment of AscF, AndB, and other meroterpenoid TCps, Pyr4 in pyrrolepyrrole biosynthesis (36), and PaxB in psallamine biosynthesis (37), revealed that the active-site E63 and D218 residues of Pyr4 (36) were also conserved in AscF (SI Appendix, Fig. S2). Although three other genes (gene-1, -2, and -3) of unknown function are located within the asc-1 cluster, we excluded them from the analysis since they exhibited little or no expression in the AF medium.

**Characterization of the asc-1 Cluster Genes.** To investigate the biosynthesis, we constructed Aspergillus oryzae transformants expressing ascA-D genes under the starch-inducible amyB promoter (38). Comparison of MS and NMR data with literature (24) indicated that 5 (m/z 355, [M – H]−) was produced in the strain expressing ascCAB (yield, 0.71 mg/L) (SI Appendix, Figs. S3 and S4 and Table S3). We further confirmed that AscA, AscC, and AscB in this order catalyzed each reaction as expected from the homology, based on the detection of the specific products 3 and 4 in the transformants expressing ascC and ascCA (SI Appendix, Figs. S4 and S5 and Table S3). Subsequently, the A. oryzae transformant harboring ascCABD yielded an additional compound (SI Appendix, Fig. S4), which was identified as ilicicolin A (6) (0.04 mg/L) by comparison with the authentic standard (SI Appendix, Fig. S6A). These data showed that AscCABD were responsible for the biosynthesis of 6.

Next, we performed an in vitro reconstruction of the succeeding steps, since the meager production of 6 complicated the analysis of the downstream metabolites in A. oryzae transformants. To prepare the AscE-G proteins, we employed an Aspergillus sojae high-copy expression system in which the pyrG selective marker with a truncated promoter enables the transformation of high-copy plasmids (SI Appendix, Fig. S7) (39). Since AscF and AscG are membrane-bound proteins and difficult to purify, a cell-free homogenate was used for the in vitro enzyme reaction. We hypothesized that 6 was epoxidized into ilicicolin A epoxide (7) by AscE or AscG before cyclization by AscF, as in the case of other fungal meroterpenoid biosynthesis in which terpene cyclization follows epoxidation of the prenyl group (25). This is also supported by the previous report of accumulation of 7 in A. egyptiacum mutant strain obtained through random chemical mutagenesis (23). However, the in vitro assay showed that 7 was not detected with either the AscE- or AscG-containing homogenate; alternatively, a compound (11) with an m/z of 423 ([M – H]−) was detected only with the AscE-containing homogenate (Fig. 3B and SI Appendix, Fig. S8 and Table S7). The m/z suggested that 11 was a diol, hydrolyzed from the epoxide 7 (m/z 405, [M – H]−), so we considered that AscE catalyzes the epoxidation to produce 7. Since 11 is unlikely to participate in the production of 1 or 2 in view of the reaction mechanisms, the diol 11 was presumed to be a shunt product formed by endogenous hydrolyse in A. sojae, as previously observed in the A. oryzae system (36, 40). These assumptions were later confirmed by our deletion experiment (see below). Another reaction product (8) was detected when a mixed homogenate containing AscE and AscF was incubated with 6 (Fig. 3B). Compound 8 was deduced to be ilicicolin C from the high-resolution (HR)-MS data (SI Appendix, Fig. S8 and Table S7), which was supported by the fact that ilicicolin C was isolated from A. egyptiacum (41). Finally, when the protein extracts containing AscE, AscF, and AscG were incubated with 6, the reaction product was identified as aschochlorin (2) (Fig. 3B). Confirmation of the structure was obtained by direct comparison of the MS/MS fragmentation pattern, and by co-injection with the authentic standard.

