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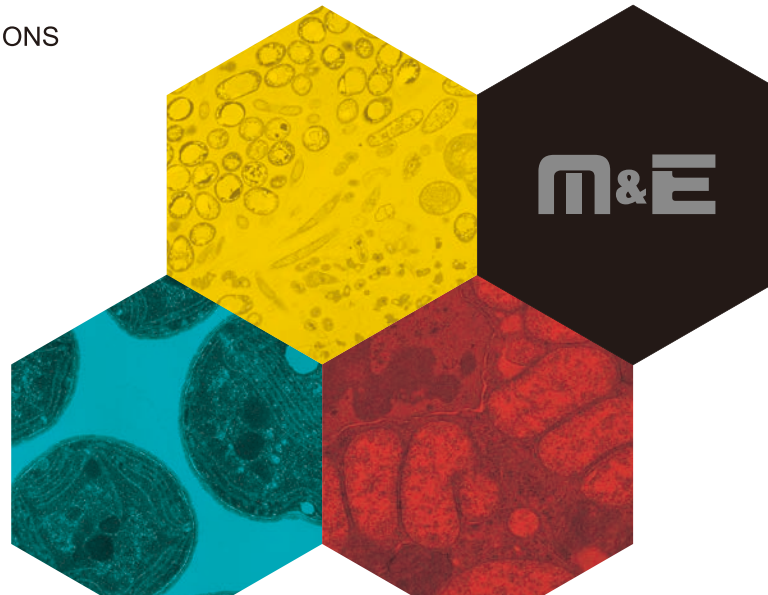

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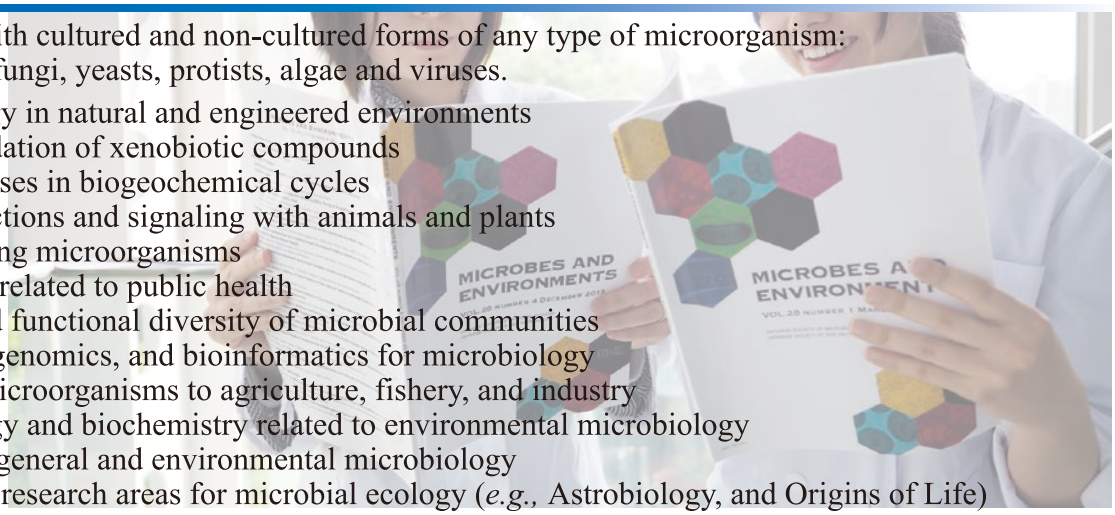

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MOST VALUABLE PAPER OF THE YEAR 2019

Antimicrobial Activities of Cysteine-rich Peptides Specific to Bacteriocytes of the Pea Aphid *Acyrthosiphon pisum*

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Aphids have a mutualistic relationship with the bacterial endosymbiont *Buchnera aphidicola*. We previously reported seven cysteine-rich peptides in the pea aphid *Acyrthosiphon pisum* and named them Bacteriocyte-specific Cysteine-Rich (BCR) peptides; these peptides are exclusively expressed in bacteriocytes, special aphid cells that harbor symbionts. Similar symbiotic organ-specific cysteine-rich peptides identified in the root nodules of leguminous plants are named Nodule-specific Cysteine-Rich (NCR) peptides. NCR peptides target rhizobia in the nodules and are essential for symbiotic nitrogen fixation. A BacA (membrane protein) mutant of *Sinorhizobium* is sensitive to NCR peptides and is unable to establish symbiosis. Based on the structural and expressional similarities between BCR peptides and NCR peptides, we hypothesized that aphid BCR peptides exhibit antimicrobial activity, similar to some NCR peptides. We herein synthesized BCR peptides and investigated their antimicrobial activities and effects on the bacterial membrane of *Escherichia coli*. The peptides BCR1, BCR3, BCR5, and BCR8 exhibited antimicrobial activities with increased membrane permeability. An *sbmA* mutant of *E. coli*, a homolog of *bacA* of *S. meliloti*, was more sensitive to BCR peptides than the wild type. Our results suggest that BCR peptides have properties that may be required to control the endosymbiont, similar to NCR peptides in legumes.

Key words: symbiosis, aphid, *Buchnera*, cysteine-rich peptide, bacteriocyte

Endosymbiosis is often essential for the survival of hosts and symbionts. A well-studied example is the mutual interdependence between aphids and *Buchnera* (30). Aphids harbor an endosymbiotic γ-proteobacterium, *Buchnera aphidicola*, within specialized cells called bacteriocytes (27). *Buchnera* provides the host with nutrients, such as essential amino acids, that aphids cannot synthesize and that are deficient in plant phloem sap, aphids' sole dietary component (13, 29). The relationship between aphids and *Buchnera* is syntrophic and obligate. *Buchnera* cells are vertically transmitted through host generations by transvariole transfer: they are exocytosed from the maternal bacteriocyte, temporarily released into the extracellular space, and endocytosed by the posterior syncytial cytoplasm of the blastula during early embryogenesis (stage 7) (4, 5, 17, 26). This symbiotic relationship is estimated to have been established 200–250 Myr ago. This long-term endosymbiotic relationship has shaped the characteristic streamlined genome, from which *Buchnera* has lost many genes, including those involved in the biosynthesis of lipopolysaccharides and phospholipids, gene regulation, and defense responses, and has, thus, lost the ability to survive outside of host bacteriocytes (2, 29).

Seven cysteine-rich peptides (CRPs) that are exclusively expressed in the bacteriocytes of the pea aphid *Acyrthosiphon pisum* have been identified and designated as "BCRs" (Bacteriocyte-specific Cysteine-Rich [BCR] peptides) (31). Each BCR peptide consists of a secretion signal peptide and mature peptide (44–84 amino acids) with 6 or 8 cysteine residues (31). Their expression was initially detected in stage 7 embryos, with *Buchnera* cells being transported from maternal bacteriocytes to the embryonic syncytium, and bacteriocyte-specific expression is then maintained throughout the rest of the aphid's life. Although this expression pattern suggests the importance of BCRs in the symbiosis with *Buchnera*, their physiological activities and functions in symbiosis currently remain unknown.

CRPs in symbiosis organs are found in other symbioses, such as that between legumes and nitrogen-fixing *α-Proteobacteria* called rhizobia (22), actinorhizal plants and nitrogen-fixing *Frankia* (6), and bean bugs and *β-Proteobacteria* of the genus *Burkholderia* (10). In the legume symbiosis, the host plant forms a specific organ, the root nodule, in which rhizobia live. Rhizobia penetrate the nodule cells and differentiate into bacteroids, the symbiotic form. By metabolic adaptation, bacteroids gain the ability to fix nitrogen (22, 33). In nodules formed on the roots of legumes, such as *Medicago*, *Pisum*, and *Trifolium*, bacteroids show cell elongation, genome amplification, cell membrane modifications, and the loss of reproductive activity (23). This terminal differentiation is mediated by nodule-specific cysteine-rich (NCR) peptides that are produced by the host plants (34, 35).

Medicago truncatula produces more than 600 NCR peptides in infected nodule cells only (28). NCR peptides are structurally similar to defensins, *i.e.*, they have signal peptides and mature peptides that conserve 4 or 6 cysteine residues (22). Some NCR peptides exhibit antimicrobial activity (34). Synthesized NCR peptides have the ability to induce cell elongation, polyploidization, and cell membrane modifications in *Sinorhizobium*

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meliloti cultured *in vitro* (34). Signal peptides are cleaved by signal peptidase, and mature NCR peptides are delivered to the microsymbionts inside host plant cells. The *DNF1* gene of *M. truncatula* encodes a subunit of a nodule-specific signal peptidase; *dnf1* mutants cannot establish effective symbiosis (32, 35). In *dnf1* mutant nodules, rhizobia remain undifferentiated, and NCR peptides localize within the endoplasmic reticulum, and, thus, are not delivered to bacteroids. These findings strongly support the view that NCR peptides are essential for effective symbiosis (34, 35).

To survive exposure to NCR peptides, *S. meliloti* requires BacA (14). The *S. meliloti bacA* mutant is hypersensitive to NCR peptides: when *S. meliloti bacA* mutant cells are released into nodule cells, they are rapidly killed (14). However, they may survive in the nodule cells of the *dnf1* mutant because NCR peptides are not transported to these cells. These findings show that BacA is essential for the chronic infection of nodules as well as bacteroid development (12, 14).

We investigated whether the BCR peptides of *A. pisum* exhibit antimicrobial activity and affect cell membrane permeability, similar to the NCR peptides of legume plants. We treated *E. coli*, a model γ-proteobacterium closely related to *Buchnera*, with chemically synthesized BCRs. We found that 4 out of the 6 BCR peptides assayed exhibited antimicrobial activities and induced cell elongation and higher intensities of 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) fluorescence. The *E. coli sbmA* mutant, a homolog of *bacA* of *S. meliloti*, was more sensitive to BCR peptides than the wild type. Similarities in the *in vitro* activities of BCR peptides to those of NCR peptides (14, 34) suggest that BCR peptides are involved in the symbiosis with *Buchnera* in the pea aphid in a similar manner to NCR peptides in legume plants.

Materials and Methods

Bacterial strains and media

E. coli wild-type strains MG1655 and BW25113 and the *sbmA*-disrupted mutant JW0368 derived from BW25113 (1) were provided by the National BioResource Project (https://shigen.nig.ac.jp/ecoli/strain) and maintained on Luria-Bertani (LB) medium. *S. meliloti* 1021 and its *bacA* mutant (8) were maintained on TY medium (3). In bioassays of the activities of BCR and NCR peptides, all strains were cultured in M9 liquid medium (25) supplemented with 0.2% glucose. In estimations of colony-forming units (cfus), *E. coli* strains were plated on LB agar plates and *Sinorhizobium* strains on TY agar plates.

Refolding of BCR peptides

BCR1, BCR2, BCR4, BCR5, and BCR8 peptides were chemically synthesized through a custom peptide synthesis service by Medical & Biological Laboratories (Nagoya, Japan) and BCR3 was synthesized by Biomatik Corporation (Cambridge, Canada). They were refolded with a Refolding CA Kit (Takara Bio, Kusatsu, Japan) according to the manufacturer's instructions. In brief, peptides were unfolded using guanidine hydrochloride with dithiothreitol, and were refolded in cycloamylose/Tween 40 with D,L-cystine. They were then passed through an Oasis HLB 3 cc column (Nihon Waters, Tokyo, Japan) and eluted with 1 mL of elution buffer (4 vol. acetonitrile : 1 vol. methanol : 5 vol. Milli-Q water containing 0.1% trifluoroacetic acid). Eluates were dried and then dissolved in Milli-Q water to a final concentration of 2 mg mL^{-1}. The refolded peptide was verified by HPLC, and the formation of disulfide bonds was confirmed by mass spectrometry. BCR6 was not assayed in the present study because BCR6 is too long (84 aa) to synthesize chemically. The NCR247 peptide was synthesized and used without refolding.

Treatment of bacterial strains with BCR or NCR peptides

All bacterial strains were cultured in liquid M9 medium. When the OD_{600} of the culture reached 0.3, cells were harvested and washed with 10 mM Tris·HCl buffer (pH 7.5) three times. Cells were then suspended in 10 mM Tris·HCl buffer (pH 7.5) to OD_{600}=0.1. Each test peptide was added to the bacterial suspension at an appropriate concentration and the suspension was incubated at 30°C for 3 h. In the analysis of cell morphology and membrane permeability, BCR peptides were added to a final concentration of 5 μM. As a control treatment, bovine serum albumin (BSA) was used at the same concentration as BCR peptides.

Detection of antimicrobial activities of BCR and NCR peptides

To estimate antimicrobial activities, we diluted the bacterial suspension treated with each peptide and spread it on LB (*E. coli*) or TY (*S. meliloti*) agar plates. The relative number of cfus was assessed in relation to the number that appeared on control (BSA-treated) plates as 100%. To investigate changes in morphology and membrane permeability, we analyzed the bacterial suspension according to previous studies (15, 34) that analyzed the activities of NCR peptides. Bacterial cells are detectable by staining with the fluorescent DNA dye DAPI. PI, a fluorescent dye that stains nucleic acids, is excluded from living cells, but enters dead cells or cells with the loss of membrane integrity. In brief, we added DAPI and PI together to the suspension to a final concentration of 10 μg mL^{-1} each and analyzed cells using a cell sorter (SH800, Sony, Tokyo, Japan) and confocal microscopy (A1, Nikon Instech, Tokyo, Japan). The cfus of *E. coli* suspensions and morphology and membrane permeability of *E. coli* cells were not affected by BSA under the experimental conditions employed in the present study (data not shown).

Results

Antimicrobial activities of synthetic BCR peptides

BCR1, BCR3, BCR5, and BCR8 at 5 μM exhibited strong antimicrobial activities against *E. coli* (MG1655) cells; the latter three prevented colony formation (Fig. 1). When *ca.* 5×10^7 cells were treated with 5 μM of BCR1, *ca.* 1×10^2 cells

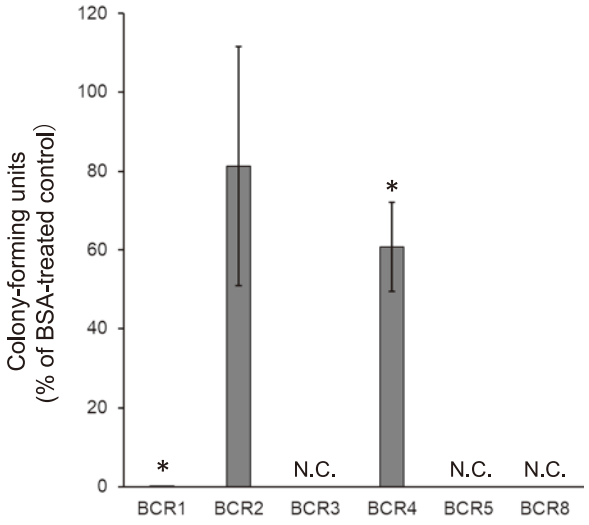

Fig. 1. Antimicrobial activities of BCR peptides. *E. coli* MG1655 was treated with 5 μM BCR peptides for 3 h and colony-forming units were estimated relative to the BSA control. Each value is the mean±SE of three independent experiments. Asterisks indicate a significant difference between BCR4 and the control (P<0.05 by the Student's t-test). N.C., no colony appeared.

survived (data not shown). No colony formed from the bacterial suspension treated with BCR3, BCR5, or BCR8. BCR4 exhibited mild antimicrobial activity, whereas BCR2 showed no significant antimicrobial activity (Fig. 1).

Effects of BCR peptides on E. coli *cells*

To reveal the effects of BCR peptides on cell morphology, we used the cell sorter to measure the forward scatter parameter (FSC), which indicates cell size, and the side scatter parameter (SSC), which indicates the complexity of granularity and internal complexity of cells, including cell formation. Histograms of FSC and SSC measured by the cell sorter are shown in Fig. 2A. BCR2, BCR3, BCR5, and BCR8 increased FSC (Fig. 2A) and SSC (Fig. S1). BCR1 and BCR4 slightly increased FSC (Fig. 2A), but not SSC (Fig. S1).

Most control cells stained with DAPI fluoresced at intensities of 20,000–30,000 (Fig. 2B). BCR1-treated and BCR3-treated cells produced a wider range of the signal than control cells, and BCR1-treated cells increased the frequency at higher intensities (Fig. 2B). Cells treated with BCR2, BCR5, and BCR8 fluoresced at higher intensities (Fig. 2B). BCR4-treated cells produced slightly higher intensities (Fig. 2B). BCR1, BCR2, BCR3, BCR5, and BCR8 clearly changed the profile of PI staining, shifting it to higher intensities and a wider distribution (Fig. 2C). BCR1 widened the distribution, shifting the main frequency to a lower intensity and slightly increasing the higher intensities (Fig. 2C). BCR4 slightly shifted the distribution to a higher intensity (Fig. 2C). In summary, cell sorter analyses revealed that some of the BCRs affected cell morphology and membrane permeability, which increased the intensities of DAPI and PI staining.

BCR-treated *E. coli* cells were also inspected by fluorescence microscopy. BCR1 and BCR3 significantly elongated cells and increased the intensities of DAPI and PI fluorescence (Fig. 2D). BCR3 and BCR8 promoted cell aggregation (Fig. 2A, 2D, and S1). BCR4 had no significant effect on either

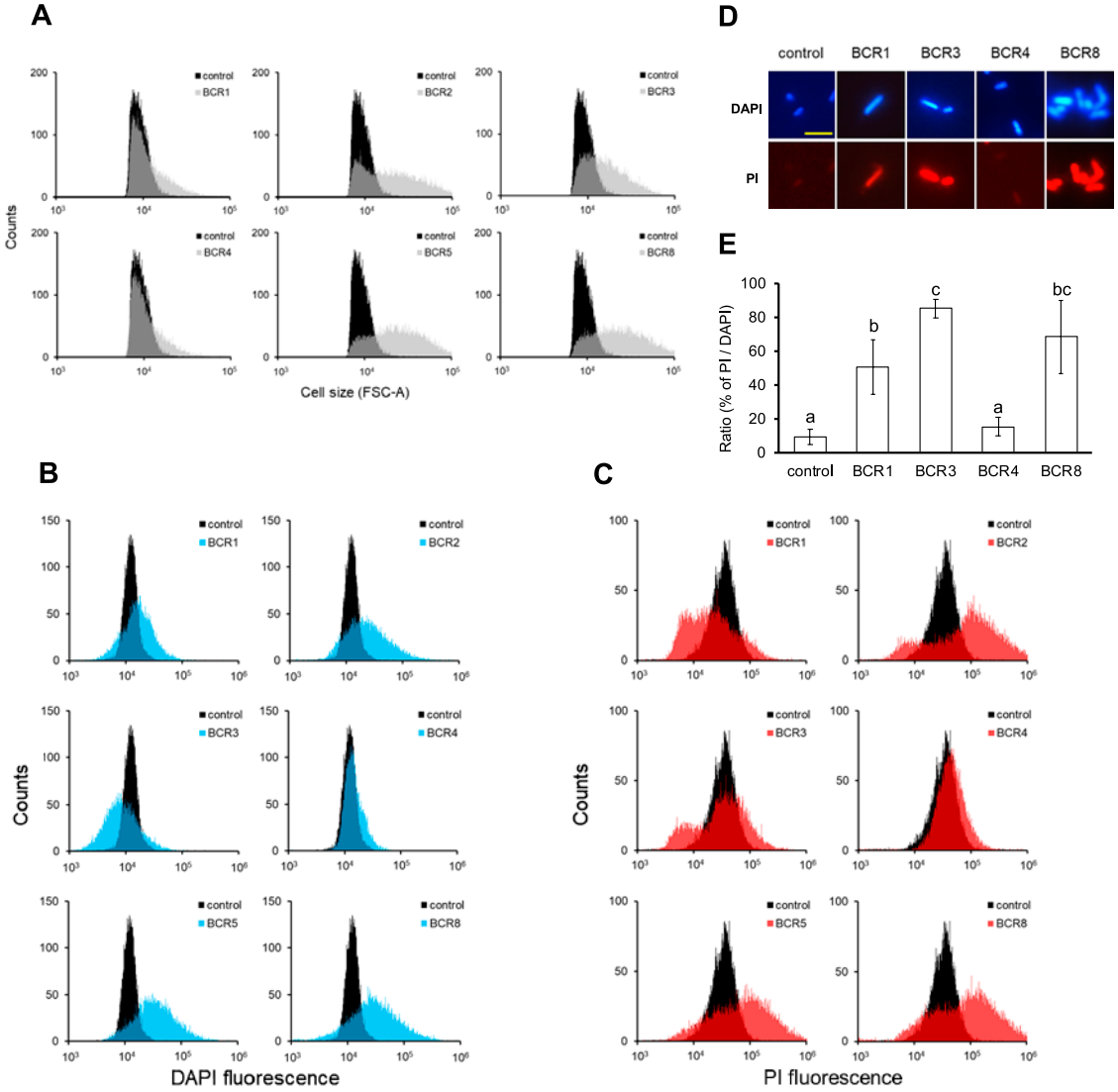

Fig. 2. Effects of BCR peptides on *E. coli* cells. *E. coli* MG1655 cells were treated for 3 h with 5 μM of the BCR peptide or BSA as the control. Cells were stained with DAPI and PI and analyzed by a cell sorter. (A) Forward scatter (FSC), (B) DAPI, (C) PI. (D) Fluorescent microscopy of MG1655 cells treated with BCR peptides and stained with DAPI and PI. Images are representative micrographs of cells treated with each peptide. Scale bars, 5 μm. (E) Ratio of PI-positive cells to DAPI-stained cells. At least 4,000 cells were counted in each treatment. Each value is the mean±SE. Means denoted by the same letter do not differ significantly (P<0.05, the Student's t-test).

morphology or fluorescence. BCR1, BCR3, and BCR8 significantly increased the fraction of PI-positive cells (Fig. 2E).

Sensitivity of the sbmA *mutant to BCR peptides and NCR247*

To reveal whether the SbmA protein is related to the antimicrobial activity of BCR peptides, BCR1, BCR3, and BCR8 were used because these BCRs exhibited strong antimicrobial activity. We treated two strains of *E. coli*, BW25113 (wild type) and JW0368 (*sbmA* mutant), with the BCR peptides at lower concentrations and calculated cfu values. Since no colonies formed when BW25113 and JW0368 were treated with 5 μM BCR1, BCR3, or BCR8 (Fig. S2), we used a sub-lethal concentration, 3 μM (Fig. 3A), in subsequent assays. BCR1 and BCR3 reduced the cfus of JW0368 to less than those of BW25113 (Fig. 3A). BCR1, BCR3, and BCR8 also caused the elongation of BW25113 and JW0368 cells (Fig. S3).

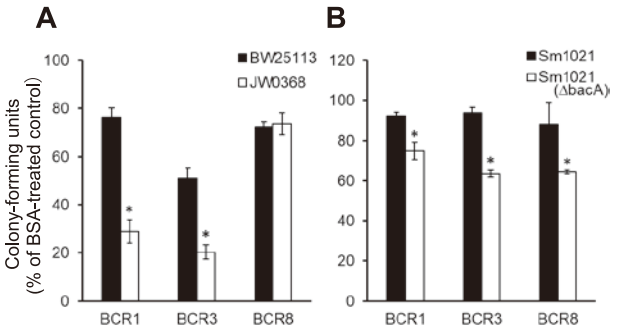

Fig. 3. Sensitivity of the *E. coli sbmA* mutant and *S. meliloti* Δ*bacA* mutant to BCR peptides. After a treatment with 3 μM BCRs for 3 h, the colony-forming units of each strain were estimated relative to the BSA control. The dataset shown is representative of three independent experiments. (A) *E. coli* BW25113 (wild type) and JW0368 (*sbmA* mutant). (B) *S. meliloti* 1021 and Δ*bacA mutant*. Each value is the mean±SE of three independent experiments. Asterisks indicate a significant difference between the wild type and mutant (P<0.01, the Student's *t*-test).

S. meliloti Δ*bacA* was significantly more sensitive to 20 μM NCR247 than wild-type *S. meliloti* 1021 (Fig. S4), as previously reported (14). JW0368, an *E. coli sbmA* mutant, was more sensitive to NCR247 at 5 μM, but not at 20 μM, than BW25113 (Fig. S4). Both *E. coli* strains were more resistant to NCR247 than *S. meliloti* (Fig. S4). No *S. meliloti* colonies formed at 5 μM BCR1, BCR3, or BCR8 (Fig. S5). Strain 1021 was resistant to 3 μM BCR1, BCR3, and BCR8, whereas the Δ*bacA* mutant was sensitive to them (Fig. 3B).

The effects of BCR1 and BCR8 on wild-type *S. meliloti* cells was analyzed using the cell sorter. BCR1 and BCR8 markedly increased FSC (Fig. 4A), SSC (Fig. S6), DAPI, and PI fluorescence (Fig. 4B). Thus, BCR1 and BCR8 exerted similar effects on *S. meliloti* as those on *E. coli*. BCR4 showed similar, but weaker effects.

The observed effects of BCR/NCR peptides on both *E. coli* and *S. meliloti* are summarized in Supplementary Tables S1 and S2.

Discussion

We investigated the antimicrobial activities of synthesized BCR peptides found in the bacteriocytes of pea aphids. Seven genes for BCR peptides (BCR1–6, 8) have been identified in the genome of *A. pisum* (31). The mRNA expression of BCRs in the embryo is initiated around the developmental stage coincident with the infection of *Buchnera* into the embryo from maternal bacteriocytes, and mRNA expression is maintained exclusively in bacteriocytes throughout the life of the aphid (31). Although expression patterns strongly suggest

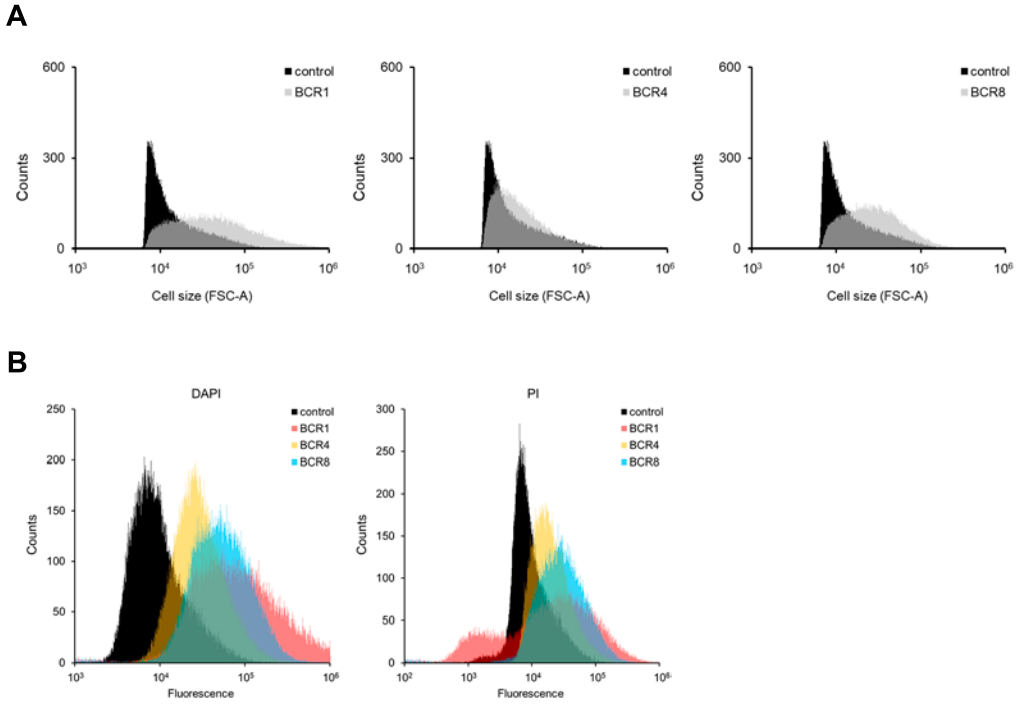

Fig. 4. Effects of BCR peptides on *S. meliloti*. *S. meliloti* 1021 cells were treated for 3 h with 5 μM BCR peptides or 5 μM BSA. Cells stained with DAPI and PI were analyzed by flow cytometry. (A) Forward scatter (FSC), (B) DAPI and PI. Data are representative of at least three independent experiments.

that BCR peptides control endosymbionts, their functions and activities have not yet been investigated. We synthesized 6 out of 7 *A. pisum* BCR peptides and examined their effects on bacteria. All peptides exhibited antimicrobial activity or permeabilized the membrane of *E. coli* cells: BCR1, BCR3, BCR5, and BCR8 exhibited strong antimicrobial activity and permeabilized the cell membrane, BCR2 only increased permeabilization, and BCR4 only showed mild antimicrobial activity (Fig. 1 and 2). Thus, at least four aphid BCR peptides were identified as antimicrobial peptides (AMPs). There was no obvious correlation detectable among the amino acid sequences, isoelectric points, and antimicrobial activities of BCR peptides. Since the sequence database search of BCR peptides returned no significant hits outside of aphid species (31), these BCR peptides constitute a novel class of AMPs specific to the aphid lineage.

We found that some BCRs exerted antimicrobial effects, whereas other did not. We also noted that each BCR peptide exerted antimicrobial effects at a different level and their effects on bacterial morphology and membrane permeability varied. In *M. truncatula*, the genes for NCR peptides are expressed exclusively in the root nodules, whereas expression patterns differ among genes (22). Each NCR peptide localizes to a different zone of the nodule; *e.g.*, NCR035 localizes to the interzone and nitrogen-fixing zone, while NCR001 only localizes to the nitrogen-fixing zone (34). NCR035 and NCR247 exhibit strong antimicrobial activities against *S. meliloti*, while some NCR peptides, such as NCR057 or NCR224, do not. The genes for all six BCR peptides used in the present study are exclusively expressed in the bacteriocyte of the pea aphid (31). Among them, BCR2 and BCR4, similar to NCR057 and NCR224, did not exhibit strong antimicrobial activities; BCR2 affected cell morphology and membrane permeability without antimicrobial activity. These results suggest that each BCR plays a different role in symbiosis, similar to NCR peptides. Although the location and target of the peptides within the bacteriocyte have not yet been reported, the pea aphid also has a diverse line-up of CRPs that may function in a different context in symbiosis, as observed in *M. truncatula*.

Legumes, such as *M. truncatula*, of the Inverted-Repeat-Lacking Clade (IRLC) use several hundred NCR peptides to control their microsymbionts. *Aeschynomene* spp. legumes, of the more ancient dalbergoid lineage, are expected to have several tens to hundreds of NCR-like peptides (7). On the other hand, only seven genes for BCR peptides have been identified on the genome of *A. pisum*. Although the reason for the marked difference in the number of CRPs between aphid–*Buchnera* and legume–rhizobia symbioses remains unclear, different systems of symbiosis may be responsible: Rhizobia are soil bacteria that may survive independently of their host plant; therefore, during symbiosis, the host plant may need to tightly control them by using NCR peptides with diverse functions. In contrast, *Buchnera* has lost the ability to survive outside of the pea aphid and, thus, may be controlled by BCR peptides with very restricted functions. Further studies are required to understand the diverse evolutionary processes of CRPs among symbiotic systems.

BacA, a membrane protein of *S. meliloti*, is essential for symbiosis with *M. sativa* (12). BacA of *S. meliloti* is involved in the modification of lipids with fatty acids (9); however, the molecular mechanisms employed by the BacA protein for resistance to AMPs remain unknown. The *bacA* mutant of *S. meliloti* is sensitive to NCR peptides and is unable to differentiate into bacteroids in host nodule cells, resulting in the abortion of symbiosis (14). *SbmA* of *E. coli* is a homolog of *BacA* of *S. meliloti* and may complement the symbiosis defect of the *S. meliloti bacA* mutant (16). Although differences were small, the significantly greater sensitivity of the *E. coli sbmA* mutant and *S. meliloti bacA* mutant to BCR1, BCR3, and NCR247 than their parent strains (Fig. 3 and S4) suggests the involvement of bacterial SbmA and BacA proteins in sensitivity to these cysteine-rich AMPs and the similar function of these BCR peptides to NCR peptides. BacA and SbmA are both transporters that import a number of structurally diverse peptides into the cell (11, 18, 20, 21, 36). In *S. meliloti*, BacA is essential for bacteroid differentiation and survival in the host plant cell (12, 14). However, we did not find any homologs of *bacA/sbmA* in the *Buchnera* genome. BCR peptides may function via a BacA/SbmA-independent mechanism and affect the membrane permeability and survival of *Buchnera*.

AMPs are common peptides that function in the innate immunity of eukaryotes (37). Besides BCR peptides and NCR peptides, AMPs that are expressed and/or function in symbiotic organs have been reported in other symbiotic relationships: *e.g.*, between bean bugs and *Burkholderia* (10) and between actinorhizal plants and *Frankia* (6). The weevil antimicrobial coleoptericin-A peptide regulates the growth of its symbiont by inhibiting cell division (19). In the symbiosis between the actinorhizal plant *Alnus* and *Frankia*, *Alnus* provides defensin-like peptide Ag5 to *Frankia*. The present study revealed that some BCR peptides exhibited antibiotic activity and increased the permeabilities of *E. coli* and *S. meliloti* cells. A possible function of antimicrobial BCR peptides is to control the growth of *Buchnera* in bacteriocytes, similar to coleoptericin-A, Ag5, and NCR, thereby controlling the size of the symbiont population within the host. These antimicrobial BCRs may also interact with secondary symbionts or invading microbes in the aphids. Another possible function of BCRs is to promote metabolite exchange (24). *In vitro*, Ag5 exhibits antimicrobial activity against *Frankia* and, at sublethal concentrations, induces the permeabilization of the vesicle membrane, resulting in the release of amino acids, particularly glutamine and glutamate, from *Frankia* cells (6, 24), which contributes to metabolic exchange between *Frankia* and nodule cells. Since the pea aphid has lost many transporter genes in its genome (24, 29), BCR peptides that permeabilize the cell membrane may be used in the exchange of metabolites between *Buchnera* and host cells. Animals and plants may both use AMPs not only as a defense against microbial attack, but also for symbiosis with microbes, representing a parallel evolution in symbiosis.

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References

1. Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K.A. Datsenko, M. Tomita, B.L. Wanner, and H. Mori. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. 2006.0008.
2. Baumann, P., L. Baumann, C.-Y. Lai, and D. Rouhbakhsh. 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: Intracellular symbionts of aphids. Annu. Rev. Microbiol. 49:55–94.
3. Beringer, J.E. 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84:188–198.
4. Braendle, C., T. Miura, R. Bickel, A.W. Shingleton, S. Kambhampati, and D.L. Stern. 2003. Developmental origin and evolution of bacteriocytes in the Aphid–*Buchnera* symbiosis. PLoS Biol. 1:e1.
5. Buchner, P. 1965. Endosymbiosis of Animals with Plant Microorganisms. Interscience, New York.
6. Carro, L., P. Pujic, N. Alloisio, *et al.* 2015. *Alnus* peptides modify membrane porosity and induce the release of nitrogen-rich metabolites from nitrogen-fixing *Frankia*. ISME J. 9:1723–1733.
7. Czernic, P., D. Gully, F. Cartieaux, *et al.* 2015. Convergent evolution of endosymbiont differentiation in Dalbergioid and inverted repeat-lacking clade legumes mediated by nodule-specific cysteine-rich peptides. Plant Physiol. 169:1254–1265.
8. Ferguson, G.P., R.M. Roop II, and G.C. Walker. 2002. Deficiency of a *Sinorhizobium meliloti* BacA mutant in alfalfa symbiosis correlates with alteration of the cell envelope. J. Bacteriol. 184:5625–5632.
9. Ferguson, G.P., A. Datta, J. Baumgartner, R.M. Roop II, R.W. Carlson, and G.C. Walker. 2004. Similarity to peroxisomal-membrane protein family reveals that *Sinorhizobium* and *Brucella* BacA affect lipid-A fatty acids. Proc. Natl. Acad. Sci. U.S.A. 101:5012–5017.
10. Futahashi, R., K. Tanaka, M. Tanahashi, N. Nikoh, Y. Kikuchi, B.L. Lee, and T. Fukatsu. 2013. Gene expression in gut symbiotic organ of stinkbug affected by extracellular bacterial symbiont. PLoS One 8:e64557.
11. Ghosal, A., A. Vitali, J.E.M. Stach, and P.E. Nielsen. 2013. Role of SbmA in the uptake of peptide nucleic acid (PNA)-peptide conjugates in *E. coli*. ACS Chem. Biol. 8:360–367.
12. Glazebrook, J., A. Ichige, and G.C. Walker. 1993. A *Rhizobium meliloti* homolog of the *Escherichia coli* peptide-antibiotic transport protein SbmA is essential for bacteroid development. Genes Dev. 7:1485–1497.
13. Gündüz, E.A., and A.E. Douglas. 2009. Symbiotic bacteria enable insect to use a nutritionally inadequate diet. Proc. R. Soc. B 276:987–991.
14. Haag, A.F., M. Baloban, M. Sani, *et al.* 2011. Protection of *Sinorhizobium* against host cysteine-rich antimicrobial peptides is critical for symbiosis. PLoS Biol. 9:e1001169.
15. Haag, A.F., B. Kerscher, S. Dall'Angelo, *et al.* 2012. Role of cysteine residues and disulfide bonds in the activity of a legume root nodule-specific, cysteine-rich peptide. J. Biol. Chem. 287:10791–10798.
16. Ichige, A., and G.C. Walker. 1997. Genetic analysis of the *Rhizobium meliloti bacA* gene: functional interchangeability with the *Escherichia coli sbmA* gene and phenotypes of mutants. J. Bacteriol. 179:209–216.
17. Koga, R., X.-Y. Meng, T. Tsuchida, and T. Fukatsu. 2012. Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. Proc. Natl. Acad. Sci. U.S.A. 109:E1230–E1237.
18. LeVier, K., and G.C. Walker. 2001. Genetic analysis of the *Sinorhizobium meliloti* BacA protein: differential effects of mutations on phenotypes. J. Bacteriol. 183:6444–6453.
19. Login, F.H., S. Balmand, A. Vallier, C. Vincent-Monégat, A. Vingneron, M. Weiss-Gayet, D. Rochat, and A. Heddi. 2011. Antimicrobial peptides keep insect endosymbionts under control. Science 334:362–365.
20. Marlow, V.L., A.F. Haag, H. Kobayashi, V. Fletcher, M. Scocchi, G.C. Walker, and G.P. Ferguson. 2009. Essential Role for the BacA protein in the uptake of a truncated eukaryotic peptide in *Sinorhizobium meliloti*. J. Bacteriol. 191:1519–1527.
21. Mattiuzzo, M., A. Bandiera, R. Gennaro, M. Benincasa, S. Pacor, N. Antcheva, and M. Scocchi. 2007. Role of the *Escherichia coli SbmA* in the antimicrobial activity of proline-rich peptides. Mol. Microbiol. 66:151–163.
22. Mergaert, P., K. Nikovics, Z. Kelemen, N. Maunoury, D. Vaubert, A. Kondorosi, and E. Kondorosi. 2003. A Novel family in *Medicago truncatula* consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs. Plant Physiol. 132:161–173.
23. Mergaert, P., T. Uchiumi, B. Alunni, *et al.* 2006. Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*-legume symbiosis. Proc. Natl. Acad. Sci. U.S.A. 103:5230–5235.
24. Mergaert, P., Y. Kikuchi, S. Shigenobu, and E.C.M. Nowack. 2017. Metabolic integration of bacterial endosymbionts through antimicrobial peptides. Trends Microbiol. 25:703–712.
25. Miller, J. 1992. A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory Press, New York.
26. Miura, T., C. Braendle, A. Shingleton, G. Sisk, S. Kambhampati, and D.L. Stern. 2003. A comparison of parthenogenetic and sexual embryogenesis of the pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphidoidea). J. Exp. Zool., Part B 295:59–81.
27. Munson, M.A., P. Baumann, and M.G. Kinsey. 1991. *Buchnera* gen. nov. and *Buchnera aphidicola* sp. nov., a taxon consisting of the mycetocyte-associated, primary endosymbiont of aphids. Int. J. Syst. Bacteriol. 41:566–568.
28. Nallu, S., K.A.T. Silverstein, D.A. Samac, Br. Bucciarelli, C.P. Vance, and K.A. VandenBosch. 2013. Regulatory patterns of a large family of defensin-like genes expressed in nodule of *Medicago truncatula*. PLoS One 8:e60355.
29. Shigenobu, S., H. Watanabe, M. Hattori, Y. Sakaki, and H. Ishikawa. 2000. Genome sequence of the endocellular bacteria symbiont of aphids *Buchnera* sp. APS. Nature 407:81–86.
30. Shigenobu, S., and A.C.C. Wilson. 2011. Genomic revelations of a mutualism: the pea aphid and its obligate bacterial symbiont. Cell Mol. Life Sci. 68:1297–1309.
31. Shigenobu, S., and D.L. Stern. 2013. Aphids evolved novel secreted proteins for symbiosis with bacterial endosymbiont. Proc. R. Soc. B. 280:20121952.
32. Starker, C.G., A.L. Parra-Colmenares, L. Smith, R.M. Mitra, and S.R. Long. 2006. Nitrogen fixation mutants of *Medicago truncatula* fail to support plant and bacterial symbiotic gene expression. Plant Physiol. 140:671–680.
33. Uchiumi, T., T. Ohwada, M. Itakura, *et al.* 2004. Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome. J. Bacteriol. 186:2439–2448.
34. Van de Velde, W., G. Zehirov, A. Szatmari, *et al.* 2010. Plant peptides govern terminal differentiation of bacteria in symbiosis. Science 327:1122–1126.
35. Wang, D., J. Griffitts, C. Starker, E. Fedorova, E. Limpens, S. Ivanov, T. Bisseling, and S. Long. 2010. A nodule-specific protein secretory pathway required for nitrogen-fixing symbiosis. Science 327:1126–1129.
36. Wehmeier, S., M.F.F. Arnold, V.L. Marlow, M. Aouida, K.K. Myka, V. Fletcher, M. Benincasa, M. Scocchi, D. Ramotar, and G.P. Ferguson. 2010. Internalization of a thiazole-modified peptide in *Sinorhizobium meliloti* occurs by BacA-dependent and -independent mechanisms. Microbiology 156:2702–2713.
37. Zasloff, M. 2002. Antimicrobial peptides of multicellular organisms. 2002. Nature 415:389–395.

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MOST VALUABLE PAPER OF THE YEAR 2020

Mechanisms of Rice Endophytic Bradyrhizobial Cell Differentiation and Its Role in Nitrogen Fixation

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Bradyrhizobium sp. strain SUTN9-2 is a symbiotic and endophytic diazotrophic bacterium found in legume and rice plants and has the potential to promote growth. The present results revealed that SUTN9-2 underwent cell enlargement, increased its DNA content, and efficiently performed nitrogen fixation in response to rice extract. Some factors in rice extract induced the expression of cell cycle and nitrogen fixation genes. According to differentially expressed genes (DEGs) from the transcriptomic analysis, SUTN9-2 was affected by rice extract and the deletion of the *bclA* gene. The up-regulated DEGs encoding a class of oxidoreductases, which act with oxygen atoms and may have a role in controlling oxygen at an appropriate level for nitrogenase activity, followed by GroESL chaperonins are required for the function of nitrogenase. These results indicate that following its exposure to rice extract, nitrogen fixation by SUTN9-2 is induced by the collective effects of GroESL and oxidoreductases. The expression of the sensitivity to antimicrobial peptides transporter (*sapDF*) was also up-regulated, resulting in cell differentiation, even when *bclA* (*sapDF*) was mutated. This result implies similarities in the production of defensin-like antimicrobial peptides (DEFs) by rice and nodule-specific cysteine-rich (NCR) peptides in legume plants, which affect bacterial cell differentiation.

Key words: rice, endophyte, *Bradyrhizobium*, cell differentiation, nitrogen fixation

Bradyrhizobium spp. from symbiotic and endophytic relationships with legumes and non-legumes, such as rice, namely, *Oryza breviligulata* (Chaintreuil *et al.*, 2000) and *Oryza sativa* L. ssp. *indica* (Teamtisong *et al.*, 2014) and *japonica* (Piromyou *et al.*, 2015a, 2015b). *Bradyrhizobium* sp. strain SUTN9-2 is capable of forming symbiotic and endophytic relationships with legume and rice plants (Piromyou *et al.*, 2017; Greetatorn *et al.*, 2019). Biological nitrogen fixation (BNF) by endophytic bradyrhizobia in rice may be caused by the activity of nitrogenase enzyme, encoded by the gene *nifH* (the nitrogenase structural component) (Teamtisong *et al.*, 2014; Okubo *et al.*, 2016). Another gene, *nifV*, is involved in the biosynthesis of homocitrate synthase, which activates the nitrogenase Fe protein in free-living diazotrophs (Howard and Rees, 1994) and *Bradyrhizobium* sp. (Pagan *et al.*, 1975; Okubo *et al.*, 2016). The *nifV* gene was also found in *Bradyrhizobium* sp. SUTN9-2 (Noisangiam *et al.*, 2012; Hashimoto *et al.*, 2019). This gene is mostly absent in *Rhizobium* sp. that efficiently perform nitrogen fixation only in symbiosis with legumes (Hakoyama *et al.*, 2009). This finding indicates its potential as a candidate for use as a biofertilizer or bioinoculant. However, there is currently no information on the rice endophytic molecular mechanisms that play important roles in plant colonization and growth promotion.

Elongated SUTN9-2 cells were recently observed in rice tissues at 7 days post-inoculation (dpi) by scanning electron microscopy (SEM). The elongation of these cells occurred between 3 (≅1–2 micrometer [μm]) and 7 (≅3 μm) dpi (Piromyou *et al.*, 2017). Based on this finding, we hypothesize that interactions between rice plants and bacterial factors may contribute to cell size enlargements and increases in nitrogen fixation efficiency by SUTN9-2 in rice plants, similar to bacteroid differentiation in legume plants. The responses of endophytic *Burkholderia kururiensis* M130 to rice macerate have been investigated using a transcriptomic analysis. The findings obtained revealed 27.7% of differentially expressed genes (DEGs) of its open reading frames in the presence of rice macerate. These genes were involved in membrane transporter and secretion systems, motility, chemotaxis, and adhesion, indicating the importance of the exchange of molecules for bacterial endophytic growth and adaptation to rice plants (Coutinho *et al.*, 2015).

The terminal bacteroid differentiation (TBD) of *Rhizobium* and *Bradyrhizobium* spp. has been extensively examined, particularly in an Inverted Repeat-Lacking Clade (IRLC) producing elongated polyploid bacterial cells that switch their cell cycle towards endoreduplication (Mergaert *et al.*, 2006; Kondorosi *et al.*, 2013; Alunni and Gourion,

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2016). TBD is assessed by host plant factors, including defensin-like antimicrobial peptides (DEFs) consisting of nodule-specific cysteine-rich (NCR) peptides, which are produced in large families of IRLC and Delbergioid legume clades, together with the BacA transporter protein in microbes (Mergaert *et al.*, 2006; Alunni and Gourion, 2016). NCR peptides and the BacA transporter protein have been shown to mediate the polyploidy of *Sinorhizobium meliloti* in *Medicago* nodules by altering the processes involved in sequential changes in the expression of cell cycle genes and cell size enlargements (De Nisco *et al.*, 2014; Penterman *et al.*, 2014). The BacA-like transporter of *Bradyrhizobium* sp. strain ORS285 also provides protection against the antimicrobial activity of NCR peptides in *Aeschynomene* spp. nodules. The BacA-like transporter has been identified in ORS285, carrying three genes (BRAO285v1_1320006, BRAO285v1_250005, and BRAO285v1_950010). These genes are characterized by the presence of the transmembrane domain pfam06472 (ABC_membrane_2) or pfam05992 (SbmA_BacA). However, only BRAO285v1_1320006 plays a key role in the symbiotic phenotype in host plants, providing protection against the antimicrobial activity of NCR peptides in *Aeschynomene* spp. nodules. The mutant produced small nodules, undifferentiated bacteroids, reduced nitrogen fixation activity in *Aeschynomene indica* and *Aeschynomene afraspera*, and some dead cells were observed in *A. indica* nodules. The other two genes formed normal symbiosis nodules, differentiated bacteroids, and nitrogen fixation, indicating that these genes are not important for ORS285 in *Aeschynomene* spp. symbiosis (Guefrachi *et al.*, 2015). These findings suggested that BacA-like proteins in ORS285 were encoded by BRAO285v1_1320006. However, *Bradyrhizobium* BacA-like (BclA), named according to BacA or the *Escherichia coli* homolog SbmA, differs from *Bradyrhizobium* proteins due to the presence of a C-terminal cytosolic ATPase domain typical for canonical ABC transporters (Guefrachi *et al.*, 2015). Furthermore, a correlation was observed between cell differentiation and nitrogen fixation activity in alfalfa (*Medicago sativa*) nodules (Vasse *et al.*, 1990). The small bacteroid size (1–2.5 µm) with a low nucleic acid content also exhibited weak acetylene reduction activity. On the other hand, the enlarged bacteroid size (5–7 µm) had a high nucleic acid content and very strong acetylene reduction activity (Paau and Cowles, 1978). This finding indicated a correlation between cell size and nitrogen fixation activity. Thus, the effects of DEFs from rice plants and the BclA of SUTN9-2 on cell size enlargement and nitrogen fixation efficiency, occurring during the interaction between SUTN9-2 and rice extract, were analyzed. The results obtained provide a better understanding of the mechanisms and factors involved in cell differentiation and nitrogen fixation in this model.

Materials and Methods

Plants and the bacterial strain

The rice plants *O. sativa* L. ssp. *indica* cv. Pathum Thani 1 and *O. sativa* L. ssp. *japonica* cv. Nipponbare were used in the present study. *Bradyrhizobium* sp. strain SUTN9-2 WT (LAXE00000000) was isolated from the root and stem nodules of *A. americana*, grown in rice field areas in Thailand (Noisangiam *et al.*, 2012). SUTN9-2 DsRed-tagged (Piromyou *et al.*, 2015b), Δ*nifV*, and Δ*bclA* were also used in the present study. SUTN9-2 WT and mutants were cultured at 30±2°C in yeast extract-mannitol (YEM) broth medium (Somasegaran and Hoben, 2012) for further analyses. The medium was supplemented with 200 µg mL^{-1} of streptomycin and spectinomycin for SUTN9-2 DsRed-tagged, 20 µg mL^{-1} of cefotaxime for Δ*nifV*, and 200 µg mL^{-1} of streptomycin for Δ*bclA*.

Rice growth and rice extract preparation

Rice seeds (*O. sativa* L. ssp. *indica* cv. Pathum Thani 1 and *O. sativa* L. ssp. *japonica* cv. Nipponbare) were dehulled and surface sterilized with 70% ethanol for 3 min, twice with 10% hydrogen peroxide for 10 min, with 3% sodium hypochlorite for 1 h, and then washed 3 times with sterilized water (Greetatorn *et al.*, 2019). To obtain a small emerging root, surface-disinfected rice seeds were germinated on 0.85% agar with YEM medium at 37°C for 1 day in the dark. Three germinated seeds (no contamination on YEM) were transplanted into glass test tubes (22×200 mm) containing a sterilized aluminum net and N-free rice nutrient solution ([mmol L^{-1}]: $NaH_2PO_4 \cdot 2H_2O$, 0.6; K_2SO_4, 0.3; $CaCl_2 \cdot 2H_2O$, 0.3; $MgCl_2 \cdot 6H_2O$, 0.6; EDTA-Fe, 0.045; H_3BO_3, 0.05; $MnSO_4 \cdot 5H_2O$, 0.009; $CuSO_4 \cdot 5H_2O$, 0.0003; $ZnSO_4 \cdot 7H_2O$, 0.0007; and $Na_2MoO_4 \cdot 2H_2O$, 0.0001) and pH adjusted to 6.8 (Mae and Ohira, 1981). Rice plants were grown for 14 days under controlled environmental conditions at 28±2°C and 70% relative humidity on a 16:8-h day:night cycle (full light, 639 microeinsteins [µE] m^{-2} S^{-1}).

Whole rice samples (root+shoot; 0.5 g $plant^{-1}$) were harvested at 14 dpi, sterilized and macerated separately with a sterilized mortar and pestle in sterilized buffered nodulation medium B (BNM-B) minimal medium, and kept at 4°C for no more than 3 days for further analyses. BNM-B is a synthetic plant growth medium (Ehrhardt *et al.*, 1992) supplemented with succinate, glutamate, and a cocktail of vitamins (Renier *et al.*, 2011). The rice extract was used by supplementing BNM-B medium (20–25 g of rice extract 400 mL^{-1} in BNM-B). The homogenate was passed through three layers comprising a miracloth (22–25 µm), membrane filter (8 µm), and syringe filter (0.2 µm) to discard plant debris and the rice extract obtained was further incubated with 10^8 SUTN9-2 cells in a 10-mL test tube with a tight cap and stable stage.

Construction of SUTN9-2 bclA *and* nifV *mutants*

The *bclA* deletion mutant (SUTN9-2Δ*bclA*) was constructed and standard molecular techniques were used in the present study. The 700-bp upstream and downstream fragments of the *bclA* gene were amplified by PCR and the primers used are listed in Table S1. The two regions were merged by overlap extension PCR and then digested by *EcoR*I/*Xba*I and cloned into the plasmid pNPTS129. This plasmid cannot replicate in *Bradyrhizobium* strains and carries *sacB* and the kanamycin resistance gene, which confer sensitivity to sucrose, thereby inducing bacterial death (Tsai and Alley, 2000). The spectinomycin and streptomycin cartridge from pHP45Ω was digested by *BamH*I and introduced into the upstream and downstream regions previously cloned in the pNPTS129 plasmid. The plasmid was then transferred into SUTN9-2 by triparental conjugation with the helper plasmid pRK2013 (Tamura *et al.*, 2011). Single recombinant clones were obtained by antibiotic selection, followed by double recombinant clones by growth on sucrose with spectinomycin and streptomycin, but not kanamycin. Candidate clones were checked for the loss of kanamycin resistance from the pNPTS129 plasmid, and the deletion of the *bclA* gene was verified by PCR. The mutation of the *nifV* gene was constructed as described by Hashimoto *et al.* (2019).

Confocal laser scanning microscopy

SUTN9-2 DsRed-tagged was treated with BNM-B and BNM-B supplemented with rice extract (*O. sativa* L. ssp. *Indica* cv. Pathum Thani) for 7, 14, 21, and 28 days. SUTN9-2 WT, Δ*nifV*, and Δ*bclA*

were treated with rice extract BNM-B and BNM-B supplemented with rice extract from *indica* and *japonica* for 7, 14, 21, and 28 days, and were then collected and stained with 30 μg mL^{-1} DAPI and 15 μg mL^{-1} FM4-64. Cell size, DNA content, and the cell membrane were observed in all treated cells under a confocal laser scanning microscope (Nikon Model Ni-E; *Nikon* Instech). The mean DNA content area was calculated from each cell (μm^2) using an ImageJ analysis (Collins, 2007), and the average enlarged cell size in each treatment was calculated from 20 cells in each replication for 3 replications.

Flow cytometry analysis

SUTN9-2 WT and DsRed-tagged cells were treated with BNM-B and BNM-B supplemented with rice extract (*O. sativa* L. ssp. *Indica* cv. Pathum Thani 1) for 7, 14, and 28 days. SUTN9-2 WT-treated cells were fixed at 4°C overnight in 70% ethanol, the fixed sample was digested with RNase (DNase free), and then stained with 50 μg mL^{-1} propidium iodide (PI) to analyze the DNA content (Deitch *et al.*, 1982). The size of SUTN9-2 DsRed-tagged cells treated as described above was assessed. The analysis of cell size and DNA content was conducted using a flow cytometer (BD FACSCalibur, BD Bioscience) with Cyflowgic software.

Acetylene reduction assay (ARA)

The effects of rice extract on the nitrogen fixation efficiency of SUTN9-2 WT (DsRed-tagged), Δ*nifV*, and Δ*bclA* cells treated with rice extract were investigated using ARA (Chaintreuil *et al.*, 2000). The reactions were performed in a 10-mL test tube containing 2 mL each of BNM-B medium and BNM-B medium supplemented with rice extract (*O. sativa* L. ssp. *indica* cv. Pathum Thani 1 and *japonica* cv. Nipponbare) as described previously. The reaction was incubated at 28±2°C for 7, 14, 21, and 28 days. Acetylene was injected to reach a final concentration of 10% (v/v) of the gas phase in the headspace (Somasegaran and Hoben, 2012). After the incubation, gas from the vessel was injected into a gas chromatograph (6′×1/8″ S.S. Hayesep T column; Valco Instruments). Ethylene gas production was analyzed, as described by Renier *et al.* (2011). Total concentrations in the cell suspension were evaluated using a plate count of colony-forming units (CFU) on YEM medium. The medium was supplemented with 200 μg mL^{-1} of streptomycin and spectinomycin for SUTN9-2 DsRed-tagged cells, 20 μg mL^{-1} of cefotaxime for Δ*nifV*, and 200 μg mL^{-1} of streptomycin for Δ*bclA*.

Rice cultivation and growth promotion

The rice seeds of *O. sativa* L. ssp. *indica* cv. Pathum Thani 1 were surface sterilized and germinated as described above. Germinated seeds were soaked overnight in YEM broth containing SUTN9-2 WT, Δ*nifV*, and Δ*bclA* (10^8 CFU mL^{-1}), and three germinated seeds were then transplanted into glass test tubes (22×200 mm) containing a sterilized aluminum net with N-free rice nutrient solution and N-free supplemented with 1 mmol L^{-1} ammonium nitrate under controlled environmental conditions, as previously described. Rice plants were grown and harvested at 7, 14, 21, and 28 dpi, whole rice samples were then dried in an oven at 65°C for 72 h, and dry weights were measured.

RNA preparation for qRT-PCR

RNA was isolated from three independent cultures of SUTN9-2 WT, Δ*bclA*, and Δ*nifV* incubated in BNM-B medium or BNM-B medium supplemented with rice extract (*indica*). The cultures were incubated in a 10-mL test tube with a tight cap at 28±2°C for 21 days. RNA was isolated from 10^8 cells using Plant RNA extraction kits (Qiagen). RNAs from rice plant samples (shoot+root) were harvested at 21 dpi, sterilized, and macerated (Greetatorn *et al.*, 2019). Total RNA extraction was performed according to the manufacturer's procedure. RNAs were treated with DNaseI (Qiagen) at 28±2°C for 30 min to prevent the contamination of genomic DNA. The purity of RNA was assessed by PCR on total RNA (250 ng) with GoTaq polymerase (Promega) using *dnaK*_SUNT9-2 primers and *EF-1*∝_rice plants (Table S1). The quality and concentration of RNA were assessed by Nanodrop (Thermo Scientific) and agarose gel electrophoresis.

qRT-PCR and analysis

Transcription levels were measured by qRT–PCR using Applied Biosystem, QuantStudio Design (Waltham). Primers for the amplification of genes involved in the cell cycle (*dnaK*, *gcrA*, *ctrA*, *dnaA*, and *GcrM*), nitrogen fixation (*nifH* and *nifV*), and rice hemoglobin (*EF-1*∝, *hb1*, and *hb5*) are listed in Table S1. PCR amplification was performed under the following cycling conditions: an initial denaturation step at 95°C for two min, 35 cycles at 95°C for 2 min and at the annealing temperature of all genes (50°C) for 30 s, followed by a final 5-min extension at 72°C. Relative gene expression was analyzed by the comparative Ct method (-ΔΔCT) normalized to the endogenous housekeeping gene, *dnaK* for bacterial SUTN9-2 and *EF-1*∝ for rice plants. Three biological replicates were pooled and analyzed.

RNA preparation for the RNAseq analysis

RNA was isolated from three independent cultures of SUTN9-2 WT and Δ*bclA* incubated in BNM-B medium and BNM-B medium supplemented with rice extract (*indica*). Cultures were incubated in a 50-mL test tube with a tight cap at 28±2°C for 21 days. RNA was isolated from 3×10^8 cells using Plant RNA extraction kits (Qiagen). Total RNA was extracted according to the manufacturer's procedure. The purity of RNA was assessed as described above.

RNA sequencing and analysis

To identify the bacterial genes that respond to rice extract, we used a comparative RNAseq analysis of SUTN9-2. RNA-Seq libraries were constructed from the RNA sample incubated with or without rice extract (20–25 g of rice extract 400 mL^{-1} in BNM-B) for SUTN9-2 WT and the Δ*bclA* mutant. Three biological replicates were prepared for each treatment. RNA samples were extracted following the same protocol for qRT-PCR. Eukaryotic rRNA from the samples was removed using the Ribo-Zero Magnetic Kit (Illumina) and stranded RNA-Seq libraries were constructed with the TruSeq Stranded mRNA kit (Illumina). Paired-end sequencing (150 bp) of the libraries was performed by the NovaSeq platform (Illumina). The libraries were constructed and sequenced at Novogene.

In the gene expression analysis, we adapted the mapping of the transcriptomic data obtained to the previously reported SUTN9-2 genome. A total of 865M reads (R1+R2) were obtained from the 12 libraries (Table S3). Adapter sequences and low-quality sequences were removed from raw single-end reads using fastp (v0.20.0) (fastp: an ultra-fast all-in-one FASTQ preprocessor [Chen *et al.*, 2018]). The trimmed paired-end reads were mapped to the SUTN9-2 genome (INSDC ID ASM312264v1) and calculated the TPM-normalized mapped read numbers by RSEM (v1.3.1) (RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome, [Li and Dewey, 2011]) with the --bowtie2 option (bowtie2 version 2.3.5.1) (Fast gapped-read alignment with Bowtie 2, [Langmead and Salzberg, 2012]). The mapped reads were counted based on the gene models of SUTN9-2 (LAXE01000000) predicted by Piromyou *et al.* (2015b). Differentially expressed genes (DEGs) were detected based on the false discovery rate (FDR) (<0.1) and log fold change (logFC) (>0.1 or <–0.1) from the RSEM analysis. In the enrichment analysis, the public gene models from LAXE01000000 were annotated with interproscan (v5.36-75) with --goterms option (for gene ontology annotation) and with KofamKOALA (downloaded on 13th Aug. 2019) (KofamKOALA: KEGG Ortholog assignment based on the profile HMM and adaptive score threshold [Aramaki *et al.*, 2020] for KEGG Orthologs annotation). Enrichment analyses were performed by goseq (v1.38.0) on R (v3.6.1) (gene ontology analysis for RNA-seq: accounting for selection bias, [Young *et al.*, 2010]).

qRT-PCR validation

To validate the results of the transcriptome analysis, significant DEGs were selected to compare their expression in SUTN9-2 WT in BNM-B with rice extract and SUTN9-2 WT in BNM-B alone, including biphenyl-2,3-diol 1,2-dioxygenase (*hpaD*), 3-(3-hydroxyphenyl) propanoate hydroxylase (*mhpA*), chaperonin GroEL (*groEL*), chaperonin GroES (*groES*), ABC transporter ATP-binding protein (*sapDF*), ABC transporter substrate-binding protein (*sapA*), and RND family efflux transporter (*cusF*). Primers for the amplification of these genes are listed in Table S1. RNA preparation, qRT-PCR, and analyses were performed as described above.

SbmA_BacA domain proteins and phylogenetic analysis

The identified protein sequences of the SbmA_BacA domain, including the BacA, *Bradyrhizobium* homologous, ExsX, and *Mycobacterium* BacA clades, were obtained from Guefrachi *et al.* (2015). The BacA-related protein sequences of SUTN9-2 (BclA; PWE81210.1, SapA; PWE77331.1, and SapDF; PWE82048.1) were obtained from the National Center for Biotechnology Information (NCBI) database. Protein sequences were aligned using the ClustalW program. The phylogenetic tree was constructed using the neighbor-joining method with confidence levels for 500 replicates using the MEGAX package (Saitou and Nei, 1987; Kumar *et al.*, 2008).

Statistical analysis

The statistical analysis of data sets was performed with SPSS software (SPSS 16.0 for Windows; SPSS) on data from three independent samples (each with three technical replicates). Experimental data were statistically analyzed according to Steel and Torrie (1980), and means were compared by Duncan's multiple range test ($P \leq 0.05$) (Duncan, 1955).

Results

*Cell size enlargement by SUTN9-2 in rice (*indica*) plants*

To confirm whether SUTN9-2 cell size enlargement occurs within rice plants, the red fluorescent-tagged SUTN9-2 strain (DsRed) was extracted from rice plant tissues (*indica*) at 21 and 28 dpi and visualized using confocal laser scanning microscopy. The cells of SUTN9-2 were longer, with average sizes of 5.6 and 5.8 μm at 21 and 28 dpi, respectively, than free-living SUTN9-2 cells (2.8 μm) (Fig. S1). These results revealed that SUTN9-2 cell size increased within rice plants. However, small numbers of SUTN9-2 cells were extracted from rice plant tissues. Therefore, rice extract was prepared and subsequent experiments were performed by incubating SUTN9-2 cells with the extract to investigate the influence of rice and its derived molecules on the differentiation of SUTN9-2 cells.

*SUTN9-2 increases its cell size and DNA content in response to the rice extract (*indica*) treatment*

Increases in cell size and DNA content in SUTN9-2 cells in response to the rice extract (*indica*) treatment were examined using a flow cytometer. The sizes of treated SUTN9-2 DsRed-tagged cells in BNM-B minimal medium and BNM-B supplemented with rice extract were analyzed. The forward scatter (FS) of a treated cell is related to its size. BNM-B with rice extract-treated cells had a higher FS than BNM-B-treated cells at 7, 14, and 28 days (Fig. 1A). Furthermore, the DNA content of treated SUTN9-2 WT cells was assessed by the fluorescent staining of nuclei using PI. The DNA content of BNM-B with rice extract-treated SUTN9-2 cells was higher than that in BNM-B-treated cells. The DNA content of BNM-B-treated cells was 1 to 2 genome complement (1C to 2C), similar to the ploidy level of free-growing cells (1C to 2C) (Czernic *et al.*, 2015), whereas BNM-B with rice extract-treated cells reached 2C to 7C ploidy levels, similar to the ploidy level of bacteroid cells (7C to 16C) (Czernic *et al.*, 2015; Guefrachi *et al.*, 2015) (Fig. 1A). These results confirmed that differentiated SUTN9-2 cells affected rice plants.

*SUTN9-2 cells increase their size and nitrogen fixation activity in response to rice extract (*indica*)*

To investigate whether an enlarged cell size increases nitrogen fixation activity in response to rice extract, SUTN9-2 DsRed-tagged cells treated with deionized water (DI), BNM-B, and BNM-B with rice extract (*indica*) were analyzed. Cell size was larger in the BNM-B with rice extract group than in the BNM-B alone and DI groups. The average sizes of BNM-B with rice extract-treated cells at 7, 14, 21, and 28 days were 2.76, 3.18, 3.38, and 3.79 μm, respectively (Fig. 1B), and average nitrogenase activities were 0.01, 0.02, 0.09, and 0.30 nmol C_2H_4 $\log_{10}^{-1}$ CFU, respectively (Fig. 1C). The average sizes of BNM-B alone-treated cells at 7, 14, 21, and 28 days were with 2.52, 2.74, 2.42, and 2.64 μm, respectively (Fig. 1B). Average nitrogenase activities in this group were 0.01 nmol C_2H_4 $\log_{10}^{-1}$ CFU at 7, 14, and 21 days and 0.02 nmol C_2H_4 $\log_{10}^{-1}$ CFU at 28 days (Fig. 1C). The average sizes of DI-treated cells at 7, 14, 21, and 28 days were 1.86, 2.04, 1.36, and 1.37 μm, respectively (Fig. 1B), and these cells only exhibited nitrogenase activity of 0.01 nmol C_2H_4 $\log_{10}^{-1}$ CFU at 14 days (Fig. 1C). These results demonstrated the influence of rice extract on the cell size and nitrogen fixation efficiency of SUTN9-2 cells. Time-dependent increases were observed in elongated cells in the BNM-B with rice extract group, but not in the BNM-B alone or DI group (Fig. 1B and C).

*SUTN9-2 WT and mutants show increases in cell size, DNA content, and nitrogen fixation activity in the presence of rice extract (*indica *and* japonica*)*

To clarify the effects of rice species variations and *bclA* and *nifV* genes on cell differentiation and nitrogen fixation, SUTN9-2 WT, Δ*bclA*, and Δ*nifV* were treated with rice extract from 2 different species of rice (*indica* and *japonica*). Cell size, DNA content, and nitrogenase activity were increased in the BNM-B with rice extract (*indica*) group at 28 days with average cell sizes of 3.28, 3.22, and 3.03 μm (Fig. 1D, E, and F), mean DNA content areas of 0.43, 0.33, and 0.32 μm^2 (Fig. S2 and S3), and nitrogenase activities of 0.28, 0.07, and 0.00 nmol C_2H_4 $\log_{10}^{-1}$ CFU in SUTN9-2 WT, Δ*bclA*, and Δ*nifV*, respectively (Fig. 1D, E, and F). The BNM-B with rice extract (*japonica*) group showed average cell sizes of 2.66, 2.56, and 2.60 μm (Fig. 1D, E, and F), mean DNA content areas of 0.32, 0.33, and 0.28 μm^2 (Fig. S2 and S3), and nitrogenase activities of 0.06, 0.03, and 0.00 nmol C_2H_4 $\log_{10}^{-1}$ CFU for SUTN9-2 WT, Δ*bclA*, and Δ*nifV*, respectively (Fig. 1D, E, and F). Average cell sizes at 28 days in BNM-B-treated cells were

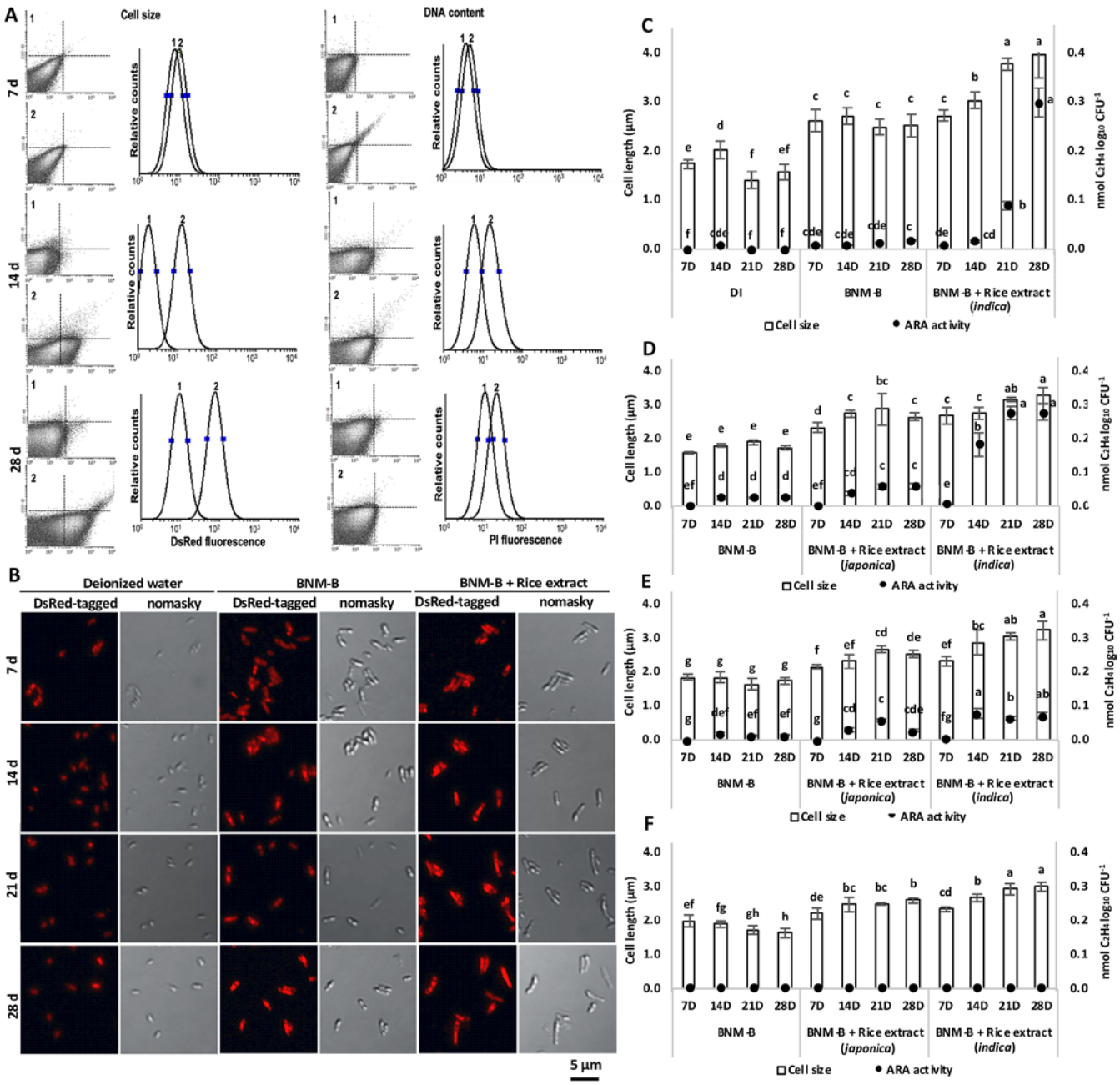

Fig. 1. Cell size, DNA content, and nitrogenase activity of SUTN9-2 in response to rice extract (*indica*). Cell size, enlarged DsRed-tagged cells, and propidium iodide (PI)-stained DNA content were analyzed by flow cytometry (1 represents free growing, and 2 represents cells treated with rice extract) (A). Cell size and enlarged DsRed-tagged cells by confocal laser scanning microscope (B). Observation of cell size by a confocal laser scanning microscope and nitrogenase activity by the acetylene reduction assay of SUTN9-2 WT cells treated with DI, BNM-B, and BNM-B +rice extract (*indica*) (C), and SUTN9-2 WT (D), Δ*bclA* (E), and Δ*nifV* (F) with BNM-B, BNM-B+rice extract (*japonica*), and BNM-B+rice extract (*indica*) at 7, 14, 21, and 28 days. Significance at $P \leq 0.05$ is indicated by the mean±standard deviation (n=3).

1.73, 1.73, and 1.63 µm (Fig. 1D, E, and F), mean DNA content areas of 0.11, 0.15, and 0.15 µm^2 (Fig. S2 and S3), and nitrogenase activities of 0.05, 0.01, and 0.00, nmol C_2H_4 log_{10}^{-1} CFU in SUTN9-2 WT, Δ*bclA*, and Δ*nifV*, respectively (Fig. 1D, E, and F). Similar results were obtained at 7, 14, and 21 days (Fig. 1D, E, and F). The effects of rice extract and Δ*bclA* and Δ*nifV* on cell sizes were observed under a transmission electron microscope (TEM). The cells of SUTN9-2 WT, Δ*bclA*, and Δ*nifV* elongated in response to the rice extract (*indica*) treatment (Fig. S4).