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**Fig. 3.** Functional characterizations of AscA-G. (A) Schematic representation of the asc-1 cluster, found by the differential expression analysis. The expression change was indicated with log20 value for each gene. (B) HPLC profiles of the in vitro reaction products of ilicicolin A (6) as a substrate when incubated with the buffer (i), the homogenate of the A. sojae wild-type strain (ii), the homogenates containing either AscE (iii) or AscG (iv), and the mixed homogenates containing AscE+AscF (v), AscE+AscG (vi), or AscE+AscF+AscG (vii), and authentic aschochlorin (viii). (C) HPLC profiles of authentic aschochlorin (i), authentic aschochlorin (ii), mycelium extracts of A. egyptiacum F-1392 (iii), ΔascE strain (iv), authentic ilicicolin A (v), and mycelium extract of ΔascE strain (vi). The yields of the compounds are summarized in SI Appendix, Table S8.
(SI Appendix, Figs. S6 B and C). These results indicated that the AscF-catalyzed terpene cyclization product 8 was oxidized into 2 by the P450 monooxygenase AscG. The absolute configuration of 8 is thought to be identical to that of (14S,15R,19R)-2, which was established by X-ray structure analysis (42).

To experimentally establish the involvement of 7 in the biosynthesis of 1 and/or 2, we constructed A. egyptiacum gene disruptants, using the kat70-deleted strain that we constructed in this study to enhance homologous recombination (43) (SI Appendix, Fig. S9). The ascF-deleted strain (ΔascF) no longer produced 2 (Fig. 3C), but instead accumulated 7 (1.22 g/L; SI Appendix, Table S8), which was isolated and structurally determined by NMR and HR-MS analyses (SI Appendix, Figs. S8 and S10 and Tables S4 and S7). In contrast, the ascE-deleted strain (ΔascE) only accumulated 6 (2.32 g/L, SI Appendix, Table S8), but neither 1 nor 2 (Fig. 3C). These results supported experimental proof that AscE epoxidized the terminal olefin of 6 to produce 7, and AscF cyclized 7 into 8 in AC biosynthesis. For AF biosynthesis, another TPC other than AscF should be involved, since ΔascF still produced 1 (0.50 g/L; SI Appendix, Table S8) (Fig. 3C). Given the fact that ΔascE no longer produced 1, the epoxide 7 was indicated as the last common precursor for the biosyntheses of 1 and 2.

Considering these results, we concluded that the seven genes, ascA-G, in the asc-1 cluster encode the biosynthetic enzymes for the production of 2. AscE is a P450 monooxygenase that catalyzes stereoselective epoxidation of the terminal double bond of the prenyl group (44). In contrast, in most of the cases, fungal meroterpenoid biosynthesis employs FAD-dependent monooxygenases for the formation of epoxide (25). Notably, AscF is the rare meroterpenoid TPC that produces a monocyclic terpene, and the cyclization reaction is proposed to be initiated by protonation of the terminal epoxide of 7 to generate a monocyclic tertiary cation, which is followed by a series of hydride and methyl shifts with abstraction of proton, leading to the formation of the (14S,15R,19R)-trimethylcyclohexane ring structure of 8 (Fig. 2B). AscA-E are also involved in AF biosynthesis, but additional enzymes are required for the later steps, after the common precursor 7, which should be encoded outside of the asc-1 cluster.

Identification of the Ascofuranone Biosynthetic Genes. Once again, the differential expression analysis was exploited to identify the genes responsible for the late stage of AF biosynthesis. Since one additional oxygen atom must be incorporated into the side chain of 7, we focused on P450s, the most abundant oxygenases in fungi. Among the gene clusters induced at least 10-fold in AF medium, only three harbored P450 monooxygenase genes other than the asc-1 cluster (SI Appendix, Table S5). One cluster on scaffold 6 encodes homologs of desmethylbassian biosynthesis enzymes, DmbA, DmbC, and DmbA (45), and thus was not likely to be involved in AF biosynthesis. Another cluster on scaffold 3 was excluded since it encodes an acetyltransferase and a peptidase, which both seemed irrelevant to AF biosynthesis. Combining the function prediction (see below), we targeted the other cluster on scaffold 3 (asc-2 cluster; DDBJ/ENA/GenBank accession no. LC406757) located on the different scaffold from asc-1 cluster (SI Appendix, Fig. S1).

The asc-2 cluster is composed of three genes, designated as ascHIJ (Fig. 4A). All three genes exhibited higher (log10 values ≥ 1.5) expression in AF medium (SI Appendix, Table S2). AscH is a P450 sharing 38% amino acid identity with the hexadecane hydroxylase P450-ALK2-A from Candida tropicalis (46). AscS is classified into the NAD(P)-dependent, short-chain alcohol dehydrogenase family, sharing 33% identity with the allatoin biosynthesis protein AthI (47). Finally, AscJ is an eight-transmembrane protein deduced by SOSUI program (48) without any Pfam motif; however, it is similar to the citreoviridin biosynthetic protein CtvD (49) and AurD in aurovertin biosynthesis (50), although it shares only 27% and 29% identities, respectively. Both enzymes are regioselective hydrolyases for cyclizing a bisepoxide to give a 3,4-dihydroxy-tetrahydrofururan structure, suggesting that AscJ is the TPC for AF biosynthesis.

Characterization of the asc-2 Cluster Genes. To link the candidate genes ascHIJ to AF biosynthesis, each candidate gene was disrupted in the A. egyptiacum ΔascF strain accumulating the precursor 7. HPLC analyses of these double disruptants showed that ΔascF/ΔascH and ΔascF/ΔascI could not produce 1, indicating that AscH and AscI are essential for AF biosynthesis, although ΔascF/ΔascC could produce it (0.41 g/L; SI Appendix, Table S8) (Fig. 4B). In contrast, the cell-free homogenate from the A. sojae strain expressing ascE, H, and J did not convert 6 into 1, while the addition of the ascH homogenate to the mixture sufficed to produce 1 (SI Appendix, Fig. S11). Taken together, AscJ is also required for the biosynthesis, although its function is compensated by the endogenous dehydrogenase in A. egyptiacum. These results clearly demonstrated that AscHIJ are responsible for AF biosynthesis.

The double disruptant ΔascF/ΔascC specifically accumulated a new compound 9 (0.05 g/L, SI Appendix, Table S8) (Fig. 4B), which was isolated from the large-scale culture, and analyzed by HR-MS and NMR. Its molecular formula was established as
C_{22}H_{31}ClO_{3} from the HR-MS data (m/z 421.1799, calc. 421.1787; SI Appendix, Fig. S8 and Table S7), indicating that it contains an additional oxygen compared with 7. The NMR data revealed that 7 and 9 are very similar to each other, but one methylene signal (δ H: 2.01/2.05 ppm; δ C: 36.4 ppm) disappeared and one oxymethine signal (δ H: 4.20 ppm; δ C: 75.5 ppm) appeared in 9 (SI Appendix, Fig. S12 and Table S4). Based on the association with the spin system of H-17/H-18, we assigned this signal as H-16. Notably, under acidic condition, 9 was non-enzymatically converted into ascosulfur (10), whose planar structure was identified by HR-MS and NMR analyses, and its absolute configuration was determined as (16S,18S)-10 by the Mosher and NOESY analyses (SI Appendix, Figs. S13 and S14 and Table S6). The absolute configurations of 7 and 9 were thus the same to be (16S)-7 and (16S,18S)-9, respectively.

We further conducted in vitro assays to attribute the succeeding reactions to the candidate genes. The microsomal fraction of the yeast expressing asc-1 efficiently converted 9 into 10, while that from the yeast harboring a blank vector did not afford any product (Fig. 4C). The \( K_m \) and \( V_{max} \) values of AscI were 50.4 ± 11.1 μM and 129 ± 13 nmol/min, respectively. In addition, we found that the cell-free homogenate of A. sojae expressing asc-1 dehydrogenizes 10 into 1 (Fig. 4D). Thus, we demonstrated that 7 is hydroxylated by the P450 monooxygenase AscH, and the resultant 9 is cyclized by AscI to 10, which is oxidized into 1 by AscJ (Fig. 2A).

Next, we investigated the reaction mechanism of the meroterpenoid TPC AscI, which lacks significant sequence similarity to any known TPCs. Sequence alignment revealed that AscI also harbors several conserved acidic residues that are thought to be essential for the catalysis (36) (SI Appendix, Fig. S15). Indeed, the alanine substitutions of AscI D61, E103, D296, E353, and D355 resulted in significant loss of activities (SI Appendix, Fig. S16). Notably, D61, D296, and E353 are also conserved in Aur D and CtV-D (SI Appendix, Fig. S15), suggesting that these residues are tight to the (16S)-7 and (16S,18S)-9, respectively.