These results demonstrated that rice extract (*indica*) exerted stronger effects on cell elongation and nitrogen fixation activity than rice extract (*japonica*) in WT and mutant SUTN9-2 cells. Increases in cell size and nitrogenase activity were smaller in Δ*bclA* than in the WT strain. Furthermore, nitrogenase activity was not detected in Δ*nifV* and their cell size was smaller than those of WT and Δ*bclA* following the treatment with both rice extracts. These results also revealed the effects of *bclA* and *nifV* genes on cell size enlargement and nitrogen fixation activity in response to different rice varieties.

*Effects of WT and mutant SUTN9-2 on rice (*indica*) plant growth*

SUTN9-2 has been reported to promote rice (*indica*) growth at the early seedling stage (Greetatorn *et al.*, 2019). Consistent results were obtained in the present study; SUTN9-2 WT, Δ*bclA*, and Δ*nifV* increased rice dry weight more than the non-inoculated control, particularly at 7 and 14 dpi, in N-free medium and ammonium nitrate-supplemented medium (Fig. S5). The significant difference observed at 7 and 14 dpi between rice plants inoculated with SUTN9-2 WT, Δ*bclA*, and Δ*nifV* and the non-inoculated control was not detected at 21 or 28 dpi (Fig. S5). However, rice dry weight did not show a significant difference between rice plants inoculated with SUTN9-2 WT, Δ*bclA*, and Δ*nifV* at 7, 14, 21, and 28 dpi (Fig. S5). These results clearly demonstrated that SUTN9-2 promoted rice plant growth at the early stage at 7 and 14 dpi under the presence or absence of supplementation with a nitrogen source. However, the effects of *bclA* and *nifV* genes on rice plant growth remain unclear.

*Expression of cell cycle genes in WT and mutant SUTN9-2 in response to rice extract (*indica*)*

SUTN9-2 cell differentiation-related genes, including *gcrA*, *ctrA*, *ccrM*, and *dnaA*, were selected based on their role in the master cell cycle. Similar results were obtained for the expression of all genes between SUTN9-2 WT and Δ*bclA*. However, Δ*bclA* showed the down-regulated expression of the genes listed above. The expression levels of *gcrA* and *ctrA* were lower in the BNM-B with rice extract (*indica*) group than in the BNM-B alone group, with 0.01- and 0.09-fold differences in WT and 0.008- and 0.01-fold differences in Δ*bclA*, respectively (Fig. 2A). However, no significant difference was noted in *gcrA* expression between the BNM-B alone and BNM-B with rice extract groups. In contrast, the expression levels of *ccrM* and *dnaA* were higher in the BNM-B with rice extract group than in the BNM-B alone group, with 0.20- and 0.22-fold differences for WT and 0.10- and 0.10-fold differences for Δ*bclA*, respectively (Fig. 2A). In spite of this result, the expression of all genes in Δ*nifV* was several-fold lower in the BNM-B with rice extract group than in the BNM-B alone group (Fig. 2A). These results suggested that cell size enlargements may also be affected by the *nifV* gene, supporting the observation of cell enlargement under the microscope. They also indicated that the *bclA* gene was disrupted, whereas other *bclA*-associated genes were still active and required for cell enlargement, showing a similar pattern of gene expression to WT. Collectively, these results demonstrated that some factors from rice extract may affect master cell-cycle regulators.

*Nitrogen fixation and BclA transporter gene expression in WT and mutant SUTN9-2 in response to rice extract (*indica*)*

To examine the effects of rice extract on BclA transporter and nitrogen fixation-related gene expression in SUTN9-2, their relative expression levels were measured in SUTN9-2 WT, Δ*bclA*, and Δ*nifV*. The expression levels of the *nifH*, *nifV*, and *bclA* genes were significantly increased in WT (0.12-, 0.12-, and 0.11-fold, respectively) and Δ*bclA* (*nifH*; 0.14-, and *nifV*; 0.14-fold) in the presence of rice extract (Fig. 2B). In contrast, the expression levels of these genes were significantly decreased in Δ*nifV* (*nifH*; 0.06-, *bclA*; 0.008-fold) in the presence of rice extract (Fig. 2B). However, the expression of the *bclA* and *nifV* genes was not detected in SUTN9-2 mutants lacking *bclA* and *nifV*, respectively (Fig. 2B). The results demonstrated that rice extract affected BclA transporter and nitrogen fixation-related gene expression, indicating the effect of Δ*nifV* on *nifH* and *bclA* gene expression.

*Expression of rice (*indica*) hemoglobin genes in response to WT and mutant SUTN9-2*

To assess the relationship between hemoglobin gene expression and nitrogen fixation efficiency by SUTN9-2 in rice plants, *hb1* and *hb5* gene expression levels were assessed in rice plants inoculated with SUTN9-2 WT, Δ*bclA*, and Δ*nifV*. The results obtained showed that *hb1* and *hb5* expression was more strongly induced in rice inoculated with SUTN9-2 WT (0.2- and 0.03-fold), Δ*bclA* (0.02- and 0.007-fold), and Δ*nifV* (0.06- and 0.01-fold) than with the non-inoculated control (Fig. 2C). The expression of these genes was suppressed in Δ*bclA*- and Δ*nifV*-inoculated rice, with the expression level of the *hb5* gene being lower than that of the *hb1* gene (Fig. 2C). These results indicated that the activity of hemoglobin in rice plants may be induced in response to the SUTN9-2 inoculation and may function as an oxygen scavenger or oxygen stock to facilitate nitrogen fixation in the endophytic state of SUTN9-2.

*SUTN9-2 transcriptome in response to rice extract (*indica*)*

To analyze the RNAseq transcriptome, total RNA was purified from SUTN9-2 incubated in BNM-B medium in the presence or absence of rice extract. The main reason for performing the transcriptome analysis was to elucidate the mechanisms and factors involved in cell differentiation and nitrogen fixation by endophytic SUTN9-2 in rice plants. The results obtained showed that the expression of a large number of genes was significantly altered (FDR value ≤0.1) in response to rice extract, with the differential expression of 365 genes being significant, representing 63.8% of DEGs (Fig. 3A). The percentage of DEGs was higher (42.1%) in BNM-B with rice extract-treated SUTN9-2 WT cells than in BNM-B-treated cells (Fig. 3A). The effects of Δ*bclA* in rice extract were also demonstrated because 42.1% of genes were differentially regulated in treated WT SUTN9-2 cells in the BNM-B with rice extract group compared to Δ*bclA* in the BNM-B with rice extract group (Fig. 3A). These results revealed the influence of rice extract and the *bclA* gene on the expression of genes in SUTN9-2.

The highest differentially up-regulated genes in SUTN9-2 WT in response to rice extract were biphenyl-2,3-diol 1,2-dioxygenase (PWE78131.1; 2.33 logFC) followed by 3-(3-hydroxyphenyl) propanoate hydroxylase (PWE78129.1; 1.85 logFC) (Fig. 3D and E). Both of these genes belong to the class of oxidoreductase catalytic enzymes involved in the degradation of plant-related compounds and interact with oxygen atoms, which are incorporated into the substrate. The third and fifth highest differentially up-regulated

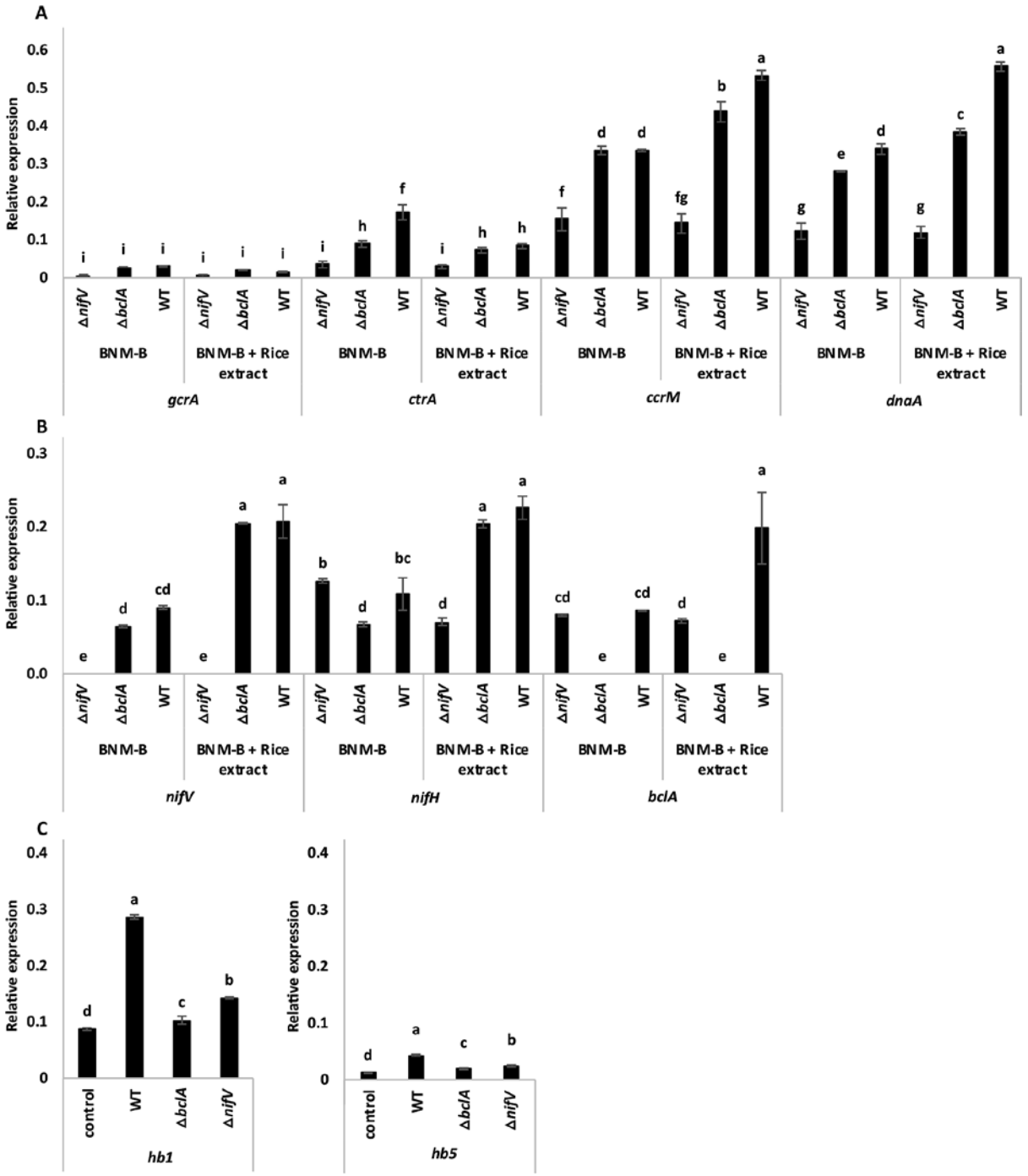

Fig. 2. Relative expression of genes involved in the master cell cycle (A), nitrogen fixation and BclA transporter (B) of SUTN9-2 Δ*nifV*, Δ*bclA*, and WT in response to rice extract (*indica*) at 21 days. Relative expression of the rice hemoglobin gene (*hb1* and *hb5*) in response to SUTN9-2 Δ*nifV*, Δ*bclA*, and WT at 21 dpi (C). Significance at $P \leq 0.05$ was indicated by the mean standard deviation ($n=3$).

genes were the molecular chaperones sGroEL (PWE76243.1; 1.42 logFC) and GroES (PWE81524.1; 1.34 logFC), which are required for the proper folding of many proteins and the function of the nitrogen fixation regulatory protein NifA. The results from the RNAseq analysis indicated that the differential expression of genes involved in nitrogen fixation and nitrogen metabolism in response to rice extract was not significant (Fig. 3E), whereas qRT-PCR results showed that nitrogen fixation genes (*nifH* and *nifV*) were up-regulated (Fig. 2B) because their expression was detected in rice plants (Piromyou *et al.*, 2017; Greetatorn *et al.*, 2019). However, a key difference between the two experiments needs to be considered. The experimental set-up for the transcriptome analysis was performed on a bigger scale than that for qRT-PCR in order to obtain a sufficiently large number of SUTN9-2 cells in rice extract for RNA purification and the transcriptome analysis. This may have been affected by the different oxygen levels in the two

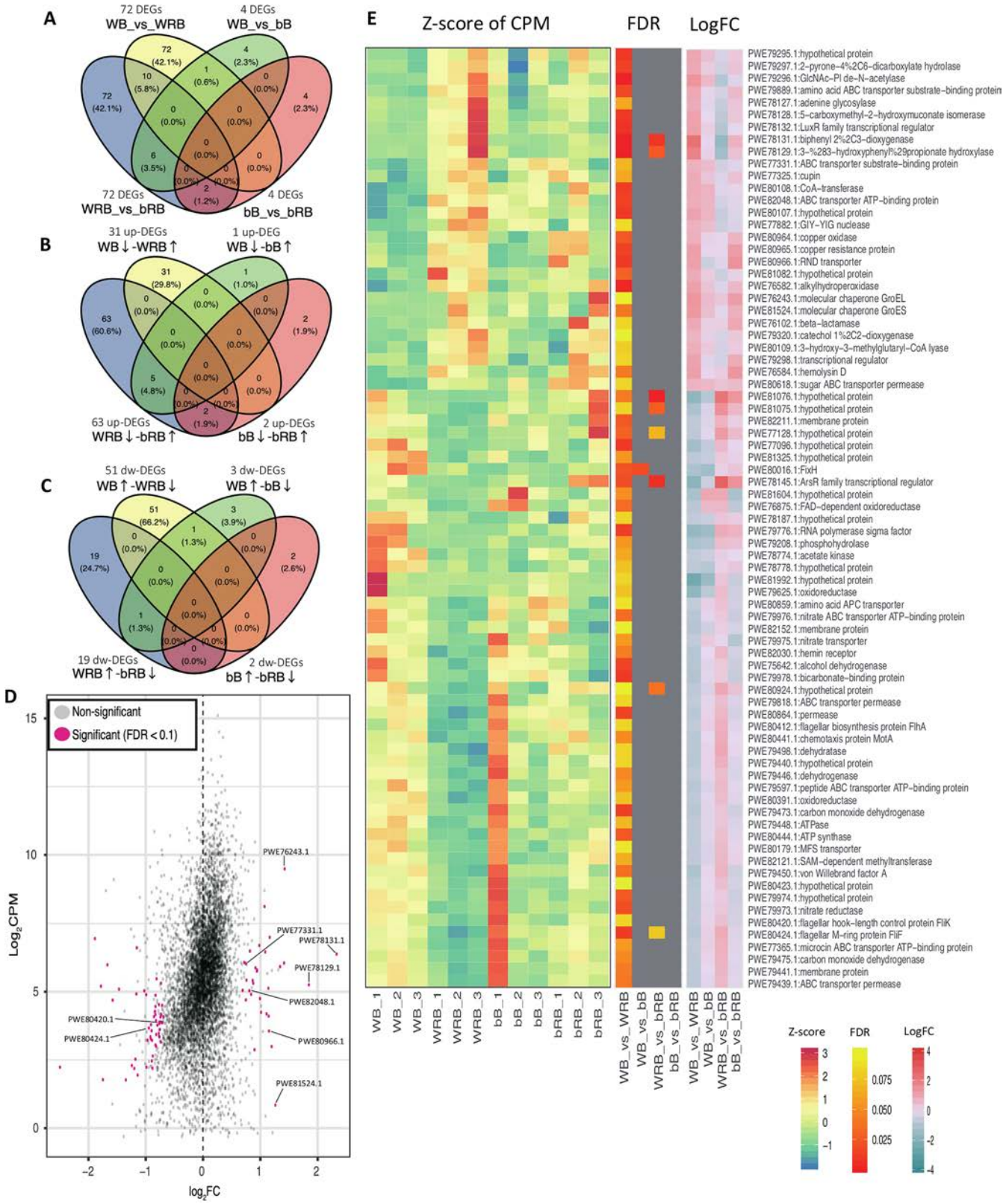

Fig. 3. Differentially expressed genes (DEGs) of SUTN9-2 WT versus Δ*bclA* in response to BNM-B (WB and bB) and BNM-B+rice extract (*indica*) (WRB and bRB). Venn diagram of all DEGs of WB_vs_WRB, WB_vs_bB, WRB_vs_bRB, and bB_vs_bRB (A), showing up-regulated genes (B), and down-regulated genes (C). MA plot of up- and down-regulated DEGs of WB_vs_WRB and genes of interest were labeled (D). Expression pattern of DEGs of WB, bB, WRB, and bRB. FDR (≤0.1) and logFC of DEGs of WB_vs_WRB, WB_vs_bB, WRB_vs_bRB, and bB_vs_bRB. The color scale bars are for normalized expression (E).

experimental set-ups, which perturbed *nif* gene expression in the transcriptome experiment.

DEGs in response to rice extract were also found to code for a protein involved in the cationic peptide transport system ATP-binding process. These DEGs include genes involved in the ABC transporter ATP-binding protein (PWE82048.1; 0.84 logFC) and ABC transporter substrate-binding protein (PWE77331.1; 7.5 logFC). The gene coding for efflux pumps was also found to be differentially up-regulated in the presence of rice extract (Fig. 3D and E). This gene belongs to the resistance nodulation and cell division family (RND) efflux system (PWE80966.1; 1.15 logFC), which may be involved in bacterial defenses against toxic plant metabolites. These results suggested the presence of cationic antimicrobial peptide (CAMP) in rice plants, which is toxic and affects bacterial cell differentiation. The significantly down-regulated DEGs of SUTN9-2 WT in the response to rice extract were related to flagella (PWE80412.1, PWE80420.1, and PWE80424.1) (Fig. 3E). In contrast, these genes were up-regulated in SUTN9-2 Δ*bclA* (PWE80424.1) in response to rice extract (Fig. 3D), indicating the influence of rice extract and Δ*bclA* on cell motility.

qRT-PCR validation

qRT-PCR was performed for DEGs, including *hpaD*, *mhpA*, *groEL*, *groES*, *sapDF*, *sapA*, and *cusF* in SUTN9-2 WT that significantly differed between the BNM-B with rice extract (*indica*) group and BNM-B alone group. The results obtained showed that the majority of genes were up-regulated in response to rice extract, which were similar to those from the transcriptome analysis. The expression of *mhpA* appeared to be more strongly up-regulated in response to rice extract (0.09-fold), followed by *sapDF* (0.07-fold), *cusF* (0.07-fold), *groEL* (0.06-fold), *hpaD* (0.05-foid), *sapA* (0.04-fold), and *groES* (0.01-fold), respectively (Fig. 4).

Discussion

The present results indicate that SUTN9-2 undergoes major changes in cell size and nitrogen fixation efficiency in response to rice extract (Fig. 1). The significant increase observed in the elongated cell size of SUTN9-2 was associated with more efficient nitrogen fixation. Similarly, a TBD process occurs before effective nitrogen fixation is established. These bacteroids are enlarged and polyploid and have lost their capacity to produce progeny (Mergaert *et al.*, 2006; Alunni and Gourion, 2016). The small bacteroid size (1–2.5 μm) in alfalfa (*Medicago sativa*) nodules had a low nucleic acid content and did not exhibit acetylene reduction activity (4.9 μmol C_2H_2 reduced $[10^{10}]^{-1}$ bacteroids). The enlarged bacteroid size (5–7 μm) had a high nucleic acid content and was very active for acetylene reduction (83.3 μmol C_2H_2 reduced $[10^{10}]^{-1}$ bacteroids) (Paau and Cowles, 1978). The bacteroids of bradyrhizobia in *A. afraspera* and *A. indica* exhibit differentiation along with a high DNA content, such that the mean DNA content of free-growing *Bradyrhizobium* sp. ORS285 was 1 to 2 genome complement (1C to 2C) and that of the bacteroid was 7C to 16C ploidy levels (Czernic *et al.*, 2015; Guefrachi *et al.*, 2015).

Cell differentiation by *Bradyrhizobium* bacteroids occurred in the interaction with defensin-like antimicrobial peptides (DEFs), which are found in the IRLC and Dalbergioids legume families with the action of BclA transporters (Guefrachi *et al.*, 2015). Based on a phylogenetic tree of SbmA_bacA domain proteins, three genes of *Bradyrhizobium* sp. strain ORS285 were revealed. Two genes (BRAO285v1_250005 and BRAO285v1_950010) are in the *Bradyrhizobium* homologous clade and one (BRAO285v1_1320006) in the BclA clade (Guefrachi *et al.*, 2015). However, only the mutant in gene BRAO285v1_1320006 had abnormal (undifferentiated) bacteroid cells, and exhibited markedly reduced nitrogen fixation activity in the host plants of *Aeschynomene* spp. (Guefrachi *et al.*, 2015). Similarly, SUTN9-2 Δ*bclA* in the present study belonged to the BclA clade. This gene was disrupted according to high homology with the BclA of ORS285 (Fig. S6). Based on the KO (KEGG [Kyoto Encyclopedia of Genes and Genomes] Orthology) annotation in the reconstruction pathway, the ABC transporter involving the CAMP resistance of SUTN9-2 Δ*bclA* was perturbed by the deletion of the ABC transporter ATP-binding domain (*bclA*; PWE81210.1) belonging to SapDF (Lopez-Solanilla *et al.*, 1998). However, the transcriptomic analysis showed that the ABC transporter substrate-binding protein consisting of SapA (PWE77331.1) and ABC transporter

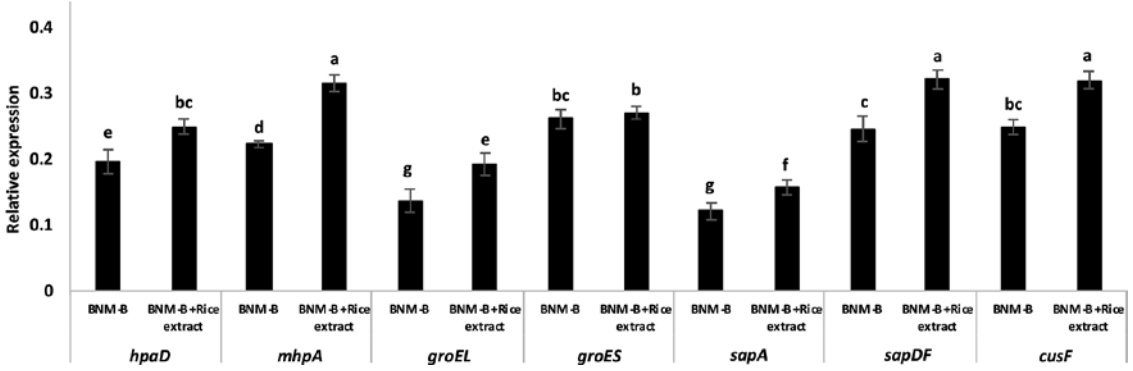

Fig. 4. qRT-PCR analysis data for significant DEGs in SUTN9-2 WT between BNM-B alone and BNM-B with rice extract (*indica*). Significance at $P \leq 0.05$ is indicated by the mean±standard deviation (n=3).

ATP-binding protein consisting of SapDF (PWE82048.1) were significantly and differentially up-regulated in response to rice extract (Fig. 3D and E, Table S2). A phylogenetic analysis based on the sequences of SbmA_BacA domain proteins showed that SapA (PWE77331.1) and SapDF (PWE82048.1) identified in SUTN9-2 were distant from the BclA and *Bradyrhizobium* homologous clades identified in *Bradyrhizobium* spp. However, both of these genes showed 97% similarity to these clades (Fig. S6) (Guefrachi *et al.*, 2015). These results indicate that SapA (PWE77331.1) and SapDF (PWE82048.1) play an important role together with SUTN9-2 Δ*bclA* (PWE81210.1; data not shown) for cell differentiation, nitrogen fixation in symbiosis, and responses to rice extract because the cell differentiation of SUTN9-2 Δ*bclA* was still maintained in both systems of bacteroids in legume plants (data not shown) and bacteria in response to rice extract (Fig. S2 and S4). However, decreases were also observed in the elongated cell sizes and nitrogen fixation activities of Δ*bclA* and Δ*nifV*. These results indicated the effects of *bclA* and *nifV* genes on the cell size and nitrogen fixation activity of SUTN9-2 cells in response to rice extract. The *sap* mutant pathogenic bacterium *Erwinia chrysanthemi* was sensitive to killing by antimicrobial peptides (AMPs) (wheat α-thionin and snaking-1) from potato tubers (Lopez-Solanilla *et al.*, 1998). The Sap transporter consists of SapABCDFZ, which shares homology to the 'ATP-binding cassette' (ABC) family of transporters that show diverse substrate binding and uptake (Hiles *et al.*, 1987; Abouhamad *et al.*, 1991; Parra-Lopez *et al.*, 1993). SapA is predicted to function as a periplasmic solute-binding protein; SapB and SapC as inner membrane permease proteins; SapD and SapF as ATPase subunits; whereas the function of SapZ remains unknown (Mason *et al.*, 2006). Moreover, previous studies identified several genes encoding cysteine-rich peptides (CRPs), also suggesting several uncharacterized AMPs in rice plants (Silverstein *et al.*, 2007), and genes encoding DEFs were also detected in rice plants (Tantong *et al.*, 2016; Li *et al.*, 2017). These results implied the production of DEFs in rice plants interacting with the Sap transporter of SUTN9-2, which affected enlarged cell sizes and high DNA contents in SUTN9-2 in response to rice extract (Fig. 5), similar to the cell differentiation and polyploidy of bacteroids in legume plants affected by NCR peptides.

Moreover, the gene coding for RND transporter efflux pumps (PWE80966.1) was differentially up-regulated in SUTN9-2 WT exposed to rice extract (Fig. 3D and E). Efflux pumps are transport proteins involved in the extrusion of toxic substrates into the external environment (Coutinho *et al.*, 2015). The RND efflux system has been recognized to play an important role in the successful colonization of the apple tree by the phytopathogen *Erwinia amylovora* (Burse *et al.*, 2004). RND efflux systems are strongly up-regulated in *B. kururiensis* M130 in the pres-

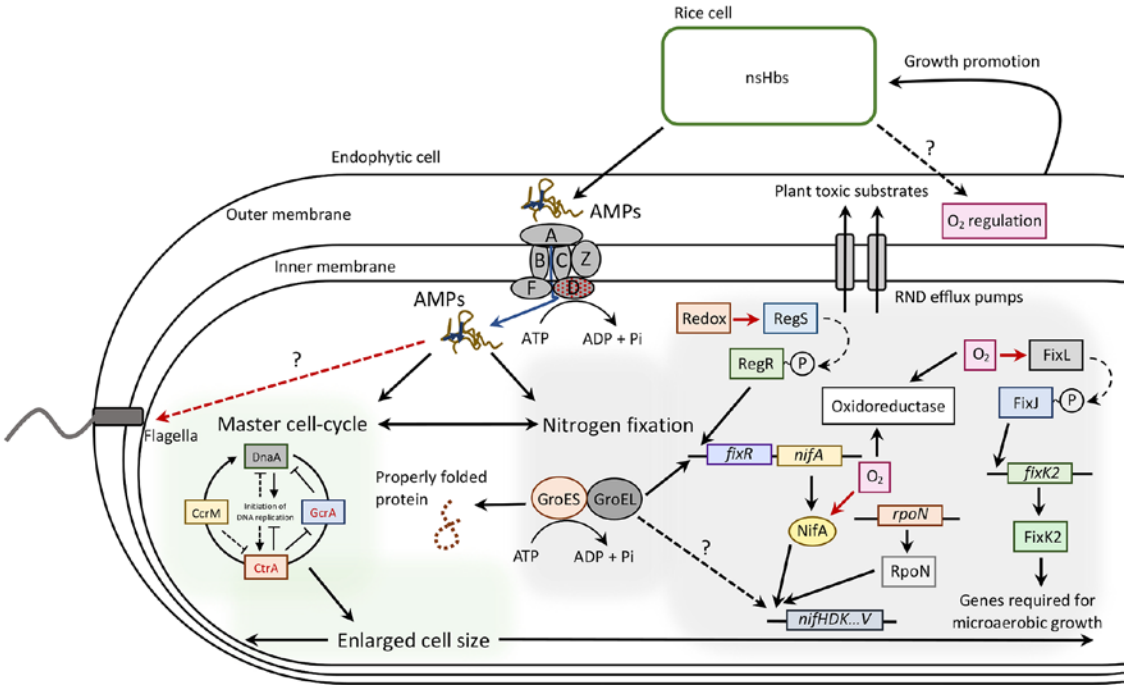

Fig. 5. Working model of cell differentiation and nitrogen fixation by SUTN9-2 exposed to rice extract. The currently implied AMPs from rice plants are predictably recognized by BclA ABC transporters belonging to Sap ABC transporter family-like AMP recognition receptors. AMPs are targeted to the cell membrane of bradyrhizobia, localizing the BclA or Sap transporter, promoting the import of AMPs and providing protection against the antimicrobial activity of these peptides. AMPs affect master cell cycle regulators by reducing GcrA activity and the CtrA autoregulation pathway, but promoting DnaA and CcrM for the initiation of DNA replication, resulting in an increase in DNA content and enlarged cell size. A defect in flagellar activity during cell differentiation was observed. This was followed by increased nitrogen fixation activity, which correlated with an enlarged cell size. Excess oxygen perturbs the nitrogen regulatory protein NifA and reduces nitrogen fixation activity. Oxidoreductase may play a role in controlling oxygen to an appropriate level for nitrogenase activity. GroESL co-regulated with the nitrogen regulatory gene *nifA* and *rpoN* RNA polymerase for the formation of a functional nitrogenase. The successful colonization of bradyrhizobia in rice plants is triggered by the RND efflux system and induced rice nsHbs then regulate low oxygen partial pressure and facilitate nitrogen-fixing endophytic cells.

ence of rice extract (Coutinho *et al.*, 2015). This type of efflux system may be important in bacterial survival defenses against toxic-plant metabolites, including DEFs (Fig. 5).

Flagellar biosynthesis is mostly down-regulated in symbiotic bacteroid cells. The transcriptome analysis of *Mesorhizobium loti* revealed that genes for flagellar formation were strongly repressed under the symbiotic condition because rhizobia under this condition do not need to be motile (Uchiumi *et al.*, 2004; Tatsukami *et al.*, 2013). In addition, the relative expression level of the SUTN9-2 flagella biosynthetic protein (*fliP*) in response to rice root exudate was lower than that without rice root exudate. The expression level of *fliP* also slightly decreased with an increase in plant age (Piromyou *et al.*, 2015b). Similarly, the differentially down-regulated expressed genes of SUTN9-2 WT were related to flagellar biosynthesis (PWE80412.1, PWE80420.1, and PWE80424.1) in response to rice extract. In contrast, these genes were up-regulated in Δ*bclA* (PWE80424.1) in response to rice extract (Fig. 3D). This up-regulation has also been observed in transcriptome experiments with *B. kururiensis* in response to rice extract and allows bacteria to escape host defense responses (Coutinho *et al.*, 2015). Therefore, the down-regulation of flagellar biosynthesis appears to be supporting the cell differentiation of SUTN9-2 in response to rice extract, similar to bacteroids in the symbiotic condition. In contrast, Δ*bclA* lacking some parts of the transporter against plant AMPs up-regulate genes for flagellar biosynthesis, thereby allowing bacteria to move faster in the plant environment in order to escape host defense responses (Fig. 5).

Rice extract (*O. sativa* L. ssp. *indica* cv. Pathum Thani 1) affected cell elongation and nitrogen fixation activity in SUTN9-2 more strongly than rice extract (*O. sativa* L. *japonica* cv. Nipponbare) in all treated SUTN9-2 WT, Δ*bclA*, and Δ*nifV* (Fig. 1D, E, F, and S2). Elongated cell size was smaller in Δ*nifV* than in WT and Δ*bclA* SUTN9-2, and also in Δ*bclA* than in WT SUTN9-2 (Fig. 1D, E, and S2). When SUTN9-2 WT was inoculated into the Thai rice cultivar Pathum Thani 1 (*indica*) and Japanese rice cultivar Nipponbare (*japonica*), the number of SUTN9-2 WT cells at 30 dpi was higher in Thai rice root tissues (*indica*) than in Japanese rice (*japonica*), with 10^3 and 10^1 CFU g^{-1} root fresh weight, respectively (Piromyou *et al.*, 2015b). The population density of SUTN 9-2 was larger in Thai rice (*indica*) than in Japanese rice (*japonica*). In addition, Thai bradyrhizobial strain SUTN9-2 was suggested to promote the total dry weight of rice (*indica*) more effectively than Japanese bradyrhizobial strains (Piromyou *et al.*, 2015a). On the other hand, rice (*indica*) responded positively only to putative Thai rice endophytic bradyrhizobia, while this phenomenon was not observed in Japanese rice (*japonica*) (Piromyou *et al.*, 2015a, 2015b). In addition, the type III secretion system (T3SS) of SUTN9-2 is involved in bradyrhizobial infections in rice plants. The density of the SUTN9-2 T3SS mutant in Thai rice (*indica)* was significantly lower than that of SUTN9-2 WT, and this property was not detected in Japanese rice (*japonica*), indicating SUTN9-2 had the ability to overcome native host rice defense responses through the function of T3SS and also that rice developed a system to protect itself from non-native soil bacteria (Piromyou *et al.*, 2015b). These results imply that the rice cultivar and bacterial strain are important factors that control the compatibility of the rice-bacterium relationship, which may contribute to bradyrhizobia-host evolution (Piromyou *et al.*, 2015b). These results support the existence of a preferable host for SUTN9-2 in rice species, which may also contribute to greater increases in the cell size enlargement and nitrogen fixation activity of SUTN9-2 in response to rice extract from *indica* than to that from *japonica*.

The attenuated pattern of *ctrA* expression in NCR-treated cells during the cell cycle may be caused by reduced GcrA activity or by defects in the CtrA autoregulation pathway. DnaA appears to be necessary for repeated initiation rounds of DNA replication during endoreduplication *in vivo* (Collier, 2012). CcrM DNA methyltransferase is present and active for a short time and essential for methylation at the start of DNA replication (Collier, 2012). In the present study, the results obtained suggested that AMPs in rice extract affect master cell-cycle regulators by suppressing the expression of *ctrA* and promoting *dnaA* and *ccrM* expression, resulting in an increase in cell size and DNA content (Fig. 2A, 5, and S3). In addition, the *nifV* gene may affect cell size enlargement following exposure to rice extract. The expression of genes involved in the nitrogen fixation activity (*nifH* and *nifV*) of SUTN9-2 has been detected in the endophytic relationship with rice plants (Piromyou *et al.*, 2017; Greetatorn *et al.*, 2019). Furthermore, the expression of *nifH*, *nifV*, and *bclA* in WT and Δ*bclA* SUTN9-2 increased in response to rice extract. In contrast, the expression of these genes in SUTN9-2 Δ*nifV* decreased in response to rice extract (Fig. 2B). These results suggest similarities in the expression model between these genes and the genes involved in the master cell cycle following exposure to rice extract. Furthermore, the effects of the interaction between the rice extract and BclA transporter on cell differentiation and nitrogen fixation were implied based on the expression of cell cycle and *nif* genes in response to rice extract (Fig. 5). Non-symbiotic hemoglobins (nsHbs) have been detected in several monocot plants. Rice (*O. sativa*) contains five copies of the *nsHb* gene, namely, *hb1-hb5* (Lira-Ruan *et al.*, 2002). *hb1*, *hb2*, and *hb5* are expressed in rice embryonic organs and vegetative organs, and their appear to function as oxygen carriers or in some aspects of oxygen metabolism (Garrocho-Villegas *et al.*, 2008; Lira-Ruan *et al.*, 2011). Hormone and stress response promoters exist upstream of the rice *hb5* gene, which was transcribed in rice organs. The amino acid sequence and protein model structure of Hb5 differ from those of rice Hbs 1 to 4 (Garrocho-Villegas *et al.*, 2008), suggesting different expression levels between *hb1* and *hb5* (Fig. 2C). However, they are present at very low levels inside host rice cells. In addition, the physiological functions of rice nsHbs are not involved in oxygen transport, but more closely resemble known oxygen sensors (Goodman and Hargrove, 2001). This finding revealed that nsHbs in rice plants may function as a regulator to maintain low oxygen partial pressure for nitrogenase activity to facilitate nitrogen-fixing endophytic cells (Fig. 5).

The RNAseq experiment provided a global view of the gene expression profile in response to rice extract. RNAseq results indicated that SUTN9-2 endophytic cells were affected by rice extract and the Δ*bclA* mutation, which is consistent with the results for significant DEGs (Fig. 3A). The expression of genes involved in the cell cycle and nitrogen fixation was up-regulated when SUTN9-2 was exposed to rice extract. These results are in contrast to those obtained from the transcriptome analysis of SUTN9-2 in the presence of rice extract. These genes did not show significant DEGs in the response to rice extract. Bacteria capable of utilizing biphenyl and phenylpropanoid compounds as carbon and energy sources are widely distributed in natural environments, and may originate from the putrefaction of proteins in soil or as breakdown products of several constituents of plants, such as lignin, various oils, and resins (Ferrandez *et al.*, 1997; Díaz *et al.*, 1998; Wesche *et al.*, 2005). The highest up-regulated DEGs involved in biphenyl-2,3-diol 1,2-dioxygenase (PWE78131.1) and 3-(3-hydroxyphenyl) propanoate hydroxylase (PWE78129.1) were observed. These genes belong to the class of oxidoreductases acting on donors with oxygen and the incorporation of atoms of oxygen into the substrate (Díaz *et al.*, 1998; Wesche *et al.*, 2005), which may have a role in controlling oxygen to an appropriate level for nitrogenase activity (Fig. 3D and E). This result suggests excess oxygen in the experimental set-up for the transcriptome analysis (50-mL tube), which affects nitrogenase activity. A larger scale experiment (50-mL tube) was performed to obtain a large amount of SUTN9-2 cells in rice extract for the transcriptome analysis, more than the experimental set-up for qRT-PCR (10-mL tube). The nitrogenase enzyme complex is highly sensitive to molecular oxygen, which irreversibly inactivates the enzyme. The inhibition of *nif* gene expression by molecular oxygen at the nitrogen regulatory protein NifA post-transcriptional stage was detected in *B. japonicum* (Fischer and Hennecke, 1987; Kullik *et al.*, 1989). Therefore, the suppression of nitrogen fixation genes observed in the transcriptome analysis may be due to the effects of excess oxygen on nitrogenase sensitivity (Fig. 5).

The molecular chaperones GroEL (PWE76243.1) and GroES (PWE81524.1) were identified as significant differentially up-regulated genes in response to rice extract (Fig. 3D and E). GroESL chaperonins are required for the formation of a functional nitrogenase in *B. japonicum*, which is co-regulated together with the symbiotic nitrogen regulatory gene *nifA* and transcribed by σ^{54} RNA polymerase (Fischer *et al.*, 1993, 1999). However, the requirement of chaperonins for nitrogen fixation does not occur at the level of RegSR-NifA-σ^{54}- or FixLJ-FixK-dependent gene regulation (Fischer *et al.*, 1999). This finding indicated that the nitrogen fixation of SUTN9-2 in response to rice extract may be affected by GroESL chaperonins with or without Nif-dependent gene regulation. Collectively, these studies imply that the nitrogen fixation of SUTN9-2 was induced in response to rice extract because the differentially up-regulated genes of GroESL chaperonins were detected (Fig. 5).

Based on the results and transcriptomic findings reported herein, a proposed model for the cell differentiation and nitrogen fixation activity of SUTN9-2 in response to rice extract was shown (Fig. 5). Rice plants are predicted to produce AMPs that are recognized during interactions by AMP recognition receptors (Sap ABC transporter family), thereby promoting the import of AMPs and protecting SUTN9-2 cells against the antimicrobial activity of these peptides. Following the recognition and transduction of these AMPs, several DEGs are induced in SUTN9-2. These AMPs predictably modulate master cell cycle regulators, thereby causing cell differentiation. These interactions induced several processes, including oxidoreductase, GroESL chaperonin, the RND efflux system, and flagellar biosynthesis, which may promote cell size enlargement, nitrogen fixation, and, ultimately, rice growth (Fig. 5). These results imply similarities in the mechanisms and factors involved in cell differentiation and nitrogen fixation between endophytic cells in rice plants and symbiotic cells in legume plants, which are based on similar mechanisms from both the bacterial side (BclA-like transporters) and plant side (AMPs). It is important to understand the mechanisms underlying the regulation of the factors, molecules, and signals of the plant as well as the bacterial cells involved in inducing cell differentiation and nitrogen fixation in endophytic cells required for *in-planta* survival and plant growth promotion. The specialized legume plant genes involved in symbiotic interactions may have arisen from a pre-existing non-symbiotic plant gene, such as rice plants, suggesting convergent coevolution in these distant plant species.

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References

Abouhamad, W.N., Manson, M., Gibson, M.M., and Higgins, C.F. (1991) Peptide transport and chemotaxis in *Escherichia coli* and *Salmonella typhimurium*: characterization of the dipeptide permease (Dpp) and the dipeptide-binding protein. *Mol Microbiol* **5**: 1035–1047.

Alunni, B., and Gourion, B. (2016) Terminal bacteroid differentiation in the legume-*Rhizobium* symbiosis: nodule-specific cysteine-rich peptides and beyond. *New Phytol* **211**: 411–417.

Aramaki, T., Blanc-Mathieu, R., Endo, H., Ohkubo, K., Kanehisa, M., Goto, S., and Ogata, H. (2020) KofamKOALA: KEGG ortholog assignment based on profile HMM and adaptive score threshold. *Bioinformatics* **36**: 2251–2252.

Burse, A., Weingart, H., and Ullrich, M.S. (2004) The phytoalexin-inducible multidrug efflux pump AcrAB contributes to virulence in the fire blight pathogen, *Erwinia amylovora*. *Mol Plant Microbe Interact* **17**: 43–54.

Chaintreuil, C., Giraud, E., Prin, Y., Lorquin, J., Bâ, A., Gillis, M., *et al.* (2000) Photosynthetic bradyrhizobia are natural endophytes of the African wild rice *Oryza breviligulata*. *Appl Environ Microbiol* **66**: 5437–5447.

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018) fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**: i884–i890.

Collier, J. (2012) Regulation of chromosomal replication in *Caulobacter crescentus*. *Plasmid* **67**: 76–87.

Collins, T.J. (2007) ImageJ for microscopy. *Biotechniques* **43**: S25–S30.

Coutinho, B.G., Licastro, D., Mendonça-Previato, L., Cámara, M., and Venturi, V. (2015) Plant-influenced gene expression in the rice endophyte *Burkholderia kururiensis* M130. *Mol Plant Microbe Interact* **28**: 10–21.

Czernic, P., Gully, D., Cartieaux, F., Moulin, L., Guefrachi, I., Patrel, D., *et al.* (2015) Convergent evolution of endosymbiont differentiation in Dalbergioid and Inverted Repeat-Lacking Clade legumes mediated by nodule-specific cysteine-rich peptides. *Plant Physiol* **169**: 1254–1265.

De Nisco, N.J., Abo, R.P., Wu, C.M., Penterman, J., and Walker, G.C. (2014) Global analysis of cell cycle gene expression of the legume symbiont *Sinorhizobium meliloti*. *Proc Natl Acad Sci U S A* **111**: 3217–3224.

Deitch, A.D., Law, H., and deVere White, R. (1982) A stable propidium iodide staining procedure for flow cytometry. *J Histochem Cytochem* **30**: 967–972.

Díaz, E., Ferrández, A., and García, J.L. (1998) Characterization of the hca Cluster encoding the dioxygenolytic pathway for initial catabolism of 3-phenylpropionic acid in *Escherichia coli* K-12. *J Bacteriol* **180**: 2915–2923.

Duncan, D.B. (1955) Multiple Range and Multiple F Tests. *Biometrics* **11**: 1–42.

Ehrhardt, D.W., Atkinson, E.M., and Long, S.R. (1992) Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. *Science* **256**: 998–1000.

Ferrández, A., Garciá, J.L., and Díaz, E. (1997) Genetic characterization and expression in heterologous hosts of the 3-(3-hydroxyphenyl) propionate catabolic pathway of *Escherichia coli* K-12. *J Bacteriol* **179**: 2573–2581.

Fischer, H.M., and Hennecke, H. (1987) Direct response of *Bradyrhizobium japonicum nifA*-mediated *nif* gene regulation to cellular oxygen status. *Mol Gen Genet* **209**: 621–626.

Fischer, H.M., Babst, M., Kaspar, T., Acuña, G., Arigoni, F., and Hennecke, H. (1993) One member of a gro-ESL-like chaperonin multigene family in *Bradyrhizobium japonicum* is co-regulated with symbiotic nitrogen fixation genes. *EMBO J* **12**: 2901–2912.

Fischer, H.M., Schneider, K., Babst, M., and Hennecke, H. (1999) GroEL chaperonins are required for the formation of a functional nitrogenase in *Bradyrhizobium japonicum*. *Arch Microbiol* **171**: 279–289.

Garrocho-Villegas, V., Bustos-Rivera, G., Gough, J., Vinogradov, S.N., and Arredondo-Peter, R. (2008) Expression and in silico structural analysis of a rice (*Oryza sativa*) hemoglobin 5. *Plant Physiol Biochem (Issy-les-Moulineaux, Fr)* **46**: 855–859.

Goodman, M.D., and Hargrove, M.S. (2001) Quaternary structure of rice nonsymbiotic hemoglobin. *J Biol Chem* **276**: 6834–6839.

Greetatorn, T., Hashimoto, S., Sarapat, S., Tittabutr, P., Boonkerd, N., Uchiumi, T., and Teaumroong, N. (2019) Empowering rice seedling growth by endophytic *Bradyrhizobium* sp. SUTN 9-2. *Lett Appl Microbiol* **68**: 258–266.

Guefrachi, I., Pierre, O., Timchenko, T., Alunni, B., Barrière, Q., Czernic, P., *et al.* (2015) *Bradyrhizobium* BclA is a peptide transporter required for bacterial differentiation in symbiosis with *Aeschynomene* legumes. *Mol Plant Microbe Interact* **28**: 1155–1166.

Hakoyama, T., Niimi, K., Watanabe, H., Tabata, R., Matsubara, J., Sato, S., *et al.* (2009) Host plant genome overcomes the lack of a bacterial gene for symbiotic nitrogen fixation. *Nature* **462**: 514–517.

Hashimoto, S., Wongdee, J., Songwattana, P., Greetatorn, T., Goto, K., Tittabutr, P., *et al.* (2019) Homocitrate synthase genes of two wide-host-range *Bradyrhizobium* strains are differently required for symbiosis depending on host plants. *Microbes Environ* **34**: 393–401.

Hiles, I.D., Gallagher, M.P., Jamieson, D.J., and Higgins, C.F. (1987) Molecular characterization of the oligopeptide permease of *Salmonella typhimurium*. *J Mol Biol* **195**: 125–142.

Howard, J.B., and Rees, D.C. (1994) Nitrogenase: a nucleotide-dependent molecular switch. *Annu Rev Biochem* **63**: 235–264.

Kondorosi, E., Mergaert, P., and Kereszt, A. (2013) A paradigm for endosymbiotic life: cell differentiation of *Rhizobium* bacteria provoked by host plant factors. *Annu Rev Microbiol* **67**: 611–628.

Kullik, I., Hennecke, H., and Fischer, H.-M. (1989) Inhibition of *Bradyrhizobium japonicum nifA*-dependent *nif* gene activation by oxygen occurs at the NifA protein level and is irreversible. *Arch Microbiol* **151**: 191–197.

Kumar, S., Nei, M., Dudley, J., and Tamura, K. (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings Bioinf* **9**: 299–306.

Langmead, B., and Salzberg, S.L. (2012) Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9**: 357–359.

Li, B., and Dewey, C.N. (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**: 323.

Li, X.M., Han, H.P., Chen, M., Yang, W., Liu, L., Li, N., *et al.* (2017) Overexpression of OsDT11, which encodes a novel cysteine-rich peptide, enhances drought tolerance and increases ABA concentration in rice. *Plant Mol Biol* **93**: 21–34.

Lira-Ruan, V., Ross, E.J.H., Sarath, G., Klucas, R.V., and Arredondo-Peter, R. (2002) Mapping and analysis of a hemoglobin gene family from *Oryza sativa*. *Plant Physiol Biochem (Issy-les-Moulineaux, Fr)* **40**: 199–202.

Lira-Ruan, V., Ruiz-Kubli, M., and Arredondo-Peter, R. (2011) Expression of non-symbiotic hemoglobin 1 and 2 genes in rice (*Oryza sativa*) embryonic organs. *Commun Integr Biol* **4**: 457–458.

López-Solanilla, E., García-Olmedo, F., and Rodríguez-Palenzuela, P. (1998) Inactivation of the *sapA* to *sapF* locus of *Erwinia chrysanthemi* reveals common features in plant and animal bacterial pathogenesis. *Plant Cell* **10**: 917–924.

Mae, T., and Ohira, K. (1981) The remobilization of nitrogen related to leaf growth and senescence in rice plants (*Oryza sativa* L.). *Plant Cell Physiol* **22**: 1067–1074.

Mason, K.M., Bruggeman, M.E., Munson, R.S., and Bakaletz, L.O. (2006) The non-typeable *Haemophilus influenzae* Sap transporter provides a mechanism of antimicrobial peptide resistance and SapD-dependent potassium acquisition. *Mol Microbiol* **62**: 1357–1372.

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., *et al.* (2006) Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*-legume symbiosis. *Proc Natl Acad Sci U S A* **103**: 5230–5235.

Noisangiam, R., Teamtisong, K., Tittabutr, P., Boonkerd, N., Toshiki, U., Minamisawa, K., and Teaumroong, N. (2012) Genetic diversity, symbiotic evolution, and proposed infection process of *Bradyrhizobium* strains isolated from root nodules of *Aeschynomene americana* L. in Thailand. *Appl Environ Microbiol* **78**: 6236–6250.

Okubo, T., Piromyou, P., Tittabutr, P., Teaumroong, N., and Minamisawa, K. (2016) Origin and evolution of nitrogen fixation genes on symbiosis islands and plasmid in *Bradyrhizobium*. *Microbes Environ* **31**: 260–267.

Paau, A.S., and Cowles, J.R. (1978) Development of bacteroids in alfalfa (*Medicago sativa*) nodules. *Plant Physiol* **62**: 526–530.

Pagan, J., Child, J., Scowcroft, W., and Gibson, A. (1975) Nitrogen fixation by *Rhizobium* cultured on a defined medium. *Nature* **256**: 406–407.

Parra-Lopez, C., Baer, M.T., and Groisman, E.A. (1993) Molecular genetic analysis of a locus required for resistance to antimicrobial peptides in *Salmonella typhimurium*. *EMBO J* **12**: 4053–4062.

Penterman, J., Abo, R.P., De Nisco, N.J., Arnold, M.F., Longhi, R., Zanda, M., and Walker, G.C. (2014) Host plant peptides elicit a transcriptional response to control the *Sinorhizobium meliloti* cell cycle during symbiosis. *Proc Natl Acad Sci U S A* **111**: 3561–3566.

Piromyou, P., Greetatorn, T., Teamtisong, K., Okubo, T., Shinoda, R., Nuntakij, A., *et al.* (2015a) Preferential association of endophytic bradyrhizobia with different rice cultivars and its implications for rice endophyte evolution. *Appl Environ Microbiol* **81**: 3049–3061.

Piromyou, P., Songwattana, P., Greetatorn, T., Okubo, T., Kakizaki, K.C., Prakamhang, J., *et al.* (2015b) The type III secretion system (T3SS) is a determinant for rice-endophyte colonization by non-photosynthetic *Bradyrhizobium*. *Microbes Environ* **30**: 291–300.

Piromyou, P., Greetatorn, T., Teamtisong, K., Tittabutr, P., Boonkerd, N., and Teaumroong, N. (2017) Potential of rice stubble as a reservoir of bradyrhizobial inoculum in rice-legume crop rotation. *Appl Environ Microbiol* **83**: e01488-01417.

Renier, A., Maillet, F., Fardoux, J., Poinsot, V., Giraud, E., and Nouwen, N. (2011) Photosynthetic *Bradyrhizobium* sp. strain ORS285 synthesizes 2-O-methylfucosylated lipochitooligosaccharides for nod gene-dependent interaction with *Aeschynomene* plants. *Mol Plant Microbe Interact* **24**: 1440–1447.

Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.

Silverstein, K.A., Moskal Jr, W.A., Wu, H.C., Underwood, B.A., Graham, M.A., Town, C.D., and VandenBosch, K.A. (2007) Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants. *Plant J* **51**: 262–280.

Somasegaran, P., and Hoben, H.J. (2012) *Handbook for Rhizobia: Methods in Legume*-Rhizobium *Technology*. New York, NY: Springer.

Steel, R.G., and Torrie, J.H. (1980) *Principles and Procedures of Statistics*. New York, NY: McGraw-Hill.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**: 2731–2739.

Tantong, S., Pringsulaka, O., Weerawanich, K., Meeprasert, A., Rungrotmongkol, T., Sarnthima, R., *et al.* (2016) Two novel antimicrobial defensins from rice identified by gene coexpression network analyses. *Peptides (N Y, NY, U S)* **84**: 7–16.

Tatsukami, Y., Nambu, M., Morisaka, H., Kuroda, K., and Ueda, M. (2013) Disclosure of the differences of *Mesorhizobium loti* under the free-living and symbiotic conditions by comparative proteome analysis without bacteroid isolation. *BMC Microbiol* **13**: 180.

Teamtisong, K., Songwattana, P., Noisangiam, R., Piromyou, P., Boonkerd, N., Tittabutr, P., *et al.* (2014) Divergent nod-containing *Bradyrhizobium* sp. DOA9 with a megaplasmid and its host range. *Microbes Environ* **29**: 370–376.

Tsai, J.-W., and Alley, M. (2000) Proteolysis of the McpA chemoreceptor does not require the *Caulobacter* major chemotaxis operon. *J Bacteriol* **182**: 504–507.

Uchiumi, T., Ohwada, T., Itakura, M., Mitsui, H., Nukui, N., Dawadi, P., *et al.* (2004) Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome. *J Bacteriol* **186**: 2439–2448.

Vasse, J., De Billy, F., Camut, S., and Truchet, G. (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. *J Bacteriol* **172**: 4295–4306.

Wesche, J., Hammer, E., Becher, D., Burchhardt, G., and Schauer, F. (2005) The *bphC* gene-encoded 2, 3-dihydroxybiphenyl-1, 2-dioxygenase is involved in complete degradation of dibenzofuran by the biphenyl-degrading bacterium *Ralstonia* sp. SBUG 290. *J Appl Microbiol* **98**: 635–645.

Young, M.D., Wakefield, M.J., Smyth, G.K., and Oshlack, A. (2010) Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol* **11**: R14.

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Taxon Richness of "Megaviridae" Exceeds those of Bacteria and Archaea in the Ocean

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Since the discovery of the giant mimivirus, evolutionarily related viruses have been isolated or identified from various environments. Phylogenetic analyses of this group of viruses, tentatively referred to as the family "Megaviridae", suggest that it has an ancient origin that may predate the emergence of major eukaryotic lineages. Environmental genomics has since revealed that Megaviridae represents one of the most abundant and diverse groups of viruses in the ocean. In the present study, we compared the taxon richness and phylogenetic diversity of Megaviridae, Bacteria, and Archaea using DNA-dependent RNA polymerase as a common marker gene. By leveraging existing microbial metagenomic data, we found higher richness and phylogenetic diversity in this single viral family than in the two prokaryotic domains. We also obtained results showing that the evolutionary rate alone cannot account for the observed high diversity of Megaviridae lineages. These results suggest that the Megaviridae family has a deep co-evolutionary history with diverse marine protists since the early "Big-Bang" radiation of the eukaryotic tree of life.

Key words: *Mimiviridae*, Megaviridae, species richness, RNA polymerase, ocean metagenome

Acanthamoeba polyphaga mimivirus (APMV), initially mistaken as a Gram-positive bacterium when it was isolated via amoeba co-culture in the 1990s (4), was recognized in 2003 as a *bone fide* virus, indeed a 'giant virus' with a large 750-nm virion including a fibril-containing rigid surface layer (40). APMV possesses a 1.2-Mb linear dsDNA genome coding for more than 1,000 genes (43, 65), which is more than those encoded on the genomes of some small prokaryotes. It is classified as a member of nucleocytoplasmic large DNA viruses (NCLDVs), the proposed order "Megavirales" (17), together with various giant viruses discovered after APMV (6, 45, 46, 63, 78). The unexpected dimensions and complexity of APMV and other exotic giant viruses triggered the reassessment of differences between cellular and viral life forms (66), fueled debates on the origin of viruses (14, 54, 56), and revived interest in re-defining the concept of viruses (13, 15, 27).

Phylogenetic studies have indicated multiple origins of APMV genes; some APMV genes appear to be of viral origin, whereas others appear to originate from cellular organisms (23, 24, 55, 71) or unknown sources (*i.e.*, ORFans). Despite the apparent mosaicity of its complex genome, one coherent finding that emerges from these studies is that the origin of APMV lineage is old, being as ancient as the emergence of the Eukarya domain in the Tree of Life (65). In particular, the ancient origin of APMV and related giant viruses has been supported by phylogenies of replication- and transcription-related genes (1, 72, 77, 89). The antiquity of giant viruses further inspired hypotheses of a putative "Fourth Domain of Life", although these are still highly controversial (7, 16, 44, 57, 59, 83, 90).

Since the discovery of APMV, numerous APMV relatives have been isolated using amoeba co-culture from different environments including marine sediment, river, soil, contact lens liquid, and sewage water (2, 31, 41, 88). These viruses are subdivided into lineage A, B and C mimiviruses (88). These amoebal mimiviruses, together with additional giant viruses infecting microzooplankton, such as *Cafeteria roenbergensis virus* (CroV) (25) and *Bodo saltans virus* (19) as well as Klosneuviruses recently identified in metagenomes (71), constitute the family *Mimiviridae* officially approved by the International Committee on Taxonomy of Viruses (ICTV). Shortly after the discovery of APMV, algal viruses isolated in the sea, such as *Chrysochromulina ericina virus* (CeV) and *Pyramimonas orientalis virus*, were found to form a strongly supported monophyletic group with APMV based on DNA polymerase phylogenies (28, 51, 52). Since then, the monophyletic group has grown with the inclusion of *Phaeocystis globosa virus* (PgV) (69), *Aureococcus anophagefferens virus* (AaV) (53), *Haptolina ericina virus* (HeV RF02), and *Prymnesium kappa viruses* (PkV RF01 and PkV RF02) (34) as well as metagenome-assembled Organic lake phycodnaviruses (OLPV1 and OLPV2) (86) and Yellowstone lake mimivirus (YSLGV) (91). Some of these viruses are officially, but inappropriately, classified in the *Phycodnaviridae* family. Arslan *et al.* proposed to reassign the family "Megaviridae" to the monophyletic group that combines the above mentioned mimiviruses, zooplankton giant viruses, and algal giant viruses (2). Gallot-Lavallée *et al.* recently proposed to classify mimiviruses and microzooplankton giant viruses of the Megaviridae family into the subfamily "Megamimivirinae" and the algal viruses into the subfamily "Mesomimivirinae" (29). The tentative Megaviridae family is the focus of the present study and it is this proposed Megaviridae nomenclature that we use henceforth.

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Megaviridae constitutes approximately 36% of giant viruses in epipelagic oceans, with their abundance being in the order of 10^3 to 10^5 genomes mL^{-1} sea water (32). A recent metatranscriptomic study also demonstrated that members of Megaviridae are active everywhere in sunlit oceans and infect eukaryotic communities of various size ranges from piconanoplankton (0.8–5 μm) up to mesoplankton (180–2,000 μm) (9). The hosts of isolated Megaviridae are still limited to a handful of eukaryotic lineages, but already encompass an extremely wide range of unicellular eukaryotes, including Amoebozoa, Stramenopiles (Cafeteriaceae and Pelagophyceae), Euglenozoa (Kinetoplastida), Haptophyceae (Phaeocystales and Prymnesiales), and Viridiplantae (Chlorophyta). Sequence similarity searches between metagenomic sequences and known Megaviridae genomes also indicated the existence of many uncultured Megaviridae lineages in marine environments (32). Taken together with the inferred antiquity of Megaviridae, these findings suggest that the host range of Megaviridae is markedly wider than currently recognized, and species richness inside the family Megaviridae may consequently be comparable with that of protists, which undoubtedly represent the major part of eukaryotic species' diversity.

DNA-dependent RNA polymerases (RNAPs) of cellular organisms are multisubunit protein complexes, the structures of which have been elucidated for all three domains of life (Bacteria, Archaea, and Eukarya) (82). The number of subunits constituting the machinery differs across the domains of life (50, 82). Among them, the two largest subunits (hereafter referred to as Rpb1 and Rpb2) are both highly conserved and mostly encoded as single copy genes in the three domains of life, although eukaryotes commonly possess distant paralogs (33). Rpb1 and Rpb2 of eukaryotes correspond to the RNA polymerase β' and β subunits of bacteria, and to the RpoA and RpoB of archaea, respectively (39, 80, 81). Archaeal RpoA is composed of two subunits encoded by two small genes. Rpb1 and Rpb2 have been selected as two of the 102 genes suitable for the assessment of phylogenetic relationships among prokaryotes (*i.e.*, 102 Nearly Universal Trees) (38). Moreover, Rpb1 and Rpb2 are conserved in all known members of Megaviridae (50). Transcriptomic and proteomic studies have indicated that Rpb1 and Rpb2 are expressed during infection and packed into mimivirus capsids (12, 42, 43, 67). Bacteriophages, such as T7 and SP6, encode single-subunit RNAPs, which are phylogenetically unrelated to multisubunit RNAPs (10, 74). Therefore, Rpb1 and Rpb2 possess the required characteristics to be used as phylogenetic markers for both Megaviridae and cellular organisms (65, 72). In the present study, we investigate the taxon richness (or lineage richness) and phylogenetic diversity (PD) of Bacteria, Archaea, and Megaviridae based on Rpb1 and Rpb2 sequences found in marine microbial metagenomes derived from prokaryotic size fractions.

Materials and Methods

Sequence data

We used the UniProt database (Release 2016_03) (79) and the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) (61) Viral Section (Release 75) database to collect Rpb1 and Rpb2 protein sequences from cellular organisms and NCLDVs. We additionally used the GenomeNet/Virus-Host Database (49) to retrieve the nucleotide sequences of Rpb1 and Rpb2.

Marine metagenomic sequence data were obtained from CAMERA (75) and the *Tara* Oceans project (32, 76) (Table S1). In addition, we obtained metagenomic data for other non-marine environments from CAMERA and KEGG/MGENES (35). Collectively, we used metagenomic data derived from 58 projects (Table S1). Based on metagenomic data, we initially prepared files for amino acid sequences for open reading frames (ORFs) that were longer than or equal to 150 codons. The total number of ORFs was 149,645,996: marine metagenomes (101,856,227 ORFs, 68%), other aquatic environmental metagenomes (8,385,210 ORFs, 5.6%), mammal-associated microbial metagenomes (38,341,510 ORFs, 26%), and other metagenomes (1,063,049 ORFs, 0.7%). Most of the analyses presented in the present study focused on data from marine metagenomes, mainly derived from two large scale oceanic microbiome projects: *Tara* Oceans and the Global Ocean Sampling project (68). Data from other environments were used to confirm marine data.

Non-synonymous and synonymous substitution rate ratio

In order to estimate the level of functional constraint on Rpb1/Rpb2 coding sequences, we computed the numbers of non-synonymous (Ka) and synonymous (Ks) substitutions per site and their ratio (ω=Ka/Ks) using a maximum likelihood method implemented in the codeml program in the PAML package (85). We used the Mann-Whitney U test to assess the significance of differences in ω values between Megaviridae and bacterial sequences.

Reference sequence alignments and phylogenetic trees

We identified Rpb1 and Rpb2 homologs in the UniProt and RefSeq databases using HMMER/HMMSEARCH (version 3.1; E-value$<1\times10^{-5}$) (20) based on profile hidden Markov models that we built from alignments of Rpb1 (COG0086) and Rpb2 homologs (COG0085) (30). We used CD-HIT version 4.6 to reduce the redundancy of the collected known Rpb1/2 sequences (47). The resulting non-redundant sequences were aligned using MAFFT v7.215 (36) with default parameters and alignment columns containing gaps were trimmed using trimAl v1.2rev59 (8). We referred to the resulting reference sequence alignments for Rpb1 and Rpb2 as RAln-Rpb1 and RAln-Rpb2, respectively. We also generated reference sequence alignments solely composed of sequences from Megaviridae, Bacteria, and Archaea, and referred to the alignments as RAln-MBA-Rpb1 and RAln-MBA-Rpb2. Maximum likelihood phylogenetic trees were constructed with the use of FastTree version 2.1.7 (64). The resulting reference trees for Rpb1 and Rpb2 were referred to as RTree-Rpb1 and RTree-Rpb2, respectively. The significance of the branches in the trees was assessed using the Shimodaira-Hasegawa test (73) implemented in FastTree. Reference alignments and trees are available at the GenomeNet ftp site (ftp://ftp.genome.jp/pub/db/community/RNAP_ref_tree).

Identification of RNAP homologs in metagenomes

In order to identify Rpb1 and Rpb2 homologs in metagenomic sequence data, we used HMMER/HMMSEARCH (version 3.1) (20) with the default parameters and built 10 HMMs for Rpb1 and 10 HMMs for Rpb2, each of which represents a group of phylogenetically related sequences in our reference phylogenetic trees. Specifically, these HMMs represent Megaviridae, other NCLDV groups 1 and 2 (group 1: *Asfarviridae*, *Poxviridae*; 2: *Ascoviridae*, *Iridoviridae*, Pandoravirus, Pithovirus), Bacteria, Archaea, Eukaryotes I to IV (I: RNAP I; II: RNAP II; III: RNAP III; IV: RNAP IV/V), and RNA polymerases of plastids. We screened metagenomic data for the Rpb1 and Rpb2 homologs (≥150 amino acid residues) using these profile HMMs with HMMSEARCH (E-value$<1\times10^{-5}$).

Taxonomic classification

Phylogenetic placement is a bioinformatics technique that is used to identify the most likely phylogenetic position for a given query sequence on a reference phylogenetic tree. Pplacer is one of the

phylogenetic placement tools that efficiently analyze large numbers of sequences, including short metagenomic sequences, within linear computation time (48). Metagenomic Rpb1/Rpb2 sequence fragments were aligned on the reference alignments (*i.e.*, RAln-Rpb1 and RAln-Rpb2) using HMMALIGN and placed on the reference phylogenetic trees (RTree-Rpb1 and RTree-Rpb2) using Pplacer with the use of the maximum likelihood mode. These Rpb1 and Rpb2 fragments were taxonomically classified into the above-mentioned 10 phylogenetic groups based on their phylogenetic placement.

Taxon richness and PD

Metagenomic Rpb1/Rpb2 fragments that were taxonomically assigned to Megaviridae, Bacteria, or Archaea were re-aligned on the RAln-MBA-Rpb1 or RAln-MBA-Rpb2 reference sequence alignments using HMMALIGN. Since metagenomic sequences were often shorter than full-length sequences in the reference alignments, we examined taxon richness (*i.e.*, the number of sequence clusters) (3) and PD (22) along the alignment using a 100-residue sliding window on the alignments (with a step size of 10 residues). Metagenomic sequences exhibiting gaps at >10% of the sites in the alignment window were discarded.

Taxon richness was computed based on sequence clustering by the ucluster_fast command of USEARCH v7.0 (21) with three cut-off values for amino acid sequence identities (*i.e.*, 70%, 80%, and 90%). The significance of differences between richness curves was assessed using a Log-rank test (70).

PD was calculated using Phylogenetic Diversity Analyzer (PDA) version 1.0 (11), based on FastTree phylogenetic trees of metagenomic sequences that were aligned inside the sliding window. In order for PD scores to be comparable between Megaviridae, Bacteria, and Archaea, we constructed a phylogenetic tree with 1,000 randomly selected sequences for each organism group and calculated the PD score.

RNAP paralogs in Megaviridae

Some of the sequenced viruses of Megaviridae, such as PgV (69), OLPV1, and OLPV2, encode two distantly related Rpb2 in their genomes. In order to eliminate the effect of the presence of these paralogs on the richness assessment of Megaviridae Rpb2, we classified Megaviridae Rpb2 metagenomic sequences into two paralogous groups based on phylogenetic reconstructions and performed additional rarefaction analyses for each of the paralogous groups.

Results

Functional constraints on Megaviridae Rpb2 and Rpb1 are higher than those on bacterial homologs

The functional constraint on a protein sequence may be estimated by the ratio (ω) of non-synonymous (Ka) and synonymous (Ks) substitution rates. A small ω value indicates an elevated level of functional constraint (*i.e.*, slow pace of amino acid sequence evolution), while a large ω value, which is typically smaller than 1 for a functional protein coding sequence, indicates a low level of functional constraint (*i.e.*, fast amino acid sequence evolution). We computed ω values for Megaviridae Rpb2/Rpb1 by comparing close homologs. We also computed ω values for bacterial Rpb2/Rpb1 by comparing genes of *Escherichia coli* K-12 MG1655 with those of other closely related bacteria (Fig. 1). The average ω value for Megaviridae Rpb2 was 0.0205, while it was 0.0582 for bacterial Rpb2. The average ω value for Megaviridae Rpb1 was 0.0105, while it was 0.0811 for bacterial Rpb1. These results suggest that functional constraints on Megaviridae Rpb2/Rpb1 were higher than those on bacterial homologs (P=0.00003 for Rpb2, P=0.003 for Rpb1); however, the sample sizes for Megaviridae were small (n=7 for Rpb2, n=3 for Rpb1).

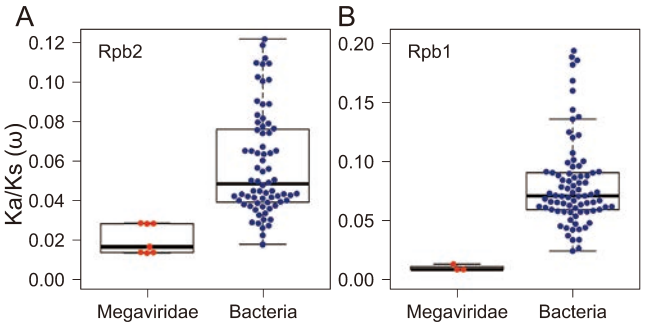

Fig. 1. Functional constraints on Megaviridae Rpb2 and Rpb1. Non-synonymous (Ka) and synonymous (Ks) substitution rate ratios (ω=Ka/Ks) are plotted for Megaviridae and bacterial Rpb2 (A) and Rpb1 (B). We selected pairs of orthologs from Megaviridae (shown in red dots) based on the following criteria: Ka>0.01, Ks<5.0, and the percent standard error of ω being below 25%. These closely related pairs of viral genes were all from amoeba infecting mimiviruses. We selected pairs of orthologs between genes from *Escherichia coli* K-12 MG1655 and genes from other bacteria (shown in blue dots) based on the following criteria: Ka>0.01, Ks<10.0, and the percent standard error of ω being below 25%. ω values were significantly lower for Megaviridae than for bacterial homologs, indicating a higher level of evolutionary constraint on Megaviridae homologs.

Reference trees and taxonomic classification of metagenomic sequences

We identified 59,938 Rpb2 and 40,534 Rpb1 homologs in the UniProt/RefSeq sequence databases using profile HMMs derived from COG0085 (Rpb2) and COG0086 (Rpb1). Viral Rpb2/Rpb1 sequences identified by this search all originated from NCLDVs. Among these sequences, 511 Rpb2 and 575 Rpb1 sequences were selected as reference sequences after reducing redundancy by clustering and discarding unusually long and short sequences. Based on the reference sequences, we built reference phylogenetic trees for Rpb2 (Fig. 2A) and Rpb1 (Fig. 2B). The reference trees were generally consistent with the classification of prokaryotes and viruses as well as eukaryotic paralogs.

Using profile HMMs built from these reference sequences, 248,101 and 252,609 sequences were obtained from metagenomes as candidates of environmental Rpb2 and Rpb1, respectively. These environmental sequences were phylogenetically classified using the reference trees described above, and specific phylogenetic groups were successfully assigned to 195,195 Rpb2 and 214,521 Rpb1 sequences (Table 1). The taxonomic assignments were dominated by Bacteria (80% for Rpb2, 81% for Rpb1), Archaea (5.7% for Rpb2, 6.6% for Rpb1), and Megaviridae (10.2% for Rpb2, 6.1% for Rpb1) as expected from the microbial size fractions (enriched with prokaryotic size organisms and viruses, Table S1) targeted by most of the analyzed metagenomes. Most of the sequences that were taxonomically assigned to Megaviridae were found in marine metagenomes (Rpb2: 18,633 [93.3%]; Rpb1: 12,225 [93.0%]), which is consistent with the previous finding of the high abundance of Megaviridae in the sea (32). The detection of eukaryotic sequences was limited (1,824 for Rpb2 and 1,276 for Rpb1 for RNA polymerase II from marine metagenomes) and likely biased towards picoeukaryotes due to the

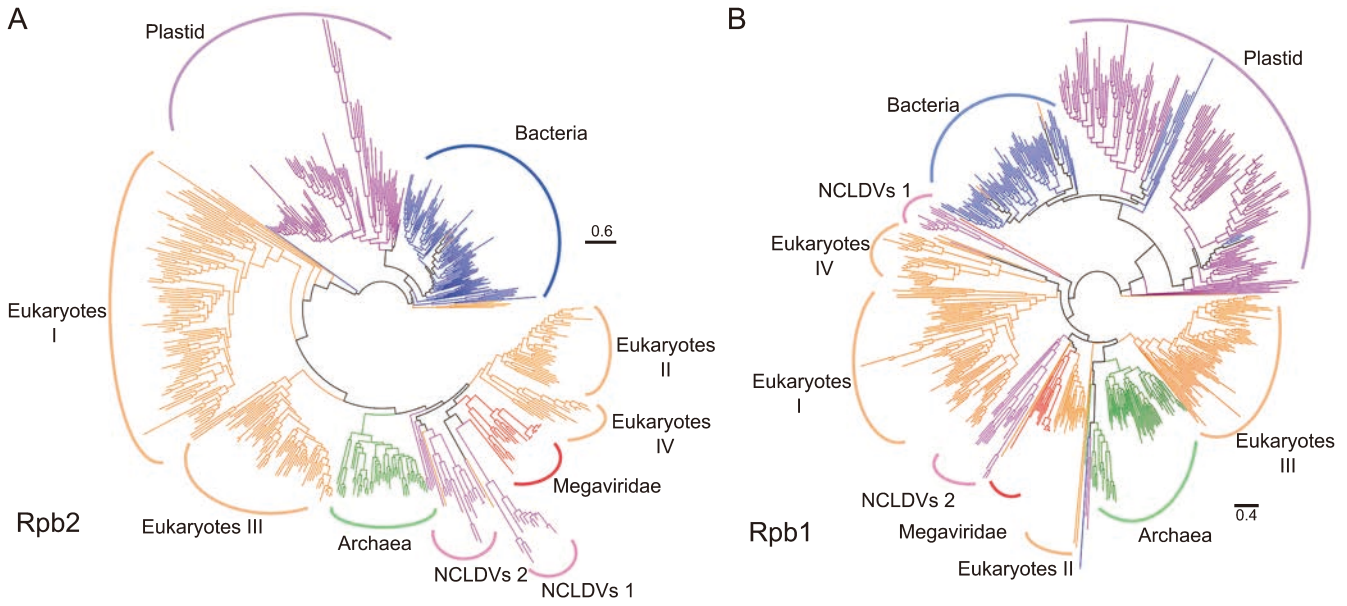

Fig. 2. Maximum likelihood phylogenetic trees of Rpb2 and Rpb1. The Rpb2 tree (RTree-Rpb2) was constructed using 511 representative sequences (A), and the Rpb1 tree (RTree-Rpb1) with 575 representative sequences (B). Branches are colored as follows: Eukaryotes I-IV (orange), Bacteria (blue), Archaea (green), Megaviridae (red), plastid (purple), and other NCLDVs (pink).

Table 1. Number of taxonomically assigned metagenome sequences.

	Environment									
	Marine		Other aquatic		Mammal associated		Other		Total	
Operational clade name	Rpb2	Rpb1	Rpb2	Rpb1	Rpb2	Rpb1	Rpb2	Rpb1	Rpb2	Rpb1
Eukaryote I	690	741	16	14	82	109	0	2	788	866
Eukaryote II	1,824	1,276	77	28	78	108	4	2	1,983	1,414
Eukaryote III	729	854	17	10	68	101	0	1	814	966
Eukaryote IV/V	82	54	7	5	0	3	0	0	89	62
Bacteria	111,124	125,874	6,387	6,740	38,192	39,798	588	625	156,291	173,037
Archaea	10,177	12,826	640	784	56	102	300	341	11,173	14,053
Megaviridae	18,633	12,225	1,330	841	10	79	0	0	19,973	13,145
Chloroplast	2,540	7,666	27	455	91	1,159	2	5	2,660	9,285
NCLDVs 1	119	80	20	6	19	9	0	0	158	95
NCLDVs 2	1,135	1,484	126	110	4	2	1	2	1,266	1,598
Total	147,053	163,080	8,647	8,993	38,600	41,470	895	978	195,195	214,521

filter size range. Therefore, we excluded eukaryotic sequences in subsequent analyses and focused on Megaviridae, Bacteria, and Archaea sequences identified in marine metagenomic data unless otherwise specified.

Taxon richness of Megaviridae RNAP is greater than those of Bacteria and Archaea

The average lengths of the Rpb2 and Rpb1 reference sequences were as follows: Megaviridae Rpb2 (1,239 aa) and Rpb1 (1,392 aa); bacterial Rpb2 (1,282 aa) and Rpb1 (1,346 aa); archaeal Rpb2 (1,132 aa) and Rpb1 (1,373 aa). In contrast, most of the metagenomic Rpb2/Rpb1 sequences were found to be partial: Megaviridae Rpb2 (314 aa) and Rpb1 (314 aa); bacterial Rpb2 (292 aa) and Rpb1 (285 aa); archaeal Rpb2 (313 aa) and Rpb1 (303 aa). These sequences were aligned on reference Rpb2 and Rpb1 alignments (RAln-MBA-Rpb2 and RAln-MBA-Rpb1) composed of complete sequences from Megaviridae, Bacteria, and Archaea (Fig. S1). We assessed taxon richness by generating operational taxonomic units (OTUs) from sequences aligned inside a 100-aa window along the reference alignments. In order to generate OTUs, clustering was performed with three amino acid sequence identity thresholds (*i.e.*, 90%, 80%, and 70% identities). Rpb2 and Rpb1 of Megaviridae showed a higher number of OTUs than those of Bacteria or Archaea at all resampling levels at each of the three arbitrarily selected sequence rich regions (Fig. 3 and Fig. S2A and S2B). Similar results were obtained when metagenomic sequences from other environments, such as freshwater and the human gastrointestinal tract, were included (Fig. S2C and S2D), and confirmed along the entire length of the reference alignments; Megaviridae exhibited a larger number of OTUs than the two cellular domains whatever the regions of Rpb2 and Rpb1 considered (Fig. 4A and B). Log-rank tests indicated that differences in the number of OTUs between Megaviridae and the two cellular domains were significant (Fig. 4C and D).

Effects of the existence of Megaviridae Rpb2 paralogs

Rpb2 and Rpb1 were encoded as a single copy in most of the sequenced bacterial and archaeal genomes; only 1.96%

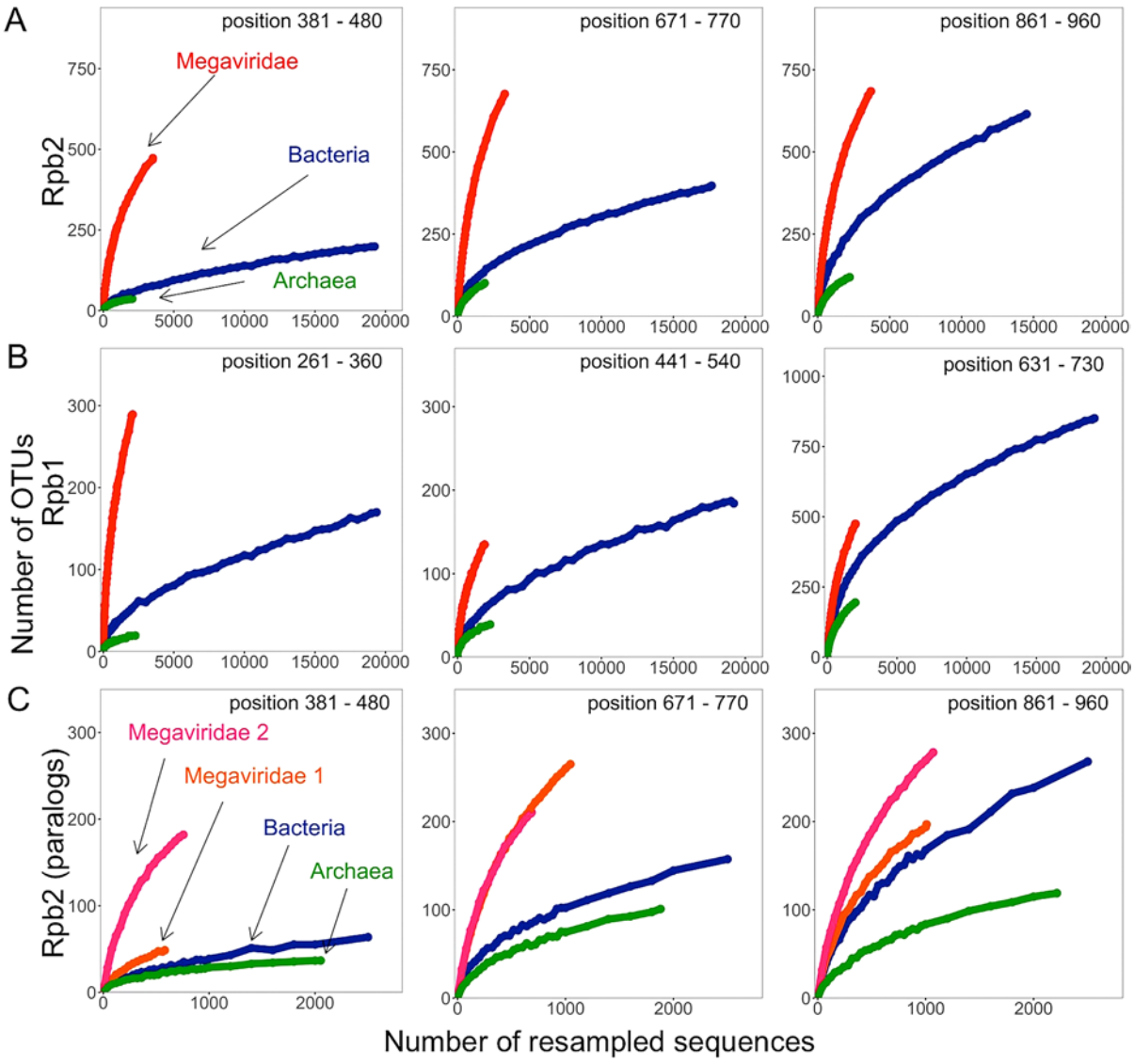

Fig. 3. Rarefaction curves of richness for metagenomic Rpb2/Rpb1 sequences. The X-axis indicates the numbers of resampled sequences for each organism group and the Y-axis indicates the average number of OTUs over 10 resamplings. Sequence clustering was performed using an 80% amino acid sequence identity cut-off. Regarding each Rpb2 (A, C) and Rpb1 (B), three positions of the reference alignment were selected for comparisons of taxon richness between Megaviridae (red), Bacteria (blue), and Archaea (green). In (C), the taxon richness of two paralogous groups of Megaviridae Rpb2 (pink/orange) were assessed separately.

(Rpb2) and 2.97% (Rpb1) of bacterial, and 1.00% (Rpb2) and 1.00% (Rpb1) of archaeal genomes presented paralogs. However, during the reconstruction of RNAP reference trees, we noted that some Megaviridae, such as PgV and OLPV1/2, encoded two copies of Rpb2 genes. The existence of these paralogs may contribute to increasing the richness of the homologous group of sequences, hence inducing bias in taxon richness interpretations. In order to investigate the evolutionary relationships of these paralogs, we reconstructed Rpb2 trees, including metagenomic sequences, based on the same three sequence rich sub-alignment regions. The results of these analyses revealed that the Rpb2 paralogs were only distantly related in the reconstructed phylogenetic trees (Fig. S3). A set of Rpb2 from PgV, OLPV1, and OLPV2 grouped together, whereas another set of Rpb2 from the same viruses formed another group. This tree topology strongly suggested a single duplication event of Rpb2 in the ancestor of these viruses. Therefore, the existence of Megaviridae Rpb2 paralogs may lead to an approximately two-fold increase in apparent richness. In order to obtain a more reasonable estimate for the taxon richness of Megaviridae based on Rpb2 sequences, we classified metagenomic Rpb2 homologs into two groups by taking putative ancient duplication into account (Fig. S3A, S3B, and S3C). Richness estimates and rarefaction curves for individually analyzed paralogous groups still indicated a larger number of OTUs for Megaviridae than for Bacteria and Archaea at any given number of resampled sequences (Fig. 3C). Paralogs were not found for Rpb1, except for a pair of Rpb1 sequences in AaV. Sequence identity between the AaV Rpb1 sequences was 33%. These sequences were found to be closely located in phylogenetic trees when metagenomic Rpb1 sequences were included (Fig. S3D). Therefore, we considered the influence of the paralogous Rpb1 groups on the taxon richness estimate to be negligible.

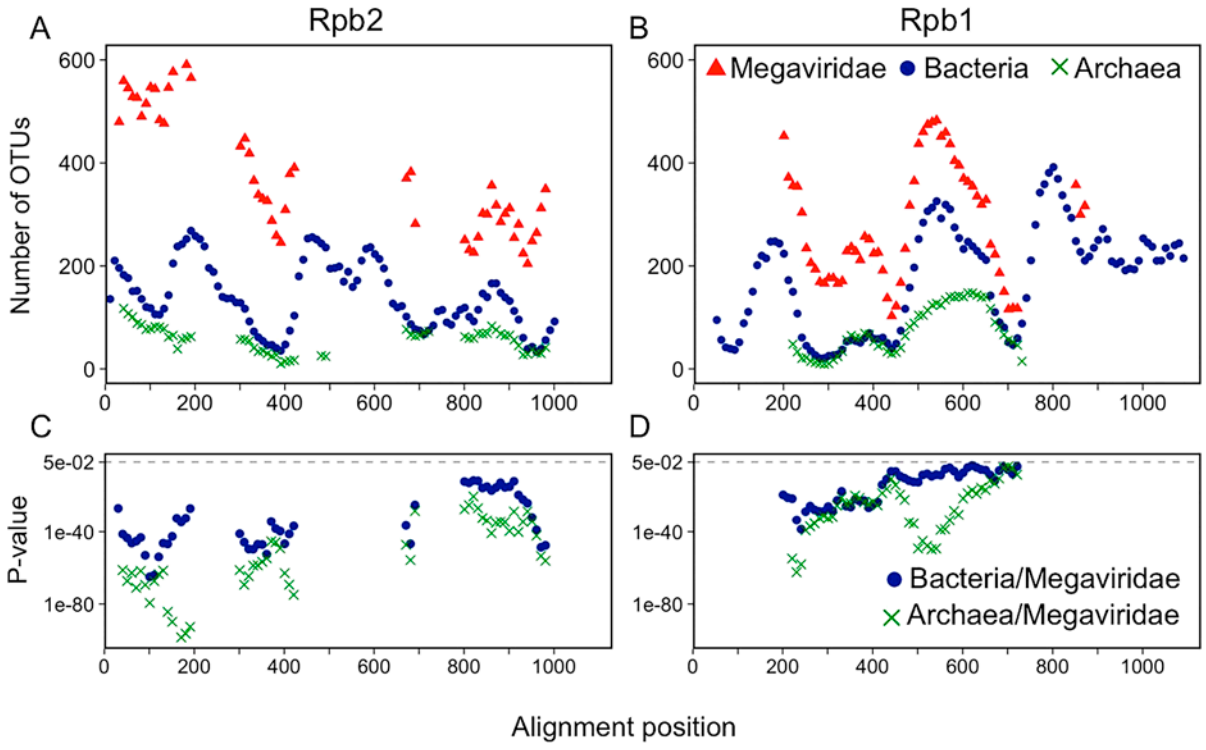

Fig. 4. Richness for 1,000 metagenomic Rpb2/Rpb1 sequences along the length of reference alignments. The numbers of OTUs after the resampling of 1,000 metagenomic sequences were plotted at each sequence region of Rpb2 (A) and Rpb1 (B). The significance (p-value) of differences between Megaviridae and prokaryotes was assessed using the Log-rank test at each sequence region of Rpb2 (C) and Rpb1 (D).

Comparison of PD between Megaviridae and Bacteria/Archaea

PD is a measure of the diversity of phylogenetically related sequences, defined as the sum of all branch lengths in the phylogenetic tree (60). We calculated PD scores for Rpb2 and Rpb1 sequences obtained from marine metagenomes using a sliding window on the Rpb alignment RAln-MBA-Rpb2 and RAln-MBA-Rpb1 (Fig. 5). Megaviridae showed higher PD scores than Bacteria and Archaea along the entire length of the alignments.

The phylogenetic distribution of Megaviridae Rpb2 sequences on the reference tree indicated that a larger number of metagenomic sequences mapped to Mesomimivirinae subfamily branches (95.1%) than to Megamimivirinae subfamily branches (4.6%) (Fig. 6). Among the Megamimivirinae branches, a larger number of environmental sequences (275 sequences) mapped to the branch leading to CroV than to the branches leading to Klosneuviruses (52 sequences) or amoebal mimiviruses (37 sequences). A notable feature of the Rpb2 phylogenetic distribution was that the deeper the branches (*i.e.*, the closer to the root), the higher the number of environmental sequences they got assigned: *e.g.* 5,414 sequences mapped to the root of one of the Mesomimivirinae Rpb2, whereas only 234, 259, 1,247, and 438 sequences mapped to the leaves representing OLPV1, OLPV2, PgV, and CeV reference genomes, respectively.

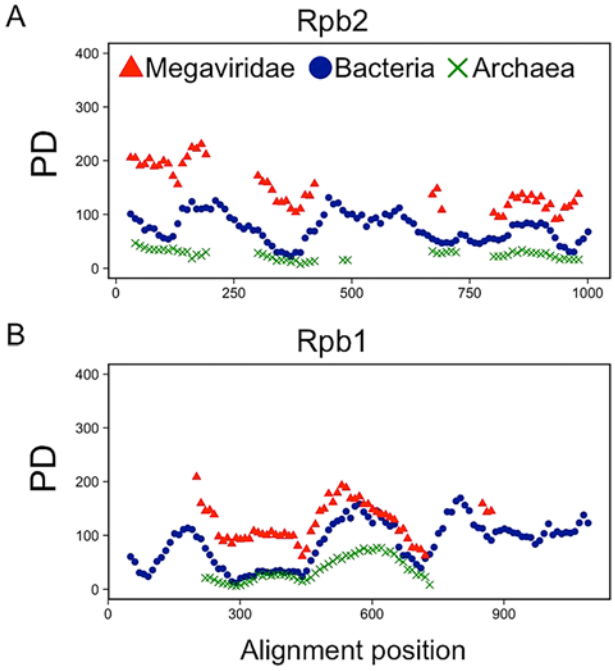

Fig. 5. Phylogenetic diversity of metagenomic Rpb2/Rpb1 sequences along the length of reference alignments. Phylogenetic diversity (PD) scores were computed with phylogenetic trees constructed using 1,000 metagenomic sequences at each sequence region of Rpb2 (A) and Rpb1 (B).

Discussion

In the present study, we extracted environmental Rpb1 and Rpb2 sequence fragments from a large set of microbial metagenomes (58 projects) and classified them into taxonomic groups using a phylogenetic placement method. Taxonomic assignments revealed a large representation of bacterial

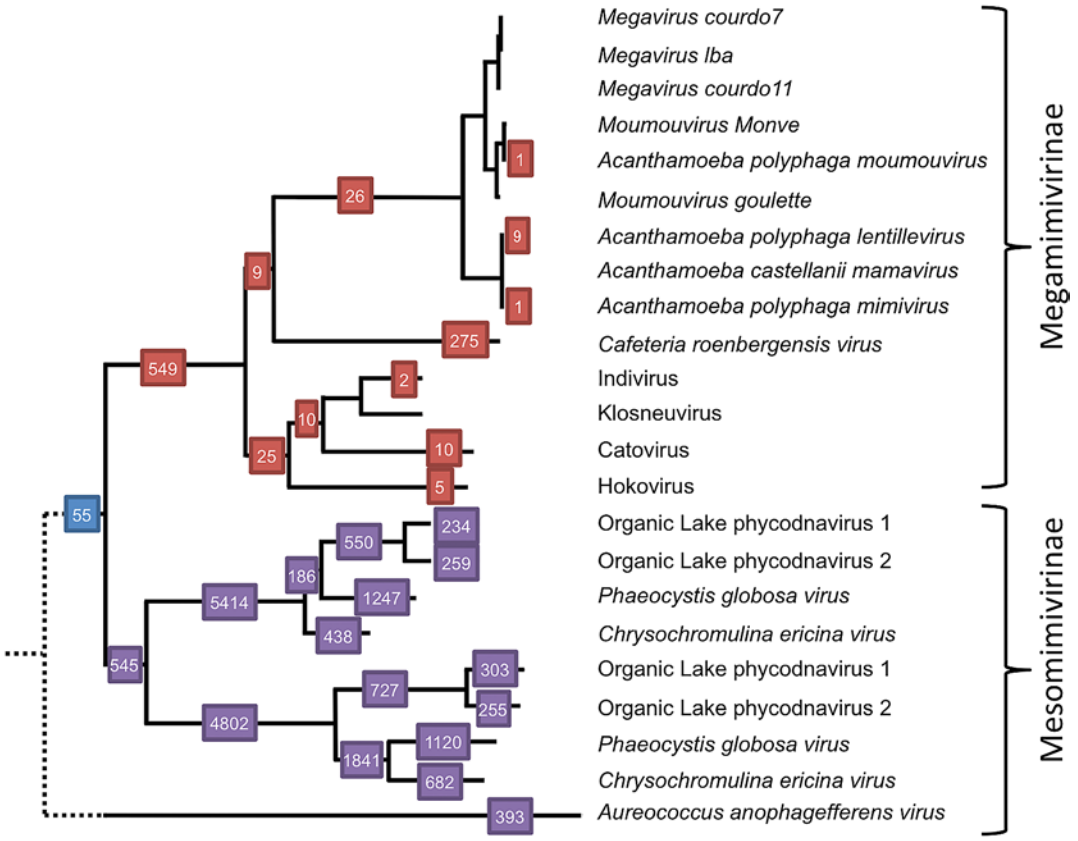

Fig. 6. Numbers of metagenomic sequences assigned to branches of Megaviridae Rbp2. This phylogenetic tree is part of the full phylogenetic tree in Fig. 2A. The numbers in red squares are the numbers of sequences in the Megamimivirinae subfamily and those in purple are the numbers of the Mesomimivirinae subfamily. The dashed branch lines near the root of the tree represent the status of AaV sequence not forming a monophyletic group with the other members of Megaviridae in the full reference tree in Fig. 2A.

Rpb1/2 sequences (~80%) and fewer archaeal and Megaviridae sequences (5–10%). Megaviridae sequences were preferentially detected from metagenomes originating from aquatic environments. This is consistent with previous findings, although members of Megaviridae have been isolated from various environments including oceans, lakes, rivers, air conditioning cooling systems, drainage, and soil (41). The over-representation of bacterial Rpb1/2 sequences in metagenomes is expected given their known dominance in various environments (26, 32). When the same sequence similarity thresholds were applied for taxon delineation for cellular organisms and viruses, Megaviridae showed significantly higher taxon richness than Bacteria and Archaea. As a more general measure that does not require sequence identity thresholds, we also examined PD. The PD indices of Megaviridae were also systematically higher than those of Bacteria and Archaea.

A possible reason for why apparent taxon richness in the Megaviridae is so vast could be a fast evolutionary rate in Megaviridae. However, our results indicated that functional constraints are higher for Megaviridae Rpb1/2 than for bacterial homologs. This result suggests that the rate of sequence evolution is lower for Megaviridae Rpb1/2 than for bacterial homologs if their mutation rates are similar. Blanc-Mathieu and Ogata (5) previously indicated that the mutation rate of giant viruses may be as low as prokaryotes based on Drake's rule, postulating that "the mutation rate per genome has evolved towards a nearly invariant value across taxa", as well as the finding that giant viruses encode many DNA repair enzymes. A recent study monitoring more than one year of experimental evolution consistently demonstrated that the mutation rates of a giant virus, *Lausannevirus*, and a bacterium remained similar over the length of the experiment (58). Therefore, the average mutation rate of Megaviridae may be similar to that of prokaryotes.

Even if a high mutation rate potentially contributed to accelerated evolution, fast evolution is not sufficient to explain the prominent radiation of evolutionarily deep lineages because radiation requires niche expansion (62). As a matter of fact, the richness of prokaryotes, which evolve faster than eukaryotes, is less than that of eukaryotes in marine environments. Recent studies revealed the presence of ~110,000 OTUs at the species level for eukaryotic plankton in the global sunlit ocean (18), but only 36,000–45,000 OTUs for prokaryotes in the same type of environment (76, 92). The markedly high taxon richness of Megaviridae revealed by our study parallels the high richness of eukaryotes, the potential hosts of Megaviridae. Based on the ancient origin of Megaviridae that has been inferred to antedate the emergence of major eukaryotic lineages, our results strongly support the Megaviridae family having a phylogenetically deep and wide co-evolutionary history with diverse marine protists. This virus-host co-evolution may have been at work from the early "Big-Bang" radiation down to the more recent diversification of the tree of eukaryotes. In other words, the long history of the diversification of

eukaryotes may have played a key role in the successive niche expansion of Megaviridae. A similar co-evolutionary history was also proposed for a family of RNA viruses (37).

Many of the Megaviridae sequences were placed in the branches leading to Mesomimivirinae (Fig. 6), which are currently represented by algae-infecting viruses, such as PgV and CeV. The host range of algal species of this clade spans from Haptophyceae (Phaeocystales and Prymnesiales) to Pelagophyceae and Chlorophyta, which are deeply separated from one another in the eukaryotic tree. It is also important to note that even haptophytes alone constitute a very rich group of unicellular eukaryotes (18). Among the Megamimivirinae subfamily, one of the most abundant lineages observed in marine metagenomes was the microzooplankton infecting CroV; however, since the deeper branches also received many sequence assignments, the inference of potential hosts for these sequences are difficult. Overall, the phylogenetic positions of these marine Megaviridae marker genes point to diverse protists, including unicellular algae and microflagellates, as the potential host of these uncultured Megaviridae. Although the amoebal co-culture method (41) has permitted many new mimiviruses to be analyzed, further efforts to isolate viruses from diverse eukaryotes are desirable in order to increase the genome sampling coverage of this diverse clade.

In the present study, we showed that the taxon richness of Megaviridae exceeded that of the prokaryotic domains in the ocean. Investigations on the as yet uncovered diversity of Megaviridae will require the development of experimental alternatives to virus isolation by co-culture method, which is a labor-intensive process depending on the culturability of eukaryotic hosts. These methods include single cell genomics (87), single virus genomics (84), the development of degenerate PCR primers, and a co-occurrence network analysis (32).

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References

1. Aherfi, S., P. Colson, B. La Scola, and D. Raoult. 2016. Giant Viruses of Amoebas: An Update. Front. Microbiol. 7:349.
2. Arslan, D., M. Legendre, V. Seltzer, C. Abergel, and J.M. Claverie. 2011. Distant Mimivirus relative with a larger genome highlights the fundamental features of Megaviridae. Proc. Natl. Acad. Sci. U.S.A. 108:17486–17491.
3. Baltanás, A. 1992. On the use of some methods for the estimation of species richness. Oikos. 65:484–492.
4. Birtles, R.J., T.J. Rowbotham, C. Storey, T.J. Marrie, and D. Raoult. 1997. Chlamydia-like obligate parasite of free-living amoebae. Lancet 349:925–926.
5. Blanc-Mathieu, R., and H. Ogata. 2016. DNA repair genes in the Megavirales pangenome. Curr. Opin. Microbiol. 31:94–100.
6. Boyer, M., N. Yutin, I. Pagnier, *et al.* 2009. Giant Marseillevirus highlights the role of amoebae as a melting pot in emergence of chimeric microorganisms. Proc. Natl. Acad. Sci. U.S.A. 106:21848–21853.
7. Boyer, M., M.A. Madoui, G. Gimenez, B. La Scola, and D. Raoult. 2010. Phylogenetic and phyletic studies of informational genes in genomes highlight existence of a 4th domain of life including giant viruses. PLoS One. 5:e15530.
8. Capella-Gutierrez, S., J.M. Silla-Martinez, and T. Gabaldon. 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics. 25:1972–1973.
9. Carradec, Q., E. Pelletier, C. Da Silva, *et al.* 2018. A global ocean atlas of eukaryotic genes. Nat. Commun. 9:373.
10. Cheetham, G.M., and T.A. Steitz. 2000. Insights into transcription: structure and function of single-subunit DNA-dependent RNA polymerases. Curr. Opin. Struct. Biol. 10:117–123.
11. Chernomor, O., B.Q. Minh, F. Forest, S. Klaere, T. Ingram, M. Henzinger, and A. von Haeseler. 2015. Split diversity in constrained conservation prioritization using integer linear programming. Methods Ecol. Evol. 6:83–91.
12. Ciaccafava, A., A. Lartigue, P. Mansuelle, S. Jeudy, and C. Abergel. 2011. Preliminary crystallographic analysis of a possible transcription factor encoded by the mimivirus L544 gene. Acta Crystallogr., Sect. F Struct. Biol. Cryst. Commun. 67:922–925.
13. Claverie, J.M. 2006. Viruses take center stage in cellular evolution. Genome Biol. 7:110.
14. Claverie, J.M., and H. Ogata. 2009. Ten good reasons not to exclude giruses from the evolutionary picture. Nat. Rev. Microbiol. 7:615; author reply 615.
15. Claverie, J.M., and C. Abergel. 2016. Giant viruses: The difficult breaking of multiple epistemological barriers. Stud. Hist. Philos. Biol. Biomed. Sci. 59:89–99.
16. Colson, P., G. Gimenez, M. Boyer, G. Fournous, and D. Raoult. 2011. The giant Cafeteria roenbergensis virus that infects a widespread marine phagocytic protist is a new member of the fourth domain of Life. PLoS One. 6:e18935.
17. Colson, P., X. De Lamballerie, N. Yutin, *et al.* 2013. "Megavirales", a proposed new order for eukaryotic nucleocytoplasmic large DNA viruses. Arch. Virol. 158:2517–2521.
18. de Vargas, C., S. Audic, N. Henry, *et al.* 2015. Ocean plankton. Eukaryotic plankton diversity in the sunlit ocean. Science 348:1261605.
19. Deeg, C.M., C.E.T. Chow, and C.A. Suttle. 2017. The kinetoplastid-infecting Bodo saltans virus (BsV), a window into the most abundant giant viruses in the sea. bioRxiv 214536.
20. Eddy, S.R. 1998. Profile hidden Markov models. Bioinformatics. 14:755–763.
21. Edgar, R.C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 26:2460–2461.
22. Faith, D.P. 1992. Conservation evaluation and phylogenetic diversity. Biol. Conserv. 61:1–10.
23. Filee, J., P. Siguier, and M. Chandler. 2007. I am what I eat and I eat what I am: acquisition of bacterial genes by giant viruses. Trends Genet. 23:10–15.
24. Filee, J. 2015. Genomic comparison of closely related Giant Viruses supports an accordion-like model of evolution. Front. Microbiol. 6:593.
25. Fischer, M.G., M.J. Allen, W.H. Wilson, and C.A. Suttle. 2010. Giant virus with a remarkable complement of genes infects marine zooplankton. Proc. Natl. Acad. Sci. U.S.A. 107:19508–19513.
26. Flaviani, F., D.C. Schroeder, C. Balestreri, J.L. Schroeder, K. Moore, K. Paszkiewicz, M.C. Pfaff, and E.P. Rybicki. 2017. A Pelagic Microbiome (Viruses to Protists) from a Small Cup of Seawater. Viruses 9:47.
27. Forterre, P. 2013. The virocell concept and environmental microbiology. ISME J. 7:233–236.
28. Gallot-Lavallee, L., A. Pagarete, M. Legendre, S. Santini, R.A. Sandaa, H. Himmelbauer, H. Ogata, G. Bratbak, and J.M. Claverie. 2015. The 474-Kilobase-Pair Complete Genome Sequence of CeV-01B, a Virus Infecting Haptolina (Chrysochromulina) ericina (Prymnesiophyceae). Genome Announc. 3.

29. Gallot-Lavallee, L., G. Blanc, and J.M. Claverie. 2017. Comparative Genomics of Chrysochromulina Ericina Virus and Other Microalga-Infecting Large DNA Viruses Highlights Their Intricate Evolutionary Relationship with the Established Mimiviridae Family. J. Virol. 91.
30. Galperin, M.Y., K.S. Makarova, Y.I. Wolf, and E.V. Koonin. 2015. Expanded microbial genome coverage and improved protein family annotation in the COG database. Nucleic Acids Res. 43:D261–D269.
31. Gaze, W.H., G. Morgan, L. Zhang, and E.M. Wellington. 2011. Mimivirus-like particles in acanthamoebae from Sewage Sludge. Emerg. Infect. Dis. 17:1127–1129.
32. Hingamp, P., N. Grimsley, S.G. Acinas, *et al.* 2013. Exploring nucleo-cytoplasmic large DNA viruses in Tara Oceans microbial metagenomes. ISME J. 7:1678–1695.
33. Iyer, L.M., E.V. Koonin, and L. Aravind. 2003. Evolutionary connection between the catalytic subunits of DNA-dependent RNA polymerases and eukaryotic RNA-dependent RNA polymerases and the origin of RNA polymerases. BMC Struct. Biol. 3:1.
34. Johannessen, T.V., G. Bratbak, A. Larsen, H. Ogata, E.S. Egge, B. Edvardsen, W. Eikrem, and R.A. Sandaa. 2015. Characterisation of three novel giant viruses reveals huge diversity among viruses infecting Prymnesiales (Haptophyta). Virology. 476:180–188.
35. Kanehisa, M., S. Goto, Y. Sato, M. Kawashima, M. Furumichi, and M. Tanabe. 2014. Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic Acids Res. 42:D199–D205.
36. Katoh, K., and D.M. Standley. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30:772–780.
37. Koonin, E.V., Y.I. Wolf, K. Nagasaki, and V.V. Dolja. 2008. The Big Bang of picorna-like virus evolution antedates the radiation of eukaryotic supergroups. Nat. Rev. Microbiol. 6:925–939.
38. Koonin, E.V., Y.I. Wolf, and P. Puigbo. 2009. The phylogenetic forest and the quest for the elusive tree of life. Cold Spring Harb. Symp. Quant. Biol. 74:205–213.
39. Kusser, A.G., M.G. Bertero, S. Naji, T. Becker, M. Thomm, R. Beckmann, and P. Cramer. 2008. Structure of an archaeal RNA polymerase. J. Mol. Biol. 376:303–307.
40. La Scola, B., S. Audic, C. Robert, L. Jungang, X. de Lamballerie, M. Drancourt, R. Birtles, J.M. Claverie, and D. Raoult. 2003. A giant virus in amoebae. Science 299:2033.
41. La Scola, B., A. Campocasso, R. N'Dong, G. Fournous, L. Barrassi, C. Flaudrops, and D. Raoult. 2010. Tentative characterization of new environmental giant viruses by MALDI-TOF mass spectrometry. Intervirology 53:344–353.
42. Legendre, M., S. Audic, O. Poirot, *et al.* 2010. mRNA deep sequencing reveals 75 new genes and a complex transcriptional landscape in Mimivirus. Genome Res. 20:664–674.
43. Legendre, M., S. Santini, A. Rico, C. Abergel, and J.M. Claverie. 2011. Breaking the 1000-gene barrier for Mimivirus using ultra-deep genome and transcriptome sequencing. Virol. J. 8:99.
44. Legendre, M., D. Arslan, C. Abergel, and J.M. Claverie. 2012. Genomics of Megavirus and the elusive fourth domain of Life. Commun. Integr. Biol. 5:102–106.
45. Legendre, M., J. Bartoli, L. Shmakova, *et al.* 2014. Thirty-thousand-year-old distant relative of giant icosahedral DNA viruses with a pandoravirus morphology. Proc. Natl. Acad. Sci. U.S.A. 111:4274–4279.
46. Legendre, M., A. Lartigue, L. Bertaux, *et al.* 2015. In-depth study of Mollivirus sibericum, a new 30,000-y-old giant virus infecting Acanthamoeba. Proc. Natl. Acad. Sci. U.S.A. 112:E5327–5335.
47. Li, W., and A. Godzik. 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 22:1658–1659.
48. Matsen, F.A., R.B. Kodner, and E.V. Armbrust. 2010. pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. BMC Bioinformatics. 11:538.
49. Mihara, T., Y. Nishimura, Y. Shimizu, H. Nishiyama, G. Yoshikawa, H. Uehara, P. Hingamp, S. Goto, and H. Ogata. 2016. Linking virus genomes with host taxonomy. Viruses. 8:66.
50. Mirzakhanyan, Y., and P.D. Gershon. 2017. Multisubunit DNA-dependent RNA polymerases from vaccinia virus and other nucleocytoplasmic large-DNA viruses: Impressions from the age of structure. Microbiol. Mol. Biol. Rev. 81.
51. Monier, A., J.M. Claverie, and H. Ogata. 2008. Taxonomic distribution of large DNA viruses in the sea. Genome Biol. 9:R106.
52. Monier, A., J.B. Larsen, R.A. Sandaa, G. Bratbak, J.M. Claverie, and H. Ogata. 2008. Marine mimivirus relatives are probably large algal viruses. Virol. J. 5:12.
53. Moniruzzaman, M., G.R. LeCleir, C.M. Brown, C.J. Gobler, K.D. Bidle, W.H. Wilson, and S.W. Wilhelm. 2014. Genome of brown tide virus (AaV), the little giant of the Megaviridae, elucidates NCLDV genome expansion and host-virus coevolution. Virology. 466–467:60–70.
54. Moreira, D., and P. Lopez-Garcia. 2005. Comment on "The 1.2-megabase genome sequence of Mimivirus". Science 308:1114; author reply 1114.
55. Moreira, D., and C. Brochier-Armanet. 2008. Giant viruses, giant chimeras: the multiple evolutionary histories of Mimivirus genes. BMC Evol. Biol. 8:12.
56. Moreira, D., and P. Lopez-Garcia. 2009. Ten reasons to exclude viruses from the tree of life. Nat. Rev. Microbiol. 7:306–311.
57. Moreira, D., and P. Lopez-Garcia. 2015. Evolution of viruses and cells: do we need a fourth domain of life to explain the origin of eukaryotes? Philos. Trans. R. Soc. Lond., B Biol. Sci. 370:20140327.
58. Mueller, L., C. Bertelli, T. Pillonel, N. Salamin, and G. Greub. 2017. One year genome evolution of Lausannevirus in allopatric versus sympatric conditions. Genome Biol. Evol. 9:1432–1449.
59. Nasir, A., K.M. Kim, and G. Caetano-Anolles. 2012. Giant viruses coexisted with the cellular ancestors and represent a distinct supergroup along with superkingdoms Archaea, Bacteria and Eukarya. BMC Evol. Biol. 12:156.
60. Nayfach, S., and K.S. Pollard. 2016. Toward accurate and quantitative comparative metagenomics. Cell 166:1103–1116.
61. O'Leary, N.A., M.W. Wright, J.R. Brister, *et al.* 2016. Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation. Nucleic Acids Res. 44:D733–745.
62. Parent, C.E., D. Agashe, and D.I. Bolnick. 2014. Intraspecific competition reduces niche width in experimental populations. Ecol. Evol. 4:3978–3990.
63. Philippe, N., M. Legendre, G. Doutre, *et al.* 2013. Pandoraviruses: amoeba viruses with genomes up to 2.5 Mb reaching that of parasitic eukaryotes. Science 341:281–286.
64. Price, M.N., P.S. Dehal, and A.P. Arkin. 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol. Biol. Evol. 26:1641–1650.
65. Raoult, D., S. Audic, C. Robert, C. Abergel, P. Renesto, H. Ogata, B. La Scola, M. Suzan, and J.M. Claverie. 2004. The 1.2-megabase genome sequence of Mimivirus. Science 306:1344–1350.
66. Raoult, D., and P. Forterre. 2008. Redefining viruses: lessons from Mimivirus. Nat. Rev. Microbiol. 6:315–319.
67. Renesto, P., C. Abergel, P. Decloquement, D. Moinier, S. Azza, H. Ogata, P. Fourquet, J.P. Gorvel, and J.M. Claverie. 2006. Mimivirus giant particles incorporate a large fraction of anonymous and unique gene products. J. Virol. 80:11678–11685.
68. Rusch, D.B., A.L. Halpern, G. Sutton, *et al.* 2007. The Sorcerer II global ocean sampling expedition: northwest Atlantic through eastern tropical Pacific. PLoS Biol. 5:e77.
69. Santini, S., S. Jeudy, J. Bartoli, *et al.* 2013. Genome of Phaeocystis globosa virus PgV-16T highlights the common ancestry of the largest known DNA viruses infecting eukaryotes. Proc. Natl. Acad. Sci. U.S.A. 110:10800–10805.
70. Schmidt, R., R. Kwiecien, A. Faldum, F. Berthold, B. Hero, and S. Ligges. 2015. Sample size calculation for the one-sample log-rank test. Stat. Med. 34:1031–1040.
71. Schulz, F., N. Yutin, N.N. Ivanova, *et al.* 2017. Giant viruses with an expanded complement of translation system components. Science 356:82–85.
72. Sharma, V., P. Colson, R. Giorgi, P. Pontarotti, and D. Raoult. 2014. DNA-dependent RNA polymerase detects hidden giant viruses in published databanks. Genome Biol. Evol. 6:1603–1610.
73. Shimodaira, H. 2002. An approximately unbiased test of phylogenetic tree selection. Syst. Biol. 51:492–508.
74. Steitz, T.A. 2009. The structural changes of T7 RNA polymerase from transcription initiation to elongation. Curr. Opin. Struct. Biol. 19:683–690.
75. Sun, S., J. Chen, W. Li, *et al.* 2011. Community cyberinfrastructure for advanced microbial ecology research and analysis: the CAMERA resource. Nucleic Acids Res. 39:D546–551.

76. Sunagawa, S., L.P. Coelho, S. Chaffron, *et al.* 2015. Ocean plankton. Structure and function of the global ocean microbiome. Science 348:1261359.
77. Takemura, M., S. Yokobori, and H. Ogata. 2015. Evolution of eukaryotic DNA polymerases via interaction between cells and large DNA viruses. J. Mol. Evol. 81:24–33.
78. Takemura, M. 2016. Morphological and taxonomic properties of Tokyovirus, the first Marseilleviridae member isolated from Japan. Microbes Environ. 31:442–448.
79. UniProt Consortium. 2015. UniProt: a hub for protein information. Nucleic Acids Res. 43:D204–212.
80. Werner, F. 2007. Structure and function of archaeal RNA polymerases. Mol. Microbiol. 65:1395–1404.
81. Werner, F. 2008. Structural evolution of multisubunit RNA polymerases. Trends Microbiol. 16:247–250.
82. Werner, F., and D. Grohmann. 2011. Evolution of multisubunit RNA polymerases in the three domains of life. Nat. Rev. Microbiol. 9:85–98.
83. Williams, T.A., T.M. Embley, and E. Heinz. 2011. Informational gene phylogenies do not support a fourth domain of life for nucleocytoplasmic large DNA viruses. PLoS One. 6:e21080.
84. Wilson, W.H., I.C. Gilg, M. Moniruzzaman, *et al.* 2017. Genomic exploration of individual giant ocean viruses. ISME J. 11:1736–1745.
85. Yang, Z. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. CABIOS, Comput. Appl. Biosci. 13:555–556.
86. Yau, S., F.M. Lauro, M.Z. DeMaere, *et al.* 2011. Virophage control of antarctic algal host-virus dynamics. Proc. Natl. Acad. Sci. U.S.A. 108:6163–6168.
87. Yoon, H.S., D.C. Price, R. Stepanauskas, V.D. Rajah, M.E. Sieracki, W.H. Wilson, E.C. Yang, S. Duffy, and D. Bhattacharya. 2011. Single-cell genomics reveals organismal interactions in uncultivated marine protists. Science 332:714–717.
88. Yoosuf, N., I. Pagnier, G. Fournous, C. Robert, D. Raoult, B. La Scola, and P. Colson. 2014. Draft genome sequences of Terra1 and Terra2 viruses, new members of the family Mimiviridae isolated from soil. Virology. 452–453:125–132.
89. Yoshida, T., J.M. Claverie, and H. Ogata. 2011. Mimivirus reveals Mre11/Rad50 fusion proteins with a sporadic distribution in eukaryotes, bacteria, viruses and plasmids. Virol. J. 8:427.
90. Yutin, N., Y.I. Wolf, and E.V. Koonin. 2014. Origin of giant viruses from smaller DNA viruses not from a fourth domain of cellular life. Virology. 466–467:38–52.
91. Zhang, W., J. Zhou, T. Liu, Y. Yu, Y. Pan, S. Yan, and Y. Wang. 2015. Four novel algal virus genomes discovered from Yellowstone Lake metagenomes. Sci. Rep. 5:15131.
92. Zinger, L., L.A. Amaral-Zettler, J.A. Fuhrman, *et al.* 2011. Global patterns of bacterial beta-diversity in seafloor and seawater ecosystems. PLoS One. 6:e24570.

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Short Communication

Intestinal Colonization by a *Lachnospiraceae* Bacterium Contributes to the Development of Diabetes in Obese Mice

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The aim of the present study was to identify bacteria that may contribute to the onset of metabolic dysfunctions. We isolated and identified a candidate bacterium belonging to *Lachnospiraceae* (strain AJ110941) in the feces of hyperglycemic obese mice. The colonization of germ-free *ob/ob* mice by AJ110941 induced significant increases in fasting blood glucose levels as well as liver and mesenteric adipose tissue weights, and decreases in plasma insulin levels and HOMA-β values. These results indicated that the specific gut commensal bacterium AJ110941 influenced the development of obesity and diabetes in *ob/ob* mice with genetic susceptibility for obesity.

Key words: gut microbiota, diabetes, T-RFLP, gnotobiotic mouse, *Lachnospiraceae*

The gut microbiota has been shown to influence host energy homeostasis, metabolism, and inflammation; thus, microbial communities within the gut are now recognized as an important environmental factor in the onset of obesity and type 2 diabetes (T2D) (5, 7, 12, 14, 23). As a consequence, interest in the development of methods to target gut microbiota as a therapy for T2D is increasing (2, 12, 18, 21).

Several studies have conducted metagenomic analyses in an attempt to identify members of the gut microbial community that contribute most significantly to the progression of T2D. Qin *et al.* performed a metagenomic analysis on fecal samples obtained from 345 Chinese men and women with or without T2D (19). Karlsson *et al.* also conducted a metagenomic analysis to compare microbial communities in fecal samples collected from 145 European women who had either T2D, impaired glucose metabolism, or were healthy (13). Both of these studies reported that butyrate-producing *Roseburia* species and *Faecalibacterium prauznitzii* were rarer in subjects with T2D; however, the identified bacteria that existed in high population in T2D subjects differed between these studies (8). Cani *et al.* proposed that lipopolysaccharide (LPS), a cell wall component of almost all Gram-negative bacteria, may be a key substance responsible for metabolic endotoxemia, low-grade systemic inflammation, and insulin resistance in mouse models (1, 3). In support of this, a previous study reported that experimental endotoxemia induced adipose inflammation and insulin resistance in human subjects (17).

We herein searched for the specific gut commensal bacterium related to metabolic syndrome using the terminal restriction fragment length polymorphism (T-RFLP) analysis of fecal samples from a mouse model of T2D. In this study, a comparison of homozygous *db/db* (diabetic) with heterozygous *db/+* (non-diabetic) mice revealed that a specific fragment of the T-RFLP analysis was present at significantly higher levels in *db/db* mice than in *db/+* mice. Even though *ob/ob* obese mice gain weight rapidly and show the symptoms of insulin resistance, most of the obese mice do not develop severe hyperglycemia compared with *db/db* mice (22). However, one of the *ob/ob* mice in the present study showed an abnormally high fasting blood glucose (FBG) level similar to *db/db* mice. The specific fragment observed in *db/db* mice was also the most prominent in the abnormally hyperglycemic *ob/ob* mouse. We hypothesized that the specific bacterium harboring the fragment may have contributed to the progression of T2D; therefore, we isolated and cultured the bacterium (strain AJ110941). We then determined that colonization by the isolate with the LPS producer *E. coli* induced hyperglycemia and the accumulation of adipose tissue in the gnotobiotic *ob/ob* mouse model.

All experimental procedures were reviewed and approved by the Animal Care Committee of Ajinomoto Co., Inc. Male 5-week-old homozygous BKS.Cg-Dock7^{m} +/+Lepr^{db}/J (*db/db*, diabetic) mice, heterozygous control (*db/+*, non-diabetic) mice, and homozygous B6.V-Lep^{ob}/J (*ob/ob*, obese) mice were obtained from Charles River Japan (Yokohama, Japan). Male 5-week-old germ-free *ob/ob* mice were obtained from Sankyo Lab Service (Tokyo, Japan). These mice were housed in a controlled environment (on a 12-h light/dark cycle with lights turning off at 19:00) with free access to standard chow CRF-1 (Oriental Yeast, Tokyo, Japan) and water and kept in specific pathogen-free (SPF) or germ-free/gnotobiotic conditions throughout the experimental period. Fresh feces and blood were collected from 5- and 11-week-old mice after fasting for 16 h.

Blood glucose (FBG) levels were determined using DRI-CHEM 7000V (Fujifilm, Tokyo, Japan). Plasma insulin levels were determined using an ultrasensitive mouse insulin kit (Morinaga Institute of Biological Science, Yokohama, Japan). Plasma glucagon levels were determined using the

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Glucagon ELISA Kit Wako (Wako Pure Chemical Industries, Osaka, Japan). Insulin resistance (HOMA-IR) and β-cell function (HOMA-β) were both calculated on the basis of the fasting levels of plasma glucose and insulin according to the homeostasis model assessment (HOMA) method (16).

Fresh fecal samples were collected from mice, added to 99% ethanol, and stored at −30°C. Bacterial DNA was extracted from the fecal samples using the FastDNA spin kit for soil (MP Biomedicals, Santa Ana, CA) using the FastPrep instrument (MP Biomedicals). T-RFLP analyses of the mouse gut microbiota were performed as previously described (10). Two universal primers, 27F labeled with 6-carboxyfluorescein (FAM): 5'-FAM-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGACTT-3' were used in PCR to amplify the 16S rRNA gene coding region. Purified PCR products of the 16S rRNA gene were digested with *Msp*I (Takara Bio, Otsu, Japan).

To isolate and culture the bacterium, fresh feces were collected and immediately weighed and transferred to an anaerobic chamber. The feces were homogenized with a 50-fold volume (v/w) of an anaerobic diluent, serially diluted, and then plated on Eggerth-Gagnon (EG) agar plates (11). The agar plates were incubated at 37°C for 4 d in the anaerobic chamber. All of the colonies were identified by their fragment size, and a target colony with a fragment size of 282 bp was then selected. The colony was passaged onto new EG agar plates, and the bacterial purity of the isolate was ensured by re-streaking and microscopic observations. The 16S rRNA gene sequence of the isolated bacterium was determined as previously described (15). The sequence was assembled using GENETYX version 7 (GENETYX, Tokyo, Japan). The identification and phylogenic tree analysis of the 16S rRNA gene sequence were carried out using the Ribosomal Database Project (RDP) (6).

When the mice were 8 weeks old, germ-free *ob/ob* mice were separated into 3 groups for the germ-free/gnotobiotic experiment: Group-1 (n=4), germ-free; Group-2 (n=4), colonization by the non-pathogenic *Escherichia coli* strain E-17, which had been isolated from SPF mice; Group-3 (n=4), colonization by AJ110941 with *E. coli* E-17. Groups-2 and -3 were then orally inoculated with *E. coli* E-17 or AJ110941 with *E. coli* E-17 (approximately 1×10^8 cells suspended in anaerobic PBS, respectively). Group-1 was orally administered PBS only. These mice were maintained under the germ-free or gnotobiotic conditions for 8 weeks.

We compared differences in gut microbiota patterns between *db/+* and *db/db* mice. Hyperglycemia was observed in 11-week-old, but not in 5-week-old mice (Fig. S1). The 282 bp fragment was significantly higher in *db/db* than *db/+* mice at both 5 and 11 weeks old (Fig. S2). We then evaluated the presence of the 282 bp fragment in another metabolic syndrome model, the *ob/ob* mouse. The highest FBG level observed among all *ob/ob* mice was in Mouse Number 4 (Fig. S3). The 282 bp fragment was also more prominent in hyperglycemic Mouse Number 4 than in the normal glycemic *ob/ob* mouse at both 5 and 11 weeks old (Fig. S4). Based on these *db/db* and *ob/ob* mice results, we focused on this 282 bp fragment bacteria.

We isolated a colony harboring the 282 bp fragment and established an axenic strain (strain AJ110941). The isolate was determined to be closely related to the genus *Anaerostipes* in the family *Lachnospiraceae* (Fig. 1).

We then generated gnotobiotic *ob/ob* mice colonized by AJ110941 with *E. coli*. Eight weeks after the inoculation, the cecum, liver, adipose tissue, and blood were collected after a 16-h fast. The gut microbiota of the cecal contents from the three groups was determined by T-RFLP analysis. No PCR amplicon was detected in Group-1. Only a single fragment (488 bp) that corresponded to *E. coli* was detected in Group-2. Only two fragments (282 bp and 488 bp) were detected in Group-3. These results clearly indicated that germ-free or gnotobiotic conditions were maintained during the experimental period. The weights of the liver and mesenteric adipose tissue significantly increased in Group-3, whereas no significant difference was observed in body weights between the three groups. FBG and plasma glucagon levels were significantly higher in Group-3, while plasma insulin levels were significantly lower. On the other hand, no significant differences were observed in these parameters between Groups-1 and -2. The homeostasis model assessment was calculated from FBG and plasma insulin levels as an index of insulin resistance (HOMA-IR) and pancreatic β cell function (HOMA-β). HOMA-β was significantly lower in Group-3, whereas no significant differences were noted in HOMA-IR between the three groups (Table 1). These results suggested that colonization by AJ110941 may have promoted the dysfunction of pancreatic β-cells.

Therefore, AJ110941 should be regarded as one of the important causative gut bacteria for the induction of T2D. A previous study reported that the relative abundance of the

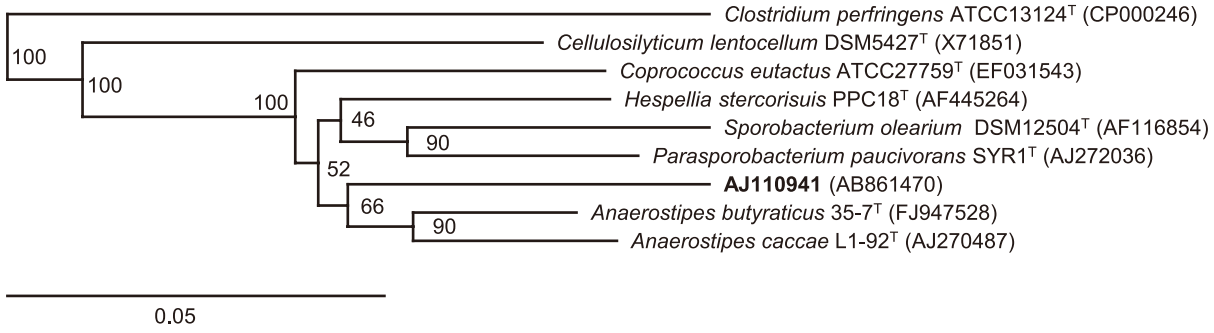

Fig. 1. Phylogenetic tree based on the 16S rRNA gene constructed by Weighbor, the weighted neighbor-joining tree building algorithm, showing AJ110941 and other strains of the family *Lachnospiraceae*. *Clostridium perfringens* ATCC13124T was used as an outgroup. The numbers close to the nodes represent bootstrap values (n=100 replicates). The scale bar represents 0.05 substitutions per nucleotide position.

Table 1. Phenotypes of germ-free and gnotobiotic *ob/ob* mice

	Group-1	Group-2	Group-3
Body weight without the caecum (g)	58.10 ± 5.98^{a}	61.75 ± 4.29^{a}	63.48 ± 10.37^{a}
Cecum (% of body weight)	11.63 ± 4.89^{a}	12.88 ± 1.94^{a}	4.47 ± 0.93^{b}
Liver (% of body weight without the cecum)	6.91 ± 0.66^{a}	7.29 ± 1.83^{a}	9.70 ± 0.46^{b}
Mesenteric adipose tissue (% of body weight without the cecum)	1.64 ± 0.46^{a}	1.81 ± 0.39^{a}	2.63 ± 0.30^{b}
Fasting blood glucose (mg dL^{-1})	216.5 ± 113.6^{a}	286.5 ± 54.1^{ab}	433.25 ± 65.4^{b}
Fasting plasma insulin (μU mL^{-1})	339.7 ± 46.44^{a}	299.8 ± 105.0^{a}	159.4 ± 50.9^{b}
Fasting plasma glucagon (pg mL^{-1})	179.9 ± 43.0^{a}	324.2 ± 42.4^{a}	529.0 ± 18.3^{b}
HOMA-IR	132.5 ± 23.3^{a}	187.5 ± 70.6^{a}	184.4 ± 64.1^{a}
HOMA-β	1350.4 ± 468.9^{a}	638.5 ± 411.9^{ab}	141.7 ± 40.7^{b}

Group-1: germ-free, Group-2: colonization by *E. coli*, Group-3: colonization by AJ110941 with *E. coli*. Data are expressed as means±SD. Values not sharing a common letter are significantly different at $p<0.05$ by the Tukey–Kramer multiple comparisons test ($n=4$ per group) using the JMP 10.0.0 statistical software package (SAS Institute, Cary, NC).

taxonomic family *Lachnospiraceae* was increased by early-life subtherapeutic antibiotic treatments in an obese mouse model (4). Additionally, a metagenomic study indicated that the taxonomic family *Lachnospiraceae* may be associated with T2D (19). However, it remains unclear whether bacteria belonging to the family *Lachnospiraceae* actually affect obesity and FBG levels *in vivo*. Therefore, we generated gnotobiotic *ob/ob* mice colonized by AJ110941 with *E. coli* to reveal a possible causal relationship. In a preliminary examination, we microscopically observed that AJ110941 did not singly colonize the intestinal tract of *ob/ob* germ-free mice. *E. coli*, which is a facultative anaerobe, may be needed to maintain an oxygen-free environment in the intestinal tract because AJ110941 needs strict anaerobic conditions for growth. In our study, a mono-association with the LPS producer *E. coli* induced neither hyperglycemia nor the accumulation of adipose tissues. In contrast, LPS derived from Gram-negative bacteria in the gastrointestinal tract was previously identified as one of the most important factors inducing the development of T2D (1, 3). Our results indicated that the presence of LPS in the intestinal tract was necessary, but not sufficient for the pathogenesis of diabetes. We speculated that AJ110941 may have assisted with the translocation of LPS into the blood from the intestinal tract. We are currently investigating the effects of AJ110941 on LPS translocation in *in vitro*/*vivo* models.

Regarding the particular gut bacterium involved in metabolic syndrome, *Methanobrevibacter smithii* and *Bacteroides thetaiotaomicron* were previously shown to enhance host energy storage in di-associated mice (20). Furthermore, Fei and Zhao reported that the strain *Enterobacter cloacae* B29, which was isolated from an obese human subject, induced obesity and insulin resistance accompanied by serum endotoxemia in mono-associated mice (9).

This is the first study to have successfully identified a specific *Lachnospiraceae* bacterium involved in metabolic disorders. Future studies are needed to elucidate the molecular mechanisms underlying the adverse effects of AJ110941 on glucose and lipid metabolism in mouse models. It remains unclear whether AJ110941 or its closely-related species inhabits the human intestinal tract. We intend to perform a preliminary epidemiological study on obese and diabetic subjects to address this question.

The 16S rRNA gene sequence of the isolated bacterium (strain AJ110941) is available in the DDBJ/EMBL/GenBank databases under the accession number AB861470.

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References

1. Cani, P.D., J. Amar, M.A. Iglesias, *et al.* 2007. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 56:1761–1772.
2. Cani, P.D., and N.M. Delzenne. 2012. The gut microbiome as therapeutic target. Pharmacol. Ther. 130:202–212.
3. Cani, P.D., S. Possemiers, T. Van de Wiele, *et al.* 2009. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 58:1091–1103.
4. Cho, I., S. Yamanishi, L. Cox, *et al.* 2012. Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature 488:621–626.
5. Clemente, J.C., L.K. Ursell, L.W. Parfrey, and R. Knight. 2012. The impact of the gut microbiota on human health: an integrative view. Cell 148:1258–1270.
6. Cole, J.R., Q. Wang, J.A. Fish, *et al.* 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42:D633–642.
7. Cox, L.M., and M.J. Blaser. 2013. Pathways in microbe-induced obesity. Cell Metab. 17:883–894.
8. de Vos, W.M., and M. Nieuwdorp. 2013. Genomics: A gut prediction. Nature 498:48–49.
9. Fei, N., and L. Zhao. 2013. An opportunistic pathogen isolated from the gut of an obese human causes obesity in germfree mice. ISME J. 7:880–884.
10. Hayashi, H., M. Sakamoto, M. Kitahara, and Y. Benno. 2003. Molecular analysis of fecal microbiota in elderly individuals using 16S rDNA library and T-RFLP. Microbiol. Immunol. 47:557–570.
11. Itoh, K., and T. Mitsuoka. 1985. Characterization of clostridia isolated from faeces of limited flora mice and their effect on caecal size when associated with germ-free mice. Lab. Anim. 19:111–118.
12. Johnson, A.M., and J.M. Olefsky. 2013. The origins and drivers of insulin resistance. Cell 152:673–684.
13. Karlsson, F.H., V. Tremaroli, I. Nookaew, G. Bergström, C.J. Behre, B. Fagerberg, J. Nielsen, and F. Bäckhed. 2013. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature 498:99–103.
14. Le Chatelier, E., T. Nielsen, J. Qin, *et al.* 2013. Richness of human gut microbiome correlates with metabolic markers. Nature 500:541–546.
15. Liu, W.T., T.L. Marsh, H. Cheng, and L.J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63:4516–4522.
16. Matthews, D.R., J.P. Hosker, A.S. Rudenski, B.A. Naylor, D.F. Treacher, and R.C. Turner. 1985. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28:412–419.
17. Mehta, N.N., F.C. McGillicuddy, P.D. Anderson, *et al.* 2010. Experimental endotoxemia induces adipose inflammation and insulin resistance in humans. Diabetes 59:172–181.

18. Panwar, H., H.M. Rashmi, V.K. Batish, and S. Grover. 2013. Probiotics as potential biotherapeutics in the management of type 2 diabetes—prospects and perspectives. Diabetes Metab. Res. Rev. 29:103–112.
19. Qin, J., Y. Li, Z. Cai, *et al.* 2012. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature 490:55–60.
20. Samuel, B.S., and J.I. Gordon. 2006. A humanized gnotobiotic mouse model of host-archaeal-bacterial mutualism. Proc. Natl. Acad. Sci. U.S.A. 103:10011–10016.
21. Sanz, Y., A. Santacruz, and P. Gauffin. 2010. Gut microbiota in obesity and metabolic disorders. Proc. Nutr. Soc. 69:434–441.
22. Shafrir, E., E. Ziv, and L. Mosthaf. 1999. Nutritionally induced insulin resistance and receptor defect leading to beta-cell failure in animal models. Ann. N. Y. Acad. Sci. 892:223–246.
23. Tremaroli, V., and F. Bäckhed. 2012. Functional interactions between the gut microbiota and host metabolism. Nature 489:242–249.

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Minireview

The Ecology, Biology and Pathogenesis of *Acinetobacter* spp.: An Overview

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Acinetobacter are a major concern because of their rapid development of resistance to a wide range of antimicrobials, and rapid profundity in transformation, surviving desiccation and persistinge in the environment for a very long time. The organisms are associated with bacteraemia, pulmonary infections, meningitis, diarrhea and notorious nosocomial infections with mortality rates of 20 to 60%. Transmission is via person-to-person contact, water and food contamination, and contaminated hospital equipment. The increasing virulence and rapid development of multidrug resistance by these organisms highlights the need to search for alternatives for chemotherapy. A poor understanding of the organisms and dearth of information about their occurrence especially in developing countries informed the need for this review paper.

Key words: *Acinetobacter*, acinetobactins, biofilms, coccobacilli, ecology, taxonomy

Introduction

The name "Acinetobacter" originates from the Greek word "akinetos" meaning "unable to move", as these bacteria are not motile yet they display a twitching kind of motility. Bacteria of the genus *Acinetobacter* have gained increasing attention in recent years first, as a result of their potential to cause severe nosocomial (Greek *nosos* disease, and *komeion* to take care of) infections (6, 7, 62, 63, 85, 108, 112, 117), second, for their profundity in developing multidrug (MDR) and extreme drug resistance (XDR) (5, 56, 86, 91, 115) third, for the ability of some strains to produce verotoxins (VA) (48), and fourth, for the role members of the genus play in enhanced biological phosphorus removal in wastewater (16, 43, 80). Recently, *Acinetobacter* spp. have demonstrated a hydrocarbon-degrading capability (74, 75, 122), that is of interest for soil bioremediation and a specific strain *Acinetobacter baylyi* ADP1 has shown remarkable competence for natural transformation irrespective of DNA source, thus making it a potentially important tool for biotechnology (2, 19, 111, 112). Possible suggested applications of *Acinetobacter* spp. are summarized in Table 1.

In addition, since the environment, soil, and animals are their natural habitats, food and water contamination exposes humans to infections. The ability of these bacteria to colonize almost any surface and to acquire antibiotic resistance distinguishes them from other infectious bacteria. Despite the huge increase in the frequency of infections caused by MDR *Acinetobacter*, there is still a lack of awareness of the importance of these microorganisms (30). This review therefore gives an overview of the biology, ecology and medical significance of the entire genus *Acinetobacter* in a broad sense with a view to providing basic general information on this group of bacteria for a better understanding and the possible adoption of proactive and effective control measures against infections associated with some of the bacteria.

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Recent taxonomy

The first strain of *Acinetobacter* spp. was isolated from soil and identified as *Micrococcus calcoaceticus* by Beijerinck in 1911 (2, 13). *Acinetobacter* group were previously insufficiently defined for a very long time and confusedly classified into more than a dozen different genera (*Achromobacter*, *Alcaligens*, *Cytophaga*, *Diplococcus*, *Bacterium*, *Herellea*, *Lingelsheimia*, *Mima*, *Micrococcus*, *Moraxella* and *Neisseria*) (2, 15, 27, 90, 97).

The genus *Acinetobacter* was first created in 1954 by Brisou and Prevot to separate the non motile from the motile members of the tribe "Achromobactereae" and was composed of non-pigmented Gram-negative saprophytic bacteria comprising both oxidase-negative and oxidase-positive species. In 1957, Brisou identified a typical species named *Acinetobacter anitratum* (15). Baumann *et al.* (3) using distinct nutritional properties later characterized the organisms as oxidase-negative and proposed to classify them under the genus *Acinetobacter*. In 1971, the subcommittee on *Moraxella* and allied bacteria accepted this proposal and the genus was limited to oxidase-negative strains (71). Three species were initially included in this genus but because of difficulties in distinguishing them based on differences in physiological characteristics, all the species were named *A. calcoaceticus* (2). In fact, *Bergy's Manual of Bacteriology* placed these bacteria in the family Neisseriaceae with only *A. calcoaceticus* as a species and the two subspecies *A. anitratum* and *Acinetobacter lwofii* (59). Several years later, Bouvet and Grimont (12) identified more than fifteen genomic species, including *Acinetobacter baumannii* (formerly *A. calcoaceticus var anitratum* and *A. glucidolytica non liquefaciens*), *Acinetobacter haemolyticus*, *Acinetobacter junii*, *Acinetobacter johnsonii* and *Acinetobacter radioresistens* (12, 13, 27).

The species' names have undergone considerable taxonomic changes over the years as molecular methods have advanced understanding of the genetic make-up of this group of organisms (109). Recent classifications which seem

Table 1. Possible applications for *Acinetobacter* spp. and their products

Bioremediation of waste waters and effluents	Bioremediation of soils and effluents contaminated with heavy metals	Production of biopolymers and biosurfactant	Biomass production	Clinical uses
i) Phosphate removal	i) Textile or tannery industrial effluent containing heavy metals	i) For prevention of dental plaque	i) Protein production	i) Production of glutaminase-sparaginase
ii) Degradation of petrochemicals	ii) Lead from digested sewage sludge	ii) For use in paper-making and other industries	ii) Manganese leaching from ores	ii) Production of L(–) carnitine
iii) Breakdown of organic pollutants	iii) Chromium-contaminated activated sludge or wastewater	iii) For efficient emulsification of oil waste pollutants	iii) Production of immune adjuvants	
	iv) Silver contaminated photographic wastewater	iv) For incorporation in cosmetics, detergents and shampoos		

(82, 107)

to have gained wide acceptance among bacterial taxonomists have recognized this group of heterogeneous bacteria as gamma proteobacteria classified in the order *Pseudomonadales* and the family *Moraxellaceae* (13). Thus the taxonomical classification is given as; Domain—*Bacteria*, Phylum—*Proteobacteria*, Class—*Gammaproteobacteria*, Order—*Pseudomonadales*, Family—*Moraxellaceae*, Genus —*Acinetobacter* (DNA G+C content 39–47%) and *species* (with *A. baumannii*, *A. haemolyticus* and *A. calcoaceticus* as species of clinical importance). Recent classifications using cell shape, absence of flagella, G+C content of DNA and nutritional properties, placed these organisms (*A. baumannii*, *A. haemolyticus* and *A. calcoaceticus* as well as other *Acinetobacters*) in the genus *Moraxella*, now known as *Acinetobacter* (2). Based on DNA-DNA hybridization studies, 32 species of *Acinetobacter* have now been recognized, with 22 assigned valid names and the rest assigned numbers and referred to as a 'genomic group' (42, 91). Among the named species, *A. baumannii* is the main species associated with clinical infections followed by the non-*A. baumannii* species *A. haemolyticus*, *A. junii*, *A. johnsonii* and *A. lwofii* (2, 49). Recently, the emergence of other species of clinical importance such as *A. ursingii* and *A. schindleri* has been reported (95).

Another difficulty associated with classification is the close resemblance between species such that phenotypic differentiation becomes very difficult. For instance, there is a close relationship between *A. baumannii* and *A. calcoaceticus*, and genomospecies 3 and 13. As a result of the difficulties in distinguishing isolates phenotypically in the former pair, the term *A. baumannii-A. calcoaceticus* complex or *Abc* complex has been used. Furthermore, some authors still report these isolates as *A. calcoaceticus* subspecies *anitratum*. This situation led to contributors to the Manual of Clinical Microbiology to conclude that the majority of species of this group of bacteria cannot be reliably distinguished based on phenotypic tests (13, 37, 94, 102).

To avoid confusion therefore, a more reliable classification based on combination of the results of DNA-DNA hybridization and on phenotypic characteristics was adopted (2). In clinical practice however, these taxonomic complications have led to the under-recognition and misclassification of the species.

Biology, cultural and biochemical characteristics of the Acinetobacter *group*

The genus *Acinetobacter* consists of strictly aerobic, non-motile, catalase-positive, indole-negative, oxidase-negative, Gram-negative, non-fermentative encapsulated coccobacilli rods (13, 111). Many strains are unable to reduce nitrates to nitrites (9). The bacteria are strictly aerobic and although they flourish on most laboratory media at temperatures of 20–30°C with the clinical isolates growing at 37 to 42°C, for most strains the optimum temperature is 33–35°C. In the exponential phase of growth, they are bacilli 0.9 to 1.6 μm in diameter and 1.5 to 2.5 μm in length, often in pairs or assembled into longer chains of varying length. *Acinetobacter* spp. are non-fastidious and can be grown on standard laboratory media (64). On blood agar (BA), colonies show typical morphology and size: Non-pigmented, white or cream colored, smooth or mucoid (when capsule is present), opaque, 1–2 mm in diameter (after 18–24 h incubation at 37°C) (9), on eosin methylene blue agar (EMB), colonies are bluish to bluish gray, on Herellea agar (HA) they are pale lavender in color (9), while on Leeds Acinetobacter Medium (LAM) the bacteria are pink on a purple background. In aged cultures the bacteria may be spherical or filamentous. The organisms can be recovered after enrichment culture from virtually all samples obtained from soil or surface water (85). The members of the *Acinetobacter* group are nutritionally versatile chemoheterotrophs and the range of substrates they use as sole carbon and energy sources parallels that of the aerobic pseudomonads.

The cell wall of *Acinetobacter* is typical of that of Gram-negative bacteria, however de-staining is difficult due to a tendency to retain crystal violet and this can lead to incorrect identification as Gram-positive cocci (1). The cells of *Acinetobacter* vary in size and arrangement. *Acinetobacter* generally form smooth and sometimes mucoid colonies on solid media, ranging in color from white to pale yellow or grayish white. Some environmental strains have been reported to produce a diffusible brown pigment (1, 85). Several clinical isolates show hemolysis on sheep blood agar plates (85).

Pathogenesis, virulence factors and resistance

Pathogenesis. In the past, *Acinetobacter* spp. were con-

sidered saprophytes of little clinical significance (7), but with the introduction of powerful new antibiotics in clinical practice and agriculture and the use of invasive procedures in hospital intensive care units (ICUs), drug resistant-related community and hospital-acquired *Acinetobacter* infections have emerged with increasing frequency (49). *A. baumannii* is an important emerging nosocomial pathogen worldwide, followed by *A. lwofii* and *A. haemolyticus*. Conditions attributable to *Acinetobacter* spp. include blood stream infections (BIs), ventilator-associated pneumonia (VAP), bacteremia, meningitis, urinary tract infections, cholangitis, peritonitis, skin and wound infections, ventriculitis, and infective endocarditis (10, 56, 117). The bacteria can also colonize the skin and respiratory tract without causing an infection. An infection results if the host first line of defence is compromised. Studies have however, revealed that colonization increased with hospital stays (82). In gastroentistenal infections with *A. lwoffii* and *H. pylori* infections for example, the normal tissue architecture of the gastric epithelium is altered leading to chronic gastritis (Fig. 1). Infections with *A. lwoffii* induce production of pro-inflammatory cytokines which increase gastrin levels that in turn promote proliferation of the gastric epithelium. Persistent inflammation including the activation of antigen-presenting cells (APCs), release of pro-inflammatory cytokines such as macrophage chemotactic protein (MCP), macrophage inflammatory protein (MIP), Toll-like receptor (TLR), somatostatin (SOM), reactive oxygen species (ROS) involved in acid secretion (Fig. 1) and changes in the number of gastric epithelial cells can lead to gastritis, peptic ulcers, and more rarely, gastric cancer (94). Though colonization occurs more frequently than infections, studies have also shown that lethal infections result from pathogenic strains in immunosuppressed animals with mortality rates of 75 to 100% (96). The bacteria have also been associated with bacteremia, sepsis in neonatal intensive care units and pediatric oncology units, as well as community acquired meningitis and endophthalmitis (25, 106, 110). Other conditions include suppuration; abscesses of the brain, lung, and the thyroid, secondary infections of wounds or surgical trauma, and purulent lesions of the eye. The organisms are ranked 9th after *S. aureus*, *E. coli*, *Klebsiella* spp. *P. aerugenosa*, *C. albicans*, *Enterococci*, *Serratia* and *Enterobacter* as agents of nosocomial BIs, and account for 34% of the mortality and 43% of deaths due to hospital-acquired infections (29). They are the second most commonly isolated nonfermenters in human specimens (81), after *Pseudomonas aeruginosa* and their incidence is on the increase and mortality rates are quite high (56, 111, 119). *A. baumannii* was found to be associated with a series of fatal cases of community pneumonia (29) and *A. haemolyticus*, with endocarditis and verotoxin production, and hence bloody diarrhea (11, 17, 47).

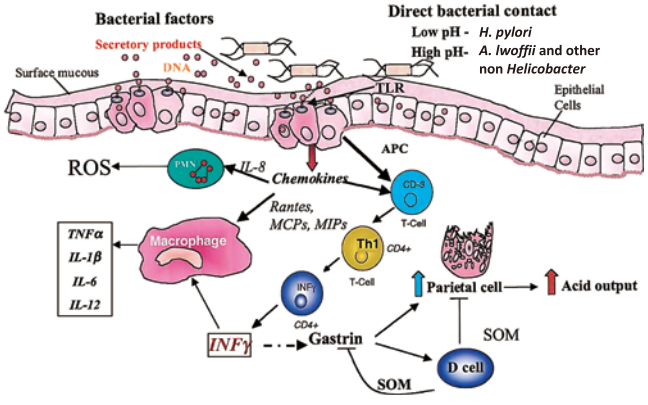

Fig. 1. Schematic model of bacterial colonization of the gastric mucosa, activation of antigen presenting cells (APC), release of pro-inflammatory cytokines and alteration in the number of gastric epithelial cells involved in acid secretion. MCP, macrophage chemotactic protein; MIP, macrophage inflammatory protein; TLR, Toll-like receptor; SOM, somatostatin; ROS, reactive oxygen species (92). (Reproduced with permission, copyright Elsevier).

Pathogenic mechanisms. The pathogenic mechanisms of *Acinetobacter* spp. are little understood or studied (84). Though the infective doses of *Acinetobacter* in human infections have yet to be determined, intraperitoneal injections in mice with 40 clinical isolates of *Acinetobacter* showed the LD_{50} to range from 103 to 106 viable cells per mouse (82). Though *A. baumannii* is the most studied species, the precise mechanisms involved in the establishment and progression of infections by this species are unclear. The organism is not known to produce either diffusible toxins or cytolysins, and few virulence factors have been identified (45). However, comparative genomic studies with *A. baumannii* and the environmental *A. baylyi* have identified genes involved in pilus biogenesis, iron uptake and metabolism, quorum sensing and a type IV secretion system as making up part of the organism's 'virulome' (106, 111). Other authors have also reported common virulence factors among the *Acinetobacters* which are discussed below. There is a need for microbiologists to further investigate these virulence mechanisms for possible discovery of more effective control measures.

Virulence factors. i) Cell surface hydrophobicity and enzymes: *Acinetobacter* spp. have been demonstrated to exhibit cell surface hydrophobicity, an important determinant bacterial adhesion. For a successful infection to occur, bacteria must successfully adhere to host cells (24). The hydrophobicity of a microorganism protects it from being phagocytosed and appears to play an important role in its attachment to various polymers. Hydrophobicity also confers the ability to adhere to plastic surfaces, such as catheters and prostheses. Through this hydrophobicity, *Acinetobacter* spp. coaggregate into flocs in sludge. Non-flocculating *A. johnsonii* S35 displays significant coaggregation with three other bacterial species, *Oligotropha carboxidovorans*, *Microbacterium esteraromaticum*, and *Xanthomonas axonopodis* (76). The degree and mechanism of coaggregation were found to be pair-dependent; and cell surface hydrophobicity was an important factor controlling the coaggregation of *A. johnsonii* S35 and its partner strains (76, 89). The hydrophobic properties of bacterial strains depend on their surface structure, which in turn determines the degree of hydrophobicity: the rougher the cell surface, the greater the hydrophobicity and vice versa. In *Acinetobacter*, the presence of protein protrusions on the cell surface confers hydrophobicity. A recent study using scanning electron microscopy showed the presence of blister-like protein protrusions on *A. johnsonii* S35 and *A. junii* S33, these bacterial

cells were able to coaggregate efficiently with other bacterial cells compared to a mutant strain *A. johnsonii* IAM1517 with smooth cell surfaces (89) which was unable to form aggregates (89).

Surface hydrophobicity is also mediated by colonization factors, complimentary cell surface receptors, fimbriae and other cell wall components (14, 60) and cell surface enzymes that facilitate the adhesion of bacterial cells to host cells. For example, the urease activity of *Acinetobacter* promotes colonization of the mouse stomach (24). Urease also helps *Acinetobacter* spp. colonize the hypochlorhydric or achlorhydric human stomach inducing inflammation (100). Polysaccharide slimes on the bacterial cell surface are reported to confer hydrophobicity (53, 61 93, 97). Other virulence-conferring enzymes secreted by the bacteria include esterases, certain amino-peptidases, and acid phosphatases (93, 108). Esterases have strong hydrolyzing activity against short-chain fatty acids, thereby causing damage to lipid tissues. Hydrolytic enzymes usually confer the bacterium with very strong hemolytic activity. The most extensively studied hydrolytic enzymes in *Ps. aerugenosa* are phospholipases C (PLC)-H, which is encoded by *plcS*, is acidic and has strong hemolytic activity, and PLC-N, which is encoded by *plcN*, is basic, and has no hemolytic activity (93). Recent studies revealed that two copies of the phospholipase C (*plc*) gene with 50% identity to that of *Pseudomonas* are found in *A. baumannii*. It is therefore assumed that these lipases serve a similar function, although this is yet to be elucidated (111). Hoštacká and Klokočníková also reported the secretion of phosphotidylethanolamine and sphingomyelin which are all cytotoxic to leucocytes (53).

ii) Toxic slime polysaccharides: Toxic slime polysaccharides have also been reported among *Acinetobacter* spp. (53). They are usually produced during the exponential phase of growth and are made up of the glucose building blocks D-glucuronic acid, D-mannose, L-ramnose and D-glucose. The slime polysaccharides are toxic to neutroplils, and inhibit their migration as well as inhibit phagocytosis, but without disrupting the host immune system (50, 53). It is important to understand these structures in order to develop effective control measures. Currently, the authors are focusing on determining the hydrophobicity of *A. haemolyticus* isolates from water and wastewater samples and the effect of stress and phytochemical extracts on this hydrophobicity.

iii) Verotoxins: Grotiuz *et al.* (47) first reported the production of verotoxins in *Acinetobacter* (from *A. haemolyticus*). Verotoxins are associated with bloody diarrheas and produced by many enteric bacteria including *E. coli* and *Shigella dysenteriae* (31). The toxins belong to a particular protein subfamily, the RNA *N*-glycosidases which directly target the cell ribosome machinery, inhibiting protein synthesis. Verotoxins can be classified into 2 antigenic groups, vtx-1 and vtx-2, which include (especially vtx-2) an important number of genotypic variants. The mechanism by which *A. haemolyticus* produces this toxin is, however, not well understood. Lambert *et al.* (65) speculated that *A. haemolyticus* acquires vtx2-producing activity via horizontal gene transfer in the gut lumen, since it can be rapidly transformed. In any case, the pathogenicity, basic structure, and chemical components of the toxins are the same as those of verotoxins from *E. coli* and other bacteria (65). The emergence of verotoxin-producing *A. haemolyticus* strains is worrisome given the high transformability of *Acinetobacter* spp. This therefore calls for intensive surveillance of these organisms especially in the environment for the development of proactive control measures. The current work therefore focuses on isolation and identification of *A. haemolyticus* from environmental samples and the determination of their verotoxin production and antimicrobial resistance profiles as well as the effect of phytochemical extracts on verotoxin production by the isolates.

iv) Siderophores: Siderophores are host iron-binding protein structures responsible for iron up take in bacteria. One possible defense mechanism against bacterial infections is the reduction of free extracellular iron concentrations via iron-binding proteins such as lactoferrin or by transfer (14, 121). The normal concentration of free iron in the body is 10^{-8} M, and the concentration required for bacteria to survive in the human body is 10^{-6} M. Bacteria meet their iron requirement by binding exogenous iron using siderophores or hemophores (61, 72, 121). Bacterial siderophores are called aerobactins. *Acinetobacter* siderophores are called acinetobactins and are chiefly made up of the amine histamine which results from histidin decarboxylation (78). Iron import into the bacterial cell is however regulated by a ferric regulator uptake protein serving as a transcription repressor to induce siderophore synthesis or degradation (111).

v) Outer membrane proteins (OMPs): Outer membrane proteins (OMPs) in some Gram-negative bacteria are known to have essential roles in pathogenesis and adaptation in host cells as well as in antibiotic resistance. Several OMPs of the OmpA family have been characterized in various *Acinetobacter* strains (28, 45). Vila *et al.* (115) reported homology between the genome sequence of OmpA of *A. radioresistens*, *A. baumannii* and *A. junii*. The OmpA proteins induce apoptosis of epithelial cells (21), stimulating gastrin and interlukin B gene expression (55). In a recent study, Vallenet *et al.* (111) showed that *A. baylyi* OmpA has emulsifying activity and that only one gene in each *Acinetobacter* strain encodes an OmpA protein. In other words, these proteins share more than 89% of their amino acids and thus have the same chromosomal context. The cells of *Acinetobacter* spp. are surrounded by OmpA, a protein to kill host cells (20). During an infection, OmpA binds to eukaryotic cells and gets translocated into the nucleus where it causes cell death (20, 28, 99).

Resistance to antibiotics and mechanisms of resistance. The major problem with *Acinetobacter* spp. is their resistance to antibiotics (66, 67). Savov *et al.* (101) reported that these organisms are most commonly resistant to ampicillin, cephalothin, carbenicillin, gentamicin, amikacin, chloramphenicol, tetracycline, co-trimoxazole, ciprofloxacin and cefoperazone. Previously ampicillin, second generation cephalosporins, quinolones, minocyline, colistin, amynoglycosides, impenim, sulbactam and gentamicin were used to treat *Acinetobacter* infections. Resistance to these antibiotics has hindered therapeutic management, causing growing concern the world over (32, 47, 87, 91, 115). *A. baumannii* has been developing resistance to all antibiotics used in treating infections. Currently, most *A. baumannii* strains are resistant

to aminoglycosides, tetracyclines, cephalosporins, ampicillins, cefotaximes, chloramphenicols, gentamicins and tobramycins (91). The activity of carbapenems is further jeopardized by the emergence of enzymatic and membrane-based mechanisms of resistance (85).

Antimicrobial resistance among *Acinetobacter* is either intrinsic or acquired via transformation. Several mechanisms of resistance including altered penicillin-binding proteins, low/decrease permeability of the outer membrane to antibiotics or increase in the active efflux of the antibiotics, target site mutations, and inactivation via modifying enzymes have been reported (56, 115). Mechanisms of resistance to antibiotics by *Acinetobacter* spp. vary with species, the type of antibiotic, and geographical location (56). Thus β-lactam antibiotics are inactivated by the production of β-lactamases or alterations of penicillin-binding proteins and decreased permeability of the outer membrane to β-lactams (92); cephalosporins by chromosomally encoded cephalosporinases and occasionally, by cell impermeability and aminoglycosides via aminoglycoside-modifying enzymes; quinolones by altering the target enzymes DNA gyrase and topoisomerase IV through chromosomal mutations, a decrease in permeability and increase in the active efflux of the drug by the microbial cell (67). Several efflux pumps acting against antibiotics have been described for *Acinetobacter* spp. grouped as (i) major facilitator superfamilies (MFSs) comprising the Tet (A)-efflux system for tetracycline, Tet (B)-efflux system for tetracycline and minocycline and Caml A-efflux system for chloramphenicol, (ii) resistance-nodulation division (RND) comprising Ade ABC (ATP binding cassettes)—efflux systems against aminoglycosides, β-lactams, chloramphenicol, erythromycin, tetracyclines, ethidium bromide, and reduced susceptibility to fluoroquinolones and (iii) multi drug and toxic compounds extrusion systems (MATEs) comprising of Abe M—efflux system against norfloxacin, ofloxacin, ciprofloxacin, gentamicin, 4,6-diamino-2-phenylindole (DAPI), triclosan, acriflavin, doxocrubicin, rhodamin 6G and ethidium bromide (114). Major mechanisms of resistance to dif-

Table 2. Major mechanisms of resistance identified for the different classes of antibiotics

Antimicrobial class/resistance mechanism	Class/family	Variants
β-Lactam		
β-Lactamases	Intrinsic cephalosporinase	AmpC (ADC1–7)
	Class A/high-prevalence $ESBL_A$	VEB-1, -2, PER-1, -2, TEM-92, -116, SHV-12, -5, CTX-M-2, -3
	Class A/low-prevalence $ESBL_A$	SCO-1
	Class D OXA enzymes/$ESBL_{M-D}$	OXA-51-like
Carbapenemases	Class D OXA enzymes/$ESBL_{CARBA-D}$,	OXA-23–27, -37, -40, -58-
	$MBLs/ESBL_{CARBA-B}$,	like, VIM, IMP, SIM
	Class A carbapenemase/$ESBL_{CARBA-A}$	GES-11
OMP loss	CarO, HMP-AB, 33–36 kDa protein, 43 kDa protein	
Efflux pump	AdeABC	
Altered PBP expression		PBP2 downregulation
Tetracyclines		
Efflux pump	MFS, RND	A, TetB, AdeABC
Ribosomal protection		TetM
Glycylcyclines		
Efflux pump	RND	AdeABC
Aminoglycosides		
Enzymatic degradation	Acetyltransferases	AacC1/2, AadA, AadB
	Nucleotidyltransferases	Ant1
	Phosphotransferases	AphA1, AphA6,
16S rDNA methyltransferases		ArmA
Quinolones		
DNA gyrase/topoisomerase mutations		GyrA/ParC
Efflux pumps	RND, MATE, BIMP	AdeABC, AdeM, AbeS
Chloramphenicol		
Efflux pumps	RND	AdeABC, AdeIJK
	MFS	CmlA, CraA
	BIMP	AbeS
Trimethoprim/sulfamethoxazole		
Efflux pump	RND	AdeABC, AdeIJK
Dihydropteroate synthase		SulI/II
Dihydrofolate reductase		FolA
Macrolides		
Efflux pumps	MATE, BIMP	AbeM, AbeS
Polymyxins	PmrAB two-component mutation	

MBL—metallo-β-lactamase; OMP—outer membrane protein; HMP—heat modifiable protein; PBP—penicillin-binding protein; MFS—major facilitator superfamily; RND—resistance–nodulation–cell division; MATE—multidrug and toxic compound extrusion; BIMP—bacterial integral membrane proteins (46).

ferent classes of antibiotics are listed in Table 2.

Owing to this escalation of multidrug resistance, deliberate efforts should be made towards investigating other sources of antibiotics with novel mechanisms of action, with a view to developing effective control measures against the recalcitrant bacteria. Investigations of phytochemicals should be considered since they form a very rich source of antibiotic substances (32) with potential activity against microbial pathogens.

Resistance to environmental and host factors. The emergence of nosocomial or community-acquired infections of *Acinetobacter* is a result of high adaptability to adverse environmental conditions, an ability to persist for several days in dry and harsh environments such as the hospital environment, the increased use of broad spectrum antibiotics, the vulnerability of individuals or patients, and the rapid transformation of organisms that results in increased multidrug resistance. To survive and multiply in the host, many bacteria produce a variety of substances that allow them avoid the defense mechanisms of the host (58). *Acinetobacter* spp. are found as natural inhabitants of human skin and repeated isolation may suggest that they are potential pathogens. *Acinetobacter* spp. are able to survive on moist and dry surfaces (118) and some strains have been found to be tolerant of soap (9, 56). The ability of *Acinetobacter* to persist in dry conditions, on inanimate objects, and in dust for several days and weeks has been reported. Recent isolates of *A. lwoffii* compared to the isolates from the 1970s are relatively resistant to irradiation. This raises concerns about the persistence of *A. lwoffii* on medical devices that are sterilized by gamma irradiation, especially in intensive care units (ICU) (93).

Acinetobacter species also survive exposure to the commonly used disinfectants like chlorhexidine, gluconate and phenols, particularly those not used in the appropriate concentrations (40). Compared with other genera of Gram-negative bacilli, *Acinetobacter* is able to survive much better on fingertips or on dry surfaces when tested under simulated environmental conditions (118). Apart from being able to grow at a very broad range of temperatures, they are also able to resist drying. The presence of more electron dense cell walls and nucleic acids are thought to be responsible for the heat (50–75°C) resistance (56). In fact, while *Ps. aerugenosa* and *E. coli* can only survive heat for a maximum of 24 h, *A. baumannii* can survive for up to 25 days (58). Survival for 157 days (*A. radioresistens*), over 30 days (*A. baumannii*) and 21 days (*A. lwoffii*) has been reported (54, 57, 88). *A. baumannii* has also demonstrated resistance to the killing action of normal human serum (NHS) and the possession of a lipopolysaccharide was thought to be partly responsible (42). King *et al.* (61) also suggested modulation of pathogen interaction with serum by a complement regulator. The complement system is the host innate immune defense comprising a series of serum proteins that initiates the death of the bacterium through either lysis or opsonization. One of the mechanisms by which bacterial cells resist killing by serum compliments is by producing surface proteins that bind human factor H (FH), and thereby inhibit the deposition of complements on the bacterial surface (41, 63). The mechanism by which *Acinetobacter* spp. resist serum compliments is however yet to be discovered.

Transfer of resistance among Acinetobacter *spp.*

Resistance to antibiotics is transferred among *Acinetobacter* spp. via plasmids and transposons. While plasmids are DNA elements that carry antibiotic and heavy metal resistance conferring genes capable of autonomous replication, transposons are sequences of DNA that can move (or transpose) themselves to new positions within the genome of a bacterium (or any other prokaryotic cell). These elements are often present in resistant bacteria and have been reported in clinical isolates of *Acinetobacter* spp. (40). Plasmids and transposons are easily transferred between bacteria via the process of genetic transformation. Transformation occurs between *Acinetobacter* spp. Due to the high frequency and degree of adaptability and transformability among some strains of *Acinetobacter* spp., species capable of colonizing multiple settings can mediate the transfer of novel antibiotic resistance genes from antibiotic-producing environmental species to clinical isolates. For example, an intermediate group including spp. 7, 8, and 9 is capable of adapting to human tissues as well as remaining in the environment thus serving as effective vehicles for conveying resistance genes between species (93). Gene transfers in *Acinetobacter* spp. also occur via conjugation and transduction. Conjugation in *Acinetobacter* involves a wide host range and chromosomal transfer, while transduction involves a large number of bacteriophages with a restricted host range (93). Owing to the high transformation ability of *Acinetobacters*, the role of genetic elements in the virulence of this group needs to be thoroughly investigated and adequately understood as in the case of *E. coli*. This will no doubt open up more frontiers for more effective control measures and the application of the organisms in biotechnology.

Epidemiology and ecology

Several studies have reported the epidemiology of *A. baumannii* infections in different parts of the world including Europe, the United States and South America (64, 104). Although these organisms are often associated with nosocomial (117) infections, community acquired diarrhea outbreaks and pneumonia have been reported with some frequency in tropical regions of the world especially during warm (summer) and humid months (18, 56). An infrequent manifestation of *Acinetobacter* is nosocomial meningitis and these cases have been reported after neurosurgical procedures (18, 56, 58). The morbidity and mortality rates of *Acinetobacter* infections are comparable to those of methicillin-resistant *Staphylococcus aureus* (MRSA), and the organisms have been termed ‘Gram-negative MRSA’, manifesting similar epidemiological behavior to MRSA. The impact in terms of morbidity and mortality is probably closer to that of coagulase-negative staphylococci and available data suggest that the mortality rate ranges from 20% to 60% (58). Thus several reports have alerted clinicians to the emergence of a potentially difficult and dangerous organism that is responsible for outbreaks of infection and can cause severe problems (58). Owing to the morphological

similarity between *Acinetobacter* and *Neisseriaceae* (both being Gram-negative diplococci), care should be taken while examining the Gram stain. *Neisseria meningitidis* is, however, far more common as an agent of meningitis. Uncommon conditions involving *Acinetobacter* are contiguous osteomyelitis, peritonitis associated with continuous ambulatory peritoneal dialysis, ophthalmic infection, skin and wound infections, abscesses, sepsis, endocarditis and burn infections. Despite the increasing significance of *Acinetobacter*, there are no significant epidemiological reports on the incidence of infections from many parts of the world particularly, developing countries. Epidemiologic investigations on *Acinetobacter* spp. of clinical significance other than *A. baumannii* as well as on the epidemiology of acinetobacteriosis in developing countries are essential.

Acinetobacters are mostly free living saprophytes found ubiquitously in nature (111). However, different species of the genus are generally associated with different habitats. *Acinetobacter* genomospecies 3 is found in water and soil, on vegetables, and on human skin; *A. johnsonii* and *A. haemolyticus* are found in water, wastewater, soil, on human skin, and in human feces; *A. lwoffii* and *A. radioresistens* are found on human skin; and *Acinetobacter* genomic species 11 is found in water, in soil, and on vegetables as well as the human intestinal tract (10, 26).

Human and animal body. *Acinetobacter* spp. are generally considered part of the normal flora of the skin and mucous membranes or the pharynx, human respiratory secretions, urine, rectum (116) and other human clinical samples (101). They are the only group of Gram-negative bacteria that may be natural residents of human skin, with carriage rates of 42.5% in healthy individuals and as high as 75% in hospitalized patients (101). In a study conducted by Seifert *et al.* (103) *Acinetobacter* spp. were isolated from various parts of the human body including the forehead, nose, ear, throat, trachea, conjunctiva axilla, hand, groin, vagina, perineum and toe web. The organisms are also found in the distal urethra of healthy people (63). Generally the species most frequently isolated are *A. johnsonii*, *A. lwoffii*, *A. radioresistens*, *A. baumannii*, *A. calcoaceticus*, *A. haemolyticus* and *Acinetobacter* genomospecies 3 and 13. Colonization of the intestinal tract by *Acinetobacter* spp. however, is controversial. While some authors suggest that it is an unusual event (46), others report that the gastrointestinal tract is the most important reservoir of resistant strains (23). The difference is probably due to the epidemiological situation *i.e.* whether there is an epidemic outbreak or not.

Acinetobacter spp. have been isolated from different animal sources including birds; fish and rainbow trout (10, 49). On several occasions, chicken septicemia (15% death rate), septicemia in turkeys and calves, mastitis and metrititis in cows, abortions in cattle, pigs and horses, keratoconjunctivitis in cattle, omphalitis in calves, ear infections in cats, and respiratory infections and Balanoposthitis in horses have been reported (38). *A. beijerinkii* and *A. baumannii* are among the species of *Acinetobacter* implicated in animal diseases (38). The organisms have also been isolated from lice collected from homeless people (68).

Food contamination. *Acinetobacter* spp. have been associated with food contamination. Several foods including vegetables have long been known to be an important source of contamination with Gram-negative bacteria such as *Escherichia coli* and *Klebsiella* spp. (85). *Acinetobacter* spp. have been recovered from vegetables, apples, melons, cabbages, cauliflowers, lettuce, cucumbers, peppers, mushrooms, radishes, carrots as well as tubers such as potatoes and cereals such as sweet corn (10, 85). *Acinetobacter* spp. have also been implicated in the spoilage of bacon, chicken, meat, fish and eggs even when stored under refrigeration or after adequate gamma irradiation (84, 108). Hospital food could also be a potential source of *A. baumannii* (10, 108). Many strains isolated from foods have lipolytic activity and some strains produce diffusible pigments.

Soil and wastewaters. Water and soil also provide a home to various microorganisms. *Acinetobacter* genomospecies 3, *A. baumannii*, *A. calcoaceticus* acinetobacters, and *A. calcoaceticus*, *A. johnsonii*, *A. haemolyticus*, and *Acinetobacter* genomic species 11 have been reported to inhabit soil and aquatic environments (86). The organisms have also been isolated from freshwater ecosystems; raw sewage and wastewater treatment plants (10) and activated sludge (16, 83, 85). *A. baylyi*, *A. bouvetii*, *A. grimontii*, *A. tjernbergiae*, *A. towneri*, and *A. tandoii* are commonly found in natural environments but occasionally isolated in activated sludge and have not been found associated with humans (19, 85). They are able to store phosphate as polyphosphates and may have potential applications in the biological removal of phosphates (2).

Biofilms. Biofilms are composed of microorganisms attached to surfaces and encased in a hydrated polymeric matrix made of polysaccharides, proteins and nucleic acids (100, 113). Biofilms function in a manner similar to tissues, using a primitive circulatory system to pump fluids and nutrients through channels in the matrix by changing the ionic strength of the extracellular milieu, causing periodic contraction of matrix polymers.

Pilus mediated biofilms (PMBs) are formed by *Acinetobacter* spp. especially *A. baumannii*, *A. haemolyticus*, *A. lwoffii* and *A. calcoaceticus* thus forming thin layers of microorganisms on glass, medical devises, metals, ceramics and other inanimate objects (28, 39). The biofilm thus constitutes a colonial niche for the bacteria from where contact with humans will result in infection. The hydrophobic surface polysaccharide and pili on the bacterial cell surface initiates adherence to human epithelial cells thus initiating the infection process (28).

Life in biofilms provides microbes with protection against assault from the outside world with barriers against penetration by antimicrobial agents, oxygen and nutrients, along with depressed growth rates and an activated adaptive stress response (99). It also enables the organisms to resist the immune host response. Vidal *et al.* (90) and Lee *et al.* (113) reported the readiness of *A. baumannii* to adhere to both biological and abiotic surfaces, on which it is able to form biofilms thus ensuring its survival (69, 112).

Unlike in other bacteria where the formation of biofilms is facilitated by intrinsic factors such as the presence of type IV pili, flagella, curli and fimbriae, in *Acinetobacter* spp.,

putative chaperon secretion membrane systems (*csms*) and putative surface adherence protein regions (*sapr*) have been reported to be responsible (39, 110). The process generally, involves reversible attachment, irreversible attachment, maturation and dispersion. *A. baumannii*-associated infections are often contracted via biofilms on Foley catheters, venous catheters, or cerebrospinal shunts.

Hospital environment. *A. baumannii* is the most important bacterial species associated with nosocomial or hospital-acquired infections. These infections are caused by organisms present in hospitals or other clinical facilities. Most of these infections emerge while the patient is in the hospital but others are not detected until the patient has been discharged. In the early 70s, nosocomial pathogens were predominantly Gram-negative bacilli especially *Ps. aeruginosa*, and *Enterobacteriaceae*, while the 90s saw the emergence of Gram-positive methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) (111, 115). In recent times however, in addition to MRSA and VRE (mainly in the USA), the introduction of broad spectrum antibiotics in hospitals has resulted in the emergence of multidrug resistant strictly Gram-negative nosocomial bacterial pathogens including *Ps. aeruginosa*, *Klebsiella pneumonia* and *A. baumannii* (33). In a hospital environment, they have been isolated from reusable medical equipments such as ventilator tubings, arterial pressure monitoring devices, humidifiers, washbasins, plastic urinals and respirometers (26, 52, 112, 118). The organisms have also been isolated from the skin of healthcare personnel, mattresses, pillows and in all types of ventilator equipment and moist situations such as sinks and tap water (8, 56). The presence of MDR and nosocomial *Acinetobacter* in the hospital environment complicates treatment since such infected patients often need to be isolated (70, 77). This underlies the need to fully study these organisms and proffer alternative chemotherapeutic solutions.

Though many *Acinetobacter* spp. are only found in certain habitats, some are distributed widely in nature. *A. calcoaceticus* is found in water and soil and on vegetables (2, 10).

Diagnosis

Infection or colonization with *Acinetobacter* is usually diagnosed by the culture of environmental and clinical samples. The environmental samples include wastewater, activated sludge, sewage, aquaculture freshwater habitats, frozen shrimps (49) soil (16, 112), vegetables (10), fresh and spoiled meat (34) animal droppings and river water while the clinical samples include blood, cerebrospinal fluid, endoctracheal aspirate, pus (91), sputum, urine, respiratory secretions (115), Catheter tips (24), wound, stool or sterile body fluid, skin, cordon of newborns, nasal swabs, hand swabs of hospital workers and hospital environments (swabs on surfaces of machines, wash-hand basins, floors, tables, UV lamps) (22). Microbiologic cultures can be processed by standard methods on routine media. Antimicrobial susceptibility can be determined by various means, with the agar-dilution method being the gold-standard.

A glance at the literature shows just how non fastidious and versatile *Acinetobacters* are in terms of growth on media. A wide range of media has been employed in cultivating organisms from different sources. For routine clinical and laboratory investigations, traditional methods have used agar (34), brain heart infusion agar (108), nutrient agar, tryptic soy agar (9), Simon's Citrate agar (29) Violet red bile agar, Luria Bertani agar (49) Eosin-methylene blue, MacConkey agar and Holton medium (34). For environmental screening, especially where *Acinetobacter* may be in very low numbers, Bauman's' Enrichment Medium has been employed (13, 49) and broths including MacConkey broth, trypton soy broth, Brain Heart Infusion broth (49) and Luria broth (29, 63). Highly contaminated samples are inoculated in a liquid mineral medium containing a single carbon source and ammonium or nitrate salt as the nitrogen source with the final pH of the medium being 5.5 to 5.6 (63). Shaking during the incubation is employed in order for the strictly aerobic acinetobacters to outgrow pseudomonads. The broths can later be transferred onto Eosin-methylene blue, MacConkey agar or a selective medium such as Herellea agar or Leeds Acinetobacter Medium in which antibiotics have been added to suppress the growth of other bacteria (51, 108).

Biochemical typing methods include the use of colorimetric based GN card ID 32 GN, API 20NE, RapID NF Plus and Vitek 2 systems (19) all of which are antibody-based agglutination tests. Serological identification has been attempted with the analysis of capsular type and lipopolysaccharide (98) molecules as well as protein profiles for taxonomy and epidemiological investigations.

Because of the widespread nature of *Acinetobacter* spp., typing methods are required for genomic characterization (49). The differences in antimicrobial efficacy against different species, and the need to select effective chemotherapeutic agents, require the accurate identification of *Acinetobacter* spp. to the species level. Thus several molecular diagnostic methods including the polymerase chain reaction (PCR) (47), PFGE, RAPD-PCR DNA fingerprinting (16, 86), fluorescent *in situ* hybridization (FISH) (112), 16S rRNA gene restriction analysis (ARDRA) (79), and 16S rRNA gene PCR-DGGE fingerprinting for genetic characterization of *Acinetobacter* spp. from environmental samples Vanbroekhoven *et al.* have been employed (112). A recent diagnostic method which was reported to have high specificity and can discriminate between *Acinetobacter* species is the microsphere-based array technique that combines an allele-specific primer extension assay and microsphere hybridization (73). The method was reported to be so efficient that 13 different species of *Acinetobacter* were discriminated in less than 9 h with 90% accuracy and precision (73). The use of DNA-DNA hybridization and sequence analysis (19, 49) is considered the gold standard, but the method is labor-intensive and impractical in most clinical laboratories.

Other methods that have been employed in the epidemiological investigation of outbreaks caused by *Acinetobacter* spp. include biotyping, phage typing, cell envelope protein typing, plasmid typing, ribotyping, restriction fragment length polymorphisms and arbitrarily primed PCR (AP-PCR) (4). These methods are however too expensive and too technical for use in unequipped laboratories. Exploration of sim-

ple laboratory culture procedures will enhance isolation of these organisms especially in developing countries where electricity and sophisticated diagnostic procedures, and trained manpower are lacking.

Factors predisposing individuals to acinetobacterioses

Though it is generally agreed that *A. baumannii* is the most medically significant *Acinetobacter* spp., there is an ongoing debate on the clinical impact of the Acinetobacters with controversial views on whether these organisms increase morbidity or mortality. While some researchers are of the opinion that *A. baumannii* infections are responsible for an increase in patient mortality, others are of the opinion that infections occur in critically ill patients and mortality is as a result of other underlying diseases (28, 35, 36). Whatever the case may be, virtually every study has concluded that there is a detrimental effect (45). The lack of a consensus on the degree of mortality may be due in part to the difficulty in distinguishing between colonization and infection, which is compounded by limited information on the pathogenesis. There is also extensive methodological heterogeneity between studies (prospective versus retrospective), and variation in the definitions of cases versus controls (*A. baumannii* infection versus other infection, polymicrobial versus monomicrobial) (48, 70). There are also problems with the accurate identification of organisms to the species level as well as assessment of the impact of specific clones, which may differ in virulence potential (45). In addition, the ability of organisms to rapidly develop multidrug resistance and to persist in harsh environmental conditions calls for the need to take Acinetobacter infections seriously.

Individuals vulnerable to *Acinetobacter* infections include those who have recently undergone major surgery, those with malignant diseases or burns, and immunosuppressed patients such as the elderly, neonates with low birth weights, and patients with prolonged illnesses (84, 107, 108). Nosocomial pneumonia occur in intensive care units (ICUs) with a frequency of 3 to 5% (even higher in patients with mechanical ventilation) and with crude mortality rates of 30 to 75% have been reported (107, 108). Bacteremia is very common in elderly immunocompromised patients. The main source of bacteremia in these patients is bacterial pneumonia, and the most important predisposing factors are malignant diseases, trauma, prolonged antibiotic treatment, prolonged intravenous lines, post operations, urinary catheterization, renal transplants chest tubes, mechanical ventilation, parenteral nutrition and long hospitalization (64, 82). Poor hygienic conditions, and contaminated food and water are common sources of infection. In addition, it's been reported that lower temperatures and an acidic pH may enhance the ability of *Acinetobacter* spp. to invade dead tissues (58).

Treatment, prevention and control

Treatment of *Acinetobacter* infections should be individualized according to susceptibility patterns as the carbapenems, some fluoroquinolones and doxycycline may retain activity. Impenim with an aminoglycoside and β-lactam/β-lactamase inhibitor with an aminoglycoside was found to be synergistic *in vivo*. Quinolone and amikacin synergy was also observed. The treatment of a serious infection with *Acinetobacter* should be combination therapy based on laboratory antimicrobial susceptibility results. Local antimicrobial prescribing habits should be critically guided by the susceptibility results. Suspected hospital outbreaks involving multidrug-resistant *Acinetobacter* infections should not be regarded with akinesis but be tackled ferociously and promptly. The prompt revision of infection control procedures such as hand-washing, patient isolation, ventilator care and good housekeeping is also pertinent. Chemotherapeutic approaches for most antimicrobial-resistant Gram-negative infections, include the use of carbapenems (imipenem and meropenem), but carbapenem-resistant *Acinetobacter* is increasingly reported (56, 101). Resistance to the carbapenem class of antibiotics complicates the treatment of multidrug-resistant *Acinetobacter* infections. However, colistin and polymyxin B have been used to treat highly resistant *Acinetobacter* infections; unfortunately renal toxicity of colistin has made its choice unattractive. *Acinetobacter* isolates resistant to colistin and polymyxin B have also been reported (44). The best approach is combination therapy where studies have demonstrated *in vitro* susceptibility of multidrug-resistant *Acinetobacter* to various synergistic combinations of antimicrobials including carbapenems, colistin, rifampin, and ampicillin-sulbactam (67, 101, 121). The clinical utility of these combinations against pan-resistant *Acinetobacter* remains to be determined (44, 56, 120).

The costs associated with controlling an outbreak of *Acinetobacter* infections can be staggering, and some institutions have even been forced to close entire units in order to interrupt the transmission of *Acinetobacter* (64, 104, 109). Therefore, there is a very compelling need to prevent transmission in the healthcare setting and keep the organism from becoming endemic in an institution. It is also important that contamination of the environment, water or food should be guarded against especially by MDR strains. Careful personal and hand-hygiene should be observed. The disinfection of hands with-alcohol based disinfectants and observation of standard hospital practices cannot be overemphasized. Laboratories should embark on active surveillance to detect cultures and patients who are colonized with multidrug-resistant *Acinetobacter* as well as a community-based surveillance to determine carriage rates. Other measures successful in the control of outbreaks include isolation precautions for infected or colonized patients, cohorting of patients, patients' relatives and staff, environmental disinfection, antimicrobial control, and unit closure (105, 118). Investigations for novel antibiotic substances with possible activity against *Acinetobacter* spp. from plants and other natural sources with a view to sourcing alternative treatment should be seriously considered by both research institutions and pharmaceutical companies.

Conclusion

Acinetobacter previously ignored as low-grade pathogens have suddenly generated interest. The emergence of multi-resistant strains, some of which are pan-resistant to antibiot-

ics, that suddenly cause an outbreak of infection, and the ability of the organism to resist desiccation and its rapid transformation potential has overwhelmed scientists worldwide. Despite an exponential rise in *A. baumannii* infections over the past decade, many questions remain unanswered (45). While knowledge of the virulence and particularly the resistance mechanisms is increasing, the populations at risk and the pathogenesis of severe infection are still poorly understood. The association of the organisms with conditions such as bacteraemia or pulmonary infections, diarrhea and nosocomial infections further highlights a major concern. Most available reports are on *A. baumannii* as the most important clinical species, while investigations are concentrated more on clinical samples. Other samples including environmental and food samples should be investigated for both *A. baumannii* and other species with a view to investigating their health implications. Control measures should not be limited to investigations of the environment (food, water *etc.*) for microbial characterization and studies of *Acinetobacter* virulence and resistance mechanisms alone but the use of animal models should also be considered. The dearth of available treatments remains a major concern and although further work on the use and efficacy of combination therapies is warranted, a more urgent priority must be the development of novel therapeutic agents (45) including studies on the effect of phytochemicals from plant sources (32).

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References

1. Allen, D., and B. Hartman. 2000. *Acinetobacter* Species, p. 215–324. *In* Mandell, G.L., J.E. Bennett, R. Dolin (ed.), Principles and Practice of Infectious Diseases. Churchill Livingstone, Philadelphia, USA.
2. Barbe, V., D. Vallene, N. Fonknechten, *et al.* 2004. Unique features revealed by the genome sequence of *Acinetobacter* sp. ADP1, a versatile and naturally transformation competent bacterium. Nucleic Acids Res. 32:5766–5779.
3. Baumann, P., M. Doudoroff, and R.Y. Stanier. 1968. A study of the *Moraxella* group. II Oxidative-negative species (genus *Acinetobacter*). J. Bacteriol. 95:1520–1541.
4. Bello, H., G. Gonzalez, M. Dominguez, R. Zemelman, A. Garcia, and S. Mella. 1997. Activity of selected β-lactams, ciprofloxacin, and amikacin against different *Acinetobacter baumannii* biotypes from Chilean hospitals. Diagn. Microbiol. Infect. Dis. 28:183–186.
5. Bergobne-Bérézin, E. 1995. The increasing incidence of out breaks of *Acinetobacter* spp.: The need for control and new agents. J. Hosp. Infect. 30:441–452.
6. Bergobne-Bérézin, E., and K.J. Towner. 1995. *Acinetobacter* spp. as nosocomial pathogens: Microbiological and epidemiological features. Clin. Microbiol. Rev. 9:148–161.
7. Bergogne-Bérézin, E., and K.J. Towner. 1996. *Acinetobacter* spp. as nosocomial pathogens: Microbiological, clinical, and epidemiological features. Clin. Microbiol. Rev. 9:148–165.
8. Bergogne-Bérézin, E. 2001. The increasing role of *Acinetobacter* species as nosocomial pathogens. Curr. Infect. Dis. Rep. 3:440–444.
9. Bergogne-Bérézin, E. 2009. Importance of *Acinetobacter* spp. Acinetobacter biology and pathogenesis. Infect. Agents Pathol. 1–18.
10. Berlau, J., H. Aucken, M. Houang, and T.L. Epitt. 1999. Isolation of *Acinetobacter* spp. including *A. baumannii* from vegetables: Implications for hospital-acquired infections. J. Hosp. Infect. 42:201–204.
11. Blanco, M., J.E. Blanco, A. Mora, *et al.* 2003. Serotypes, virulence genes, and intimin types of shiga toxin (verotoxin)-producing *Escherichia coli* Isolates from healthy sheep in Spain. J. Clin. Microbiol. 41:1351–1356.
12. Bouvet, P., and P. Grimont. 1986. Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumanni* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Aninetobacter johnsonii* sp. nov., and amended description of *Acinetobacter calcoaceticus* and *Acinetobacter lwofii*. Int. J. Syst. Bacteriol. 36:228–240.
13. Bouvet, P.J.M., and M.L. Joly-Guillou. 2000. *Acinetobacter* spp. p. 34–86. *In* J. Freney, F. Renaud, W. Hansen, and C. Bollet (ed.), Summary of Clinical Bacteriology. Eska, Paris.
14. Braun, G. 2009. Acinetobacter biology and pathogenesis. Infect. Agents. Pathol. 1–10.
15. Brisou, J. 1957. Contribution á l'étude des *Pseudomonadaceae* Précisions taxonomiques sur le genre *Acinetobacter*. Ann. Inst. Pastuer. 93:397–404.
16. Carr, E., H. Eason, S. Feng, A. Hoogenraad, R. Croome, J. Soddell, K. Lindrea, and R. Seviour. 2001. RAPD-PCR typing of *Acinetobacter* isolates from activated sludge systems designed to remove phosphorus microbiologically. J. Appl. Microbiol. 90:309–319.
17. Castellanos, M.E., M.T. Asensio, V.R. Blanco, M.R. Suarez, A.M. Torrico, and A.C. Llosa. 1995. Infective endocarditis of an intraventricular patch caused by *Acinetobacter haemelyticus*. Infect. 23:243–245.
18. Chen, M.Z., P.R. Hsueh, L.N. Lee, C.L. Yu, P.C. Yang, and K.T. Luh. 2001. Severe community acquired pneumonia due to *Acinetobacter baumannii*. Chest. 120:1072–1077.
19. Chen, T., L. Siu, Y. Lee, C. Chen, L. Huang, R.C. Wu, W. Cho, and C. Fung. 2008. *Acinetobacter baylyi* as a pathogen for opportunistic Infection. J. Clin. Microbiol. 46:2938–2944.
20. Choi, C.H., J.S. Lee, Y.C. Lee, T.I. Park, and J.C. Lee. 2008. *Acinetobacter baumannii* invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. BMC Microbiol. 8:216.
21. Choi, C.H., E.Y. Lee, Y.C. Lee, T.I. Park, H.J. Kim, S.H. Hyun, S.A. Kim, S.K. Lee, and J.C. Lee. 2005. Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and induces apoptosis of epithelial cells. Cell Microbiol. 7:1127–1138.
22. Constantiniu, S., A. Romaniuc, L.S. Iancu, R. Filimon, and J. Taraşi. 2004. Cultural and biochemical characteristics of *Acinetobacter* spp. strains isolated from hospital units. J Prev. Med. 12:35–42.
23. Corbella, X., M. Pujol, and J. Ayats. 1996. Relevance of digestive tract colonization in the epidemiology of nosocomial infections due to multi-resistant *Acinetobacter baumannii*. Clin. Infect. Dis. 23:329–334.
24. Costa, G.F.M., M.C.B. Tognim, C.L. Cardoso, F.E. Carrara-Marrone, C. Marrone, and L.B. Garcia. 2006. Preliminary evaluation of adherence on abiotic and cellular surfaces of *Acinetobacter baumannii* strains isolated from catheter tips. Braz J. Infect. Dis. 10:346–351.
25. Crawford, P.M., M.D. Conway, and G.A. Peyman. 1997. Trauma-induced *Acinetobacter lwoffi* endophthalmitis with muli-organism occurrence: Strategies with intra-vitreal treatment. Eye. 11:863–864.
26. Cunha, B., J. Klimek, J. Gracewski, J. McLaughlin, and R. Quitiliani. 1980. A common source outbreak of pulmonary *Acinetobacter* iinfections traced to Wright respirometers. Postgrad. Med. J. 56:169–172.
27. De Bord, G. 1939. Organisms invalidating the diagnosis of gonorrhea by the smear method. J. Bacteriol. 39:119.
28. Dijkshoorn, L., A. Nemec, and H. Seifert. 2007. An increasing threat in hospitals: Multi-drug resistant *Acinetobacter baumannii*. Nat. Rev. Microbiol. 5:939–951.
29. Dorsey, C.W., A.P. Tomaras, P.L. Connerly, M.E. Tolmasky, J.H. Crosa, and L.A. Actis. 2004. The siderophore-mediated iron acquisition systems of *Acinetobacter baumannii* ATCC 19606 and *Vibrio anguillarum* 775 are structurally and functionally related. Microbiol. 150:3657–3667.
30. Doughari, J.H., P.A. Ndakidemi, I.S. Human, and S. Benade. 2010. Verocytotoxic diarrhogenic bacteria and food and water contamination in developing countries: A challenge to the scientific and health community. Rev. Infect. 1:202–210.
31. Doughari, J.H., P.A. Ndakidemi, I.S. Human, and S. Bennade. 2009a. Shiga toxins (Verocytotoxins). Afr. J. Microbiol. Res. 3:681–693.

32. Doughari, J.H., P.A. Ndakidemi, I.S. Human, and S. Bennade. 2009b. Phytochemicals as chemotherapeutic agents and antioxidants: Possible solution to the control of antibiotic resistant verocytotoxin producing bacteria. J. Med. Plants Res. 3:839–848.
33. Endimiani, A., F. Luzzaro, R. Migliavacca, E. Mantengoli, A. Hujer, K. Hujer, L. Pagani, R. Bonomo, G. Rossolini, and A. Toniolo. 2007. Spread in an Italian hospital of a clonal *Acinetobacter baumanni* strain producing the TEM-92 extended-spectrum β-lactamase. Antimicrob. Agents. Chempther. 51:2211–2214.
34. Eribo, B., and J.M. Jay. 1985. Incidence of *Acinetobacter* spp. and other Gram-negative, Oxidase-negative bacteria in fresh and spoiled ground beef. Appl. Environ. Microbiol. 49:256–257.
35. Falagas, M.E., I.A. Bliziotis, and I.I. Siempos. 2006. Attributable mortality of *Acinetobacter baumannii* infections in critically ill patients: A systematic review of matched cohort and Case-control studies. Critical Care. 10:48–55.
36. Falagas, M.E., and I.A. Bliziotis. 2007. Pandrug-resistant Gram-negative bacteria: The dawn of the post-antibiotic era? Int. J. Antimicrob. Agents. 29:630–636.
37. Fournier, P.E., D. Vallenet, V. Barbe, *et al.* 2006. Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. PLoS Gen. 2:7.
38. Francey, T., F. Gaschen, J. Nicolet, and A.P. Burnens. 2000. The role of *Acinetobacter baumannii* as nosocomial pathogen for dogs and cats in intensive care unit year. J. Vet. Int. Med. 14:177–183.
39. Gaddy, J.A., and L.A. Actis. 2009. Regulation of *Acinetobacter baumannii* biofilm formation. Future Microbiol. 4:273–278.
40. Gallego, L., and K.J. Towner. 2001. Carriage of class 1 integrons and antibiotic resistance in clinical isolates of *Acinetobacter baumannii* from northern Spain. J. Med. Microbiol. 50:71–77.
41. Garcia, A., H. Solar, C. Gonzalez, and R. Zemelman. 2000. Effect of EDTA on the resistance of clinical isolates of *Acinetobacter baumannii* to the bactericidal activity of normal human serum. J. Med. Microbiol. 49:1047–1050.
42. Gerischer, U. 2008. *Acinetobacter* spp. p. 101–108. *In* U.V Gerischer, L. Mukua, and K. Mirgja (ed.). *Acinetobacter* Molecular Biology. Caister Acad. Press, Norfolk, UK.
43. Ghigliazza, R., A. Lodi, and M. Rovatti. 1998. Study on biological phosphorus removal process by *Acinetobacter lwoffi*: Possibility to by-pass the anaerobic phase. Bioprocess Eng. 18:207–211.
44. Giamarellos-Bourboulis, E.J., E. Xirouchaki, and H. Giamarellou. 2001. Interactions of colistin and rifampin on multidrug-resistant *Acinetobacter baumannii*. Diagn. Microbiol. Infect. Dis. 40:117–120.
45. Gordon, N.C., and D.W. Wareham. 2010. Multidrug-resistant *Acinetobacter baumannii*: Mechanisms of virulence and resistance. Int. J. Antimicrob. Agents. 35:219–226.
46. Grehn, M., and A. von Graevetnitz. 1978. Search for *Acinetobacter calcoaceticus* subsp. *anitratus*: Enrichment of faecal samples. J. Clin. Microbiol. 8:342–343.
47. Grotiuz, G., A. Sirok, P. Gadea, G. Varela, and F. Schelotto. 2006. Shiga toxin 2-producing *Acinetobacter haemolyticus* associated with a case of bloody diarrhea. J. Clin. Microbiol. 44:3838–3841.
48. Grupper, M., H. Sprecher, T. Mashiach, and R. Finkelstein. 2007. Attributable mortality of nosocomial *Acinetobacter* bacteremia. Infect. Control Hosp. Epidemiol. 28:293–298.
49. Guardabassi, L., A. Dalsgaard, and J.E. Olsen. 1999. Phenotypic characterization and antibiotic resistance of *Acinetobacter* spp. isolated from aquatic sources. J. Appl. Microbiol. 87:659–667.
50. Heidelberger, M., A. Das, and E. Juni. 1969. Immunochemistry of the capsular polysaccharide of an *Acinetobacter*. Pub. Nat. Acad. Sci. 63:47–50.
51. Holton, J. 1983. A note on the preparation and use of selective medium for the isolation of *Acinetobacter* spp. From clinical sources. J. Appl. Bacteriol. 54:141–142.
52. Horrevorts, A., K. Bergman, L. Kollee, I. Breuker, I. Tjernberg, and L. Dijkshoorn. 1995. Clinical and epidemiological investigations of *Acinetobacter* genomospecies 3 in neonatal intensive care unit. J. Clin. Microbiol. 3:1567–1572.
53. Hoštacká, A., and L.' Klokočníková. 2002. Characteristics of clinical *Acinetobacter* spp. Strains. Folia Microbiologica. 47:579–582.
54. Houang, E.T.S., R.T. Sormunen, L. Lai, C.Y. Chan, and A.S.Y. Leong. 1998. Effect of desiccation on the ultrastructural appearances of *Acinetobacter baumannii* and *Acinetobacter lwoffii*. J. Clin. Microbiol. 51:786–788.
55. Janssen, P., R. Maquelin, R. Coopman, I. Tjernberg, and P. Bouvert. 1997. Determination of *Acinetobacter* genomic species by AFLP finger printing. Int. J. Syst. Bacteriol. 47:1179–1187.
56. Jain, R., and L.H. Danziger. 2004. Multi-drug resistant *Acinetobacter* infections: An emerging challenge to clinicians. Ann. Pharmacother. 38:1449–1459.
57. Jawad, A., A.M. Snelling, J. Heritage, and P.M. Hawkey. 1998. Exceptional desiccation tolerance of *Acinetobacter radioresistens*. J. Hosp. Infect. 39:235–240.
58. Joly-Guillou, M.L. 2005. Clinical impact and pathogenicity of *Acinetobacter*. Eur. Soc. Clin. Microbiol. Infect. Dis. 11:868–873.
59. Juni, E. 1984. Genus III. *Acinetobacter* brisou et prévot. 1954. p. 303–307. *In*: N. Krieg, and J. Holt (ed.), Bergy's Manual of Systemic Bacteriology, vol 1. The Williams and Wilkins Co., Baltimore, USA.
60. Kaplan, N., E. Rosenberg, B. Jann, and K. Jann. 1985. Structural studies of the capsular polysaccharide of *Acinetobacter calcoaceticus* BD4. Eur. J. Biochem. 152:453–458.
61. King, L.B., E. Swiatlo, A. Swiatlo, and L.S. McDaniel. 2009. Serum resistance and biofilm formation in clinical isolates of *Acinetobacter baumannii*. FEMS Immunol. Med. Microbiol. 55:414–421.
62. Knapp, S., C.W. Wieland, S. Florquin, R. Pantophlet, L. Dijkshoorn, N. Tshimbalanga, S. Akira, and T. van der Poll. 2006. Differential roles of CD14 and toll-like receptors 4 and 2 in murine *Acinetobacter* pneumonia. Am. J. Resp. Critic Care Med. 173:122–129.
63. Koneman, E.M.D., and S.M.D. 1997. *Acinetobacter* characteristics. p. 332–415. *In* W.M. Allen, P.C. Janda, W.M.D. Schreckenberger, and D. Winn Jr (ed.), Color Atlas and Text Book of Diagnostic Microbiology. Fifth edition. Lippincott Williams and Wilkins, New York, USA.
64. Kurcik-Trajkovska, B. 2009. *Acinetobacter spp.*—a serious enemy threatening hospitals worldwide. Macedonian. J. Med. Sci. 2:157–162.
65. Lambert, T., D.M. Gerbaud, M. Galimand, and P. Caurvalin. 1993. Characterization of *Acinetobacter haemolyticus aac(6')-Ig* gene encoding an aminoglycoside 6-*N*-acetyltransferase which modifies amikacin. Antimicrob. Agents Chemother. 37:2093–2100.
66. Landman, D.C., C. Georgescu, D. Martin, and J. Quale. 2002. Polymixins revisited. Clin. Microbiol. Rev. 21:449–465.
67. Landman, D., J.M. Quale, D. Mayonga, A. Adedeji, K. Vangala, and J. Ravishankar. 2002. City ward clonal outbreak of multi-resistant *Acinetobcter baumanni* and *Pseudomonas aeruginosa* in Brooklyn NY: The pre-antibiotic era has returned. Arch. Int. Med. 162:1515–1520.
68. La Scola, B., and D. Raoult. 2004. *Acinetobacter baumannii* in human body louse. Emerg Infect. Dis. 10:1671–1673.
69. Lee, H.W., Y.M. Kah, J. Kim, J.C. Lee, S.Y. Seol, and D.T. Cho. 2008. Capacity of multi-drug resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. Clin. Microbiol. Infect. 14:49–54.
70. Lee, N.Y., H.C. Lee, N.Y. Ko, C.M. Chang, H.I. Shih, C.J. Wu, and W.C. Ko. 2007. Clinical and economic impact of multidrug resistance in nosocomial *Acinetobacter baumanni* bacteremia. Infect. Control Hosp. Epidemiol. 28:713–719.
71. Lessel, E. 1971. Minutes of the subcommittee on the taxonomy of Moraxella and allied bacteria. Inter. J. Syst. Bacteriol. 21:213–214.
72. Lesouhaitier, O., W. Veron, A. Chapalain, A. Madi, A. Blier, A. Dagorn, N. Connil, S. Chevalier, N. Orange, and M. Feuilloley. 2009. Gram-negative bacterial sensors for eukaryotic signal molecules. Sensors. 9:6967–6990.
73. Lin, Y., W. Sheng, S. Chang, J. Wang, Y. Chen, R. Wu, K. Hsia, and S. Li. 2008. Application of microsphere-based array for rapid identification of *Acinetobacter* spp. with distinct antimicrobial susceptibilities. J. Clin. Microbiol. 46:612–617.
74. Mandri, T., and J. Lin. 2007. Isolation and characterization of engine oil degrading indigenous microrganisms in Kwazulu-Natal, South Africa. Afr. J. Biotechnol. 6:023–027.
75. Margesin, R., D. Labbé, F. Schinner, C.W. Greer, and L.G. Whyte. 2003. Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. Appl. Environ. Microbiol. 69:3085–3092.
76. Martinez, M.A., M.E. Pinto, R. Zemelman, L.A. Cifras, G. Lira, and C. Ramirez. 1998. Surface characteristics and antimicrobial sensitivity of clinical strains of *Acinetobacter* spp. Rev. Med. Chile. 126:1079–1084.
77. McGowan, J.E. 2006. Resistance in non fermenting Gram-negative bacteria: Multidrug resistance to the maximum. Am. J. Med. 119:S29–S36.
78. Mihara, K., T. Tanabe, Y. Yamakawa, T. Funahashi, H. Nakao, S. Narimatsu, and S. Yamamoto. 2004. Identification and transcriptional organization of a gene cluster involved in biosynthesis and transport of acinetobactin, a siderophore produced by *Acinetobacter baumannii* ATCC 19606^T. Microbiology 150:2587.

79. Nemec, A., L. Dijkshoorn, and P. Jezek. 2000. Recognition of two novel phenons of the genus *Acinetobacter* among non-glucose-acidifying isolates from human specimens. J. Clin. Microbiol. 38:3947–3951.
80. Nichols, H.A., and D.W. Osborn. 1979. Bacterial stress prerequisite for biological removal of phosphorus. J. Water Pollut. Control Fed. 51:557–569.
81. Oberoi, A., A. Aggarwal, and M. Lal. 2009. A decade of an underestimated nosocomal pathogen-*Acinetobacter* in a tertiary care hospital in Punjab. Jkscience. 11:24–26.
82. OECD. 2008. Consensus document on information used in the assessment of environmental applications involving *Acinetobacter*. OECD Environment, Health and Safety Publications Series on Harmonisation of Regulatory Oversight in Biotechnology. ENV/JM/MONO. No. 46:37.
83. Okabe, S., M. Oshiki, Y. Kamagata, *et al.* 2010. A great leap forward in microbial ecology. Microbes Environ. 25:230–240.
84. Peleg, A.Y., S. Jara, D. Monga, G.M. Eliopoulos, C. Robert, Jr. Moellering, and E. Mylonakis. 2009. *Galleria mellonella* as a model system to study *Acinetobacter baumannii*. Pathogenesis and therapeutics. Antimicrob. Agents Chemother. 53:2605–2609.
85. Peleg, A., H. Seifert, and D. Paterson. 2008. *Acinetobacter baumannii*: Emergence of a successful pathogen. Clin. Microbiol. Rev. 21:538–582.
86. Peleg, A.Y., B.A. Potoski, R. Rea, J. Adams, J. Sethi, B. Capitano, S. Husain, E.J. Kwak, S.V. Bhat, and D.L. Paterson. 2007. *Acinetobacter baumannii* bloodstream infection while receiving tigecycline: a cautionary report. J. Antimicrob. Chemother. 59:128–131.
87. Perez, F., A.M. Hujer, K.M. Hujer, B.K. Decker, P.N. Rather, and R.A. Bonomo. 2007. Global challenge of multidrug-resistant *Acinetobacter baumannii*. Antimicrob. Agents Chemother. 51:3471–3484.
88. Peterson, L. 2001. Quinolone molecular structure-activity relationship: What we have learned about improving antimicrobial activity. Clin. Infect. Dis. 33:S180–S186.
89. Phuong, K., K. Kakii, and T. Nikata. 2009. Intergeneric coaggregation of non-flocculating *Acinetobacter* spp. isolates with other sludge-constituting bacteria. J. Biosci. Bioeng. 107:394–400.
90. Piéchaud, D., M. Piéchaud, and L. Second. 1956. Variéties protéolitiques de *Moraxella lwofii* et de *Moraxella glucidolytica*. Ann. Inst. Pastuer. 90:517–522.
91. Prashanth, K., and S. Badrinath. 2005. Epidemiological investigation of nosocomial *Acinetobacter* infections using arbitrarily primed PCR and pulse field gel electrophoresis. Indian J. Med. Res. 122:408–418.
92. Poirel, L., O. Menuteau, N. Agoli, C. Cattoen, and P. Nordmann. 2003. Outbreak of extended-spectrum beta-lactamase VEB-1-producing isolates of *Acinetobacter baumannii* in a French hospital. J. Clin. Microbiol. 41:3542–3547.
93. Rathinavelu, S., Y. Zavros, and J.L. Merchant. 2003. *Acinetobacter lwoffii* infection and gastritis. Microbes Infect. 5:651–657.
94. Richet, H., and P.E. Fournier. 2006. Nosocomial infections caused by *Acinetobacter baumannii*: A major threat worldwide. Infect. Control Hosp. Epidemiol. 27:245–247.
95. Robinson, A., A.J. Brzoska, K.M. Turner, R. Withers, E.J. Harry, P.J. Lewis, and N.E. Dixon. 2010. Essential biological processes of an emerging pathogen: DNA replication, transcription, and cell division in *Acinetobacter* spp. Microbiol. Mol. Bio. Rev. 74:273–297.
96. Rodríguez-Hernández, M.J., J. Pachón, C. Pichardo, L. Cuberos, J. Ibáñez-Martínez, A., García-Curiel, F. Caballero, I. Moreno, and M. Jiménez-Mejías. 2000. Impenem, doxycyclin, and amikacin in monotherapy and in combination in *Acinetobacter baumanni* experimental pneumonia. J. Antimicrob. Chemother. 45:493–501.
97. Rossau, R., M. Vanlandschoot, M. Gillis, and J. Deey. 1991. Taxonomy of *Moraxellaceae*\fam. nov., a new bacterial family to accommodate the genera *Moraxella*, *Acinetobacter*, and *Psychrobacter* and related organisms. Int. J. Syst. Bacteriol. 41:310–319.
98. Russo, T.A., N.R. Luke, J.M. Beanan, R. Olson, S.L. Sauberan, U.C. MacDonald, L.W. Schultz, T.C. Umland, and A.A. Campagnari. 2010. The K1 capsular polysaccharide of *Acinetobacter baumannii* Strain 307-0294 is a major virulence factor. Infect. Immun. 78:3993–4000.
99. Saint, N., C.E. Hamel, E. De, and G. Molle. 2000. Ion channel formation by N-terminal domain: A common feature of OprFs of *Pseudomonas* and OmpA of *Escherichia coli*. FEMS Microbiol. Lett. 190:261–265.
100. Sauer, K., A.H. Rickard, and D.G. Davies. 2007. Biofilms and biocomplexity. Microbe. 2:347–353.
101. Savov, E., D. Chankova, R. Vatcheva, and N. Dinev. 2002. *In vitro* investigation of the susceptibility of *Acinetobacter baumannii* strains isolated from clinical specimens to ampicillin/sulbactam alone and in combination with amikacin. Int J. Antimicrob. Agents. 20:390–392.
102. Schreckenberger, P., M. Daneshvar, R. Weyant, and D. Hollis. 2003. *Acinetobacter*, *Achromobacter*, *Chryseobacterium*, *Moraxella* and other non-fermentative Gram-negative rods. p. 86–99. *In* Murray, P., Baron, E., Jorgensen, J., Pfaller, M. and Yolken, R. (ed.), Manual of Clinical Microbiology. ASM Press, Washington D.C., USA.
103. Seifert, H., L. Dijkshoorn, P. Gerner-Smidt, N. Pelzer, I. Tjernberg, and M. Vaneechoutte. 2007. Distribution of *Acinetobacter* species on human skin: Comparison of phenotypic and genotypic identification methods. J. Clin. Microbiol. 35:2819–2825.
104. Siau, H., K.Y. Yuen, P.L. Ho, S.S. Wong, and P.C. Woo. 1999. *Acinetobacter* bacteremia in Hong Kong: Prospective study and review. Clin. Infect. Dis. 28:26–30.
105. Simor, A.E., M. Lee, M. Vearncombe, L. Jones-Paul, C. Barry, and M. Gomez. 2002. An outbreak due to multiresistant *Acinetobacter baumannii* in a burn unit: Risk factors for acquisition and management. Infect. Control Hosp. Epidemiol. 23:261–267.
106. Smith, M.G., T. Glanoulis, S. Pukatzki, J. Mekalanos, L. Ornston, M. Gertstein, and M. Snyder. 2007. New insights into *Acinetobacter baumanni* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. Genes Dev. 21:601–614.
107. Torres, A., R. Aznar, J.M. Gatell, P. Jiminez, J. Gonzales, A. Ferrer, R. Celis, and R. Rodrigues-Roisin. 1990. Incidence risk and prognosis factors of nosocomial pneumonia in mechanically ventilated patients. Am. Rev. Resp. Dis. 142:522–528.
108. Towner, K. 2006. The genus *Acinetobacter*. Prok. 6:746–758.
109. Urban, C., S. Segal-Maurer, and J.J. Rahal. 2003. Considerations in control and treatment of nosocomial infections due to multidrug-resistant *Acinetobacter baumannii*. Clin. Infect. Dis. 36:1268–1274.
110. Valero, C., M. Fariñas, D. Gárcia-Palomo, J. Mazarrasa, and J. González-Macías. 1999. Endocarditis due to *Acinetobacter lwoffi* on native mitral valve. Int. J. Cardiol. 69:97–99.
111. Vallenet, D., P. Nordmann, V. Barbe, *et al.* 2008. Comparative analysis of *Acinetobacters*: Three genomes for three lifestyles. PLoS One 3:1805e.
112. Vanbroekhoven, K., A. Ryngaert, P. Wattiau, R. Demot, and D. Springael. 2004. *Acinetobacter* diversity in environmental samples by 16S rRNA gene PCR-DGGE fingerprinting. FEMS Mibrobiol. Ecol. 50:37–50.
113. Vidal, R., M. Dominguez, H. Urrutia, H. Bello, G. Gonzalez, and A. Garcia. 1996. Biofilm formation by *Acinetobacter baumannii*. Microbios. 86:49–58.
114. Vila, J., S. Marti, and J. Sanchez-Cedespes. 2007. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. J. Antimicrob. Chemother. 59:1210–1215.
115. Vila, J., A. Ribera, F. Marco, J. Ruiz, J. Mensa, J. Chaves, G. Hernandez, and M.T.J. De Anta. 2002. Activity of clinafloxacin, compared with six other quinolones, against *Acinetobacter baumanni* clinical isolates. J. Antimicrob. Chemother. 49:471–477.
116. Villegas, M.V., and A.I. Hartstein. 2003. *Acinetobacter* outbreaks, 1977–2000. Infect. Control Hosp. Epidemiol. 24:284–295.
117. Weinstein, R.A. 1998. Nosocomial infection update. Emerg. Infect. Dis. 4:415–420.
118. Wendt, C., B. Dietze, E. Dietz, and H. Ruden. 1997. Survival of *Acinetobacter baumannii* on dry surfaces. J. Clin. Microbiol. 35:1394–1397.
119. Wisplinghoff, H., T. Bischoff, S.M. Tallent, H. Seifert, R.P. Wenzel, and M.B. Edmond. 2004. Nosocomial bloodstream infections in US hospitals: Analysis of 24,179 cases from a prospective nationwide surveillance study. Clin. Infect. Dis. 39:309–317.
120. Yoon, J., C. Urban, C. Terzian, N. Mariano, and J.J. Rahal. 2004. *In vitro* double and triple synergistic activities of Polymyxin B, imipenem, and rifampin against multidrug-resistant *Acinetobacter baumannii*. Antimicrob. Agents Chemother. 48:753–757.
121. Yu, H.B., Y.L. Zhang, Y.L. Lau, F. Yao, S. Vilches, S. Merino, J.M. Tomas, S.P. Howard, and K.Y. Leung. 2005. Identification and characterization of putative virulence genes and gene clusters in *Aeromonas hydrophila* PPD134/91. Appl. Environ. Microbiol. 71: 4469–4477.
122. Zanaroli, G., S. Di Toro, D. Todaro, G.C. Varese, A. Bertolotto, and F. Fava. 2010. Characterization of two diesel fuel degrading microbial consortia enriched from a non acclimated, complex source of microorganisms. Microb. Cell Fact. 9:1–13.

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Minireview

Microbial Ecology along the Gastrointestinal Tract

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The ecosystem of the human gastrointestinal (GI) tract traverses a number of environmental, chemical, and physical conditions because it runs from the oral cavity to the anus. These differences in conditions along with food or other ingested substrates affect the composition and density of the microbiota as well as their functional roles by selecting those that are the most suitable for that environment. Previous studies have mostly focused on *Bacteria*, with the number of studies conducted on *Archaea*, *Eukarya*, and *Viruses* being limited despite their important roles in this ecosystem. Furthermore, due to the challenges associated with collecting samples directly from the inside of humans, many studies are still exploratory, with a primary focus on the composition of microbiomes. Thus, mechanistic studies to investigate functions are conducted using animal models. However, differences in physiology and microbiomes need to be clarified in order to aid in the translation of animal model findings into the context of humans. This review will highlight *Bacteria*, *Archaea*, *Fungi*, and *Viruses*, discuss differences along the GI tract of healthy humans, and perform comparisons with three common animal models: rats, mice, and pigs.

Key words: Microbiome, mycobiome, virome, human gastrointestinal (GI) tract, animal models, diet

Researchers have been investigating the ecology of the intestinal microbiota for decades (120, 165) in order to identify, characterize, and count their numbers. These extensive efforts are due to the important roles the intestinal microbiota play in digestion, the production of essential vitamins, and protection of the gastrointestinal (GI) tract from pathogen colonization (141). In the past few decades, molecular techniques targeting the 16S rRNA gene and other genetic markers have been developed to characterize and analyze bacterial communities. These methods have been used to reveal the important roles played by microbes in the GI tract (23, 180, 183, 184, 189, 212). In healthy individuals, the microbiome (microbial community) and host have a mutualistic relationship in which both partners benefit; however, pathogens may invade and cause disease under certain conditions. The initial aim of most studies was to elucidate the role of the microbiome in disease. More recently, surveys have been performed on healthy individuals in order to assess the contribution of the microbiota to health, particularly in response to dietary changes/supplementation with probiotics and/or prebiotics.

The human GI tract is a complex system that starts from the oral cavity, continues through the stomach and intestines, and finally ends at the anus (Fig. 1). The density and composition of the microbiome change along the GI tract, with major populations being selected by the functions performed at the various locations. Bacteria along the GI tract have several possible functions, many of which are beneficial for health including vitamin production, the absorption of ions (Ca, Mg, and Fe), protection against pathogens, histological development, enhancement of the immune system, and the fermentation of "non-digestible foods" to short chain fatty acids (SCFA) and other metabolites (19, 58, 63, 77, 138). The roles of fungi and viruses have not been examined in as much detail; however, they are known to play important roles in microbiota dynamics and host physiology/immunity related to health and disease (45, 94, 133).

Food passes through the GI tract and the absorption rate of nutrients is largely dependent on the activities of various enzymes in the digestive system, such as amylase in saliva, pepsin in the stomach, and pancreatic enzymes in the small intestine. These mechanisms have been extensively examined (61, 62), particularly in the stomach. However, many food components cannot be digested in the upper GI tract and are passed into the lower intestinal tract, in which they are fermented by microbes. Functional studies commonly use animal models in order to obtain a better understanding of the processes in the GI tract that may lead to better health or decrease disease. However, information from animal models may not be directly translatable to humans. Therefore, researchers need to consider the limitations of the selected animal model when extrapolating findings to humans.

Although microbiome studies often include an ecological component, most of the research performed to date has focused on *Bacteria* and not all of the biota. This represents a logical approach because *Bacteria* comprise most of the microbiome. However, even biota representing a small proportion of the microbiome may play important roles in the ecosystem (133). Therefore, researchers need to start shifting their approach to include eukaryotic, prokaryotic, and viral (33, 133) interactions in efforts to elucidate the roles of all components of the microbiome.

In recent years, a number of reviews have summarized findings from the increasing number of studies being performed in this field (36, 73, 176, 188). While most studies have focused on disease, the microbiome is also important for maintaining

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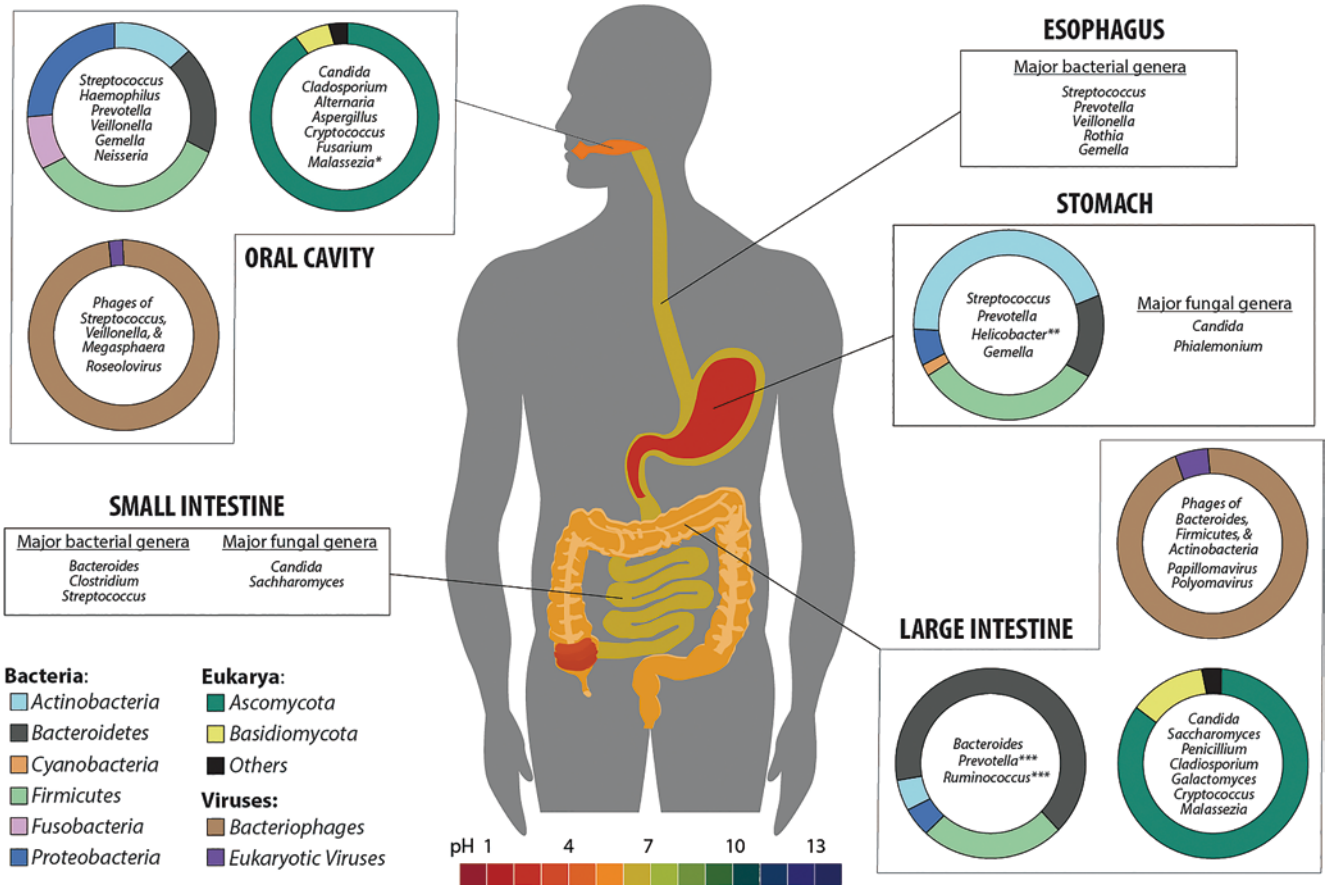

Fig. 1. Microbiome composition of *Bacteria* (1, 5, 20, 21, 43, 147, 156, 223), *Eukarya* (52, 85, 114, 126, 182, 197), and *Viruses* (45, 134, 151, 215) among the physiological niches of the human gastrointestinal (GI) tract. Phylum level compositional data are presented where available along with the most common genera in each GI tract location. The colors on the doughnut plots correspond to the legend in the lower left corner; the GI tract is colored according to the pH scale shown at the bottom of Fig. 1. (* *Malassezia* was very abundant in one study and was not detected in another study. ** The abundance of *Helicobacter* may vary greatly between individuals. *** Proportions of these and other colon genera vary with age, diet, & geographical location.)

health. We herein highlight differences in the microbiome (*Bacteria*, *Archaea*, *Fungi*, and *Viruses*) along the GI tract of healthy humans, and how it compares to those of typical animal models used in research. One finding that is consistent to most studies is that the microbiome of healthy individuals is unique; however, there are still some generalities that will be discussed in this review.

Microbiome diversity

Many factors contribute to the diversity of microbiomes, and most studies have demonstrated the individuality of microbiomes among subjects. Previous findings support microbial communities being more similar in subjects that are genetically related (191), of a similar age (135, 213), or with common diets (including the influences of ethnicity and geography) (63). Diseases will also have an impact on microbiome diversity, including autoimmune and neoplastic diseases, such as inflammatory bowel disease, diabetes, obesity, cardiovascular diseases, allergies, and cancer (37, 121). Treatments