Collectively, the three genes, ascH-J, in the asc-2 cluster encode the late-step biosynthetic enzymes, indicating that AF biosynthesis represents another rare example of a fungal multifaceted biosynthetic cluster. Although there have been a few reports (31–54), there is still no established strategy for exploring such clusters. In the cases of prenyl xanthone and astacinol, seeking the gene candidates based on the homology of the characteristic PKSs, a prenyl transferase that is proposed to have dehydrogenized the enone into a ketone in vitro, we assume that ascH ortholog contains the ascH ortholog (PSR83571; 50% amino acid identity), whereas the fungus does not encode a plausible ascH ortholog. Since the orthologs of the ascH and asc-1 cluster genes are widely distributed among Sordariomycetes, ascH is likely to play another role in the biosynthesis of AC-related compounds. Given that the report of AF-producing fungi (13) and the distribution of ascH orthologs were biased to the class Eurotiomycetes, we assume that ascH was horizontally transferred and grafted upon the preexisting ascH to make a coregulated as-2 cluster, although the elucidation will require further detailed investigation including synteny and phylogeny analyses using a wide range of fungal species.

Conclusions

We clarified the entire biosynthetic pathways of ascosulfurane (1) and ascosulfolin (2) in A. egyptiacum (Fig. 2). The biosyntheses of 1 and 2 share the common pathway up to the generation of ilicicolin A epoxide (7). Notably, the biosynthetic genes of 1 and 2 are localized at distinct chromosomal regions, but all of their promoters share a common conserved motif, and they are probably regulated by the transcriptional factor, AscR. This study thus contributes to increasing the knowledge on meroterpenoid biosynthesis and demonstrates the power of a differential expression analysis for exploring multifaceted biosynthetic clusters. From a clinical viewpoint, the elucidated genes, as well as the established method for the genetic manipulation of the strain F-1392, will be the keys for the drug development of 1. We have already established the ascF-deleted strain producing exclusively 1, but not 2 (Fig. 3C), and further manipulation will lead to the cost-effective industrial production of ascosulfurane.

Materials and Methods

A. egyptiacum (synonym: A. sclerotigenum) strain F-1392 (13, 14) is a derivative of the nitrosoguanidine-induced mutant no. 34, characterized in the original paper reporting 1 (2). The differential expression analysis was performed with an ion PGM system, and the raw sequence reads and the expression profile per gene were deposited in the DDBJ under the accession nos. DRA006136 and E-GEAD-282. Aspergillus species, A. egyptiacum gene disruptions were obtained from the newly established Dku70Δapsr6 strain by homologous recombination, after transformation by the protoplasting-polyethylene glycol method (58). Aspergillus oryzae NSAR1 (59) (nuA', C', ΔargB, Ade') was used as the host for the heterologous coexpression of ascA-D, and Aspergillus sojae P6-1 (ΔapyrG) (39) was used as the host for the high-copy heterologous expression of ascJ. The products from mycelia of each transformant were extracted with acetone, and the in vitro reaction products were extracted with ethyl acetate. The extracts were analyzed by UV-HPLC and liquid chromatography–MS, using ODS columns with standard chromatographic methods. The purified products were monitored by NMR analyses, including 1H, 13C, heteronuclear multiple bond coherence,
heteronuclear multiple quantum coherence, and COSY. Full experimental procedures are described in SI Appendix.

ACKNOWLEDGMENTS. We thank Prof. Katsuya Gomi (Tohoku University) and Prof. Katsuhiko Kitamoto (The University of Tokyo) for kindly providing the expression vectors and the fungal strain. We also thank Dr. Keiko Gomi and Dr. Ryoichi Sakae (Kikkoman Corporation) for their helpful advice. This work is partially supported by Ministry of Education, Culture, Sports, Science, and Technology (METI)/Japan Society for the Promotion of Science (JSPS) KAKENHI Grants JP17H04763 (to T.A.), JP17K70099 (to T.A.), JP17K15769 (to Y.K.), 23117004 (to M.M.), JP16H06443 (to I.A.), JP18K19139 (to I.A.), and 26253025 (to K.K.). Japanese Initiative for Project of Research on Infectious Disease for Global Epidemic [Grant JP18H0208020 (to Y.K.); Japan Science and Technology Agency/National Science Foundation of China Strategic International Collaborative Research Program (to I.A.); and Research Program on Emerging and Reemerging Infectious Diseases Grant 17k010819j0001 (to K.K.). We also acknowledge support from the Program for Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry and from the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries, and Food Industry.

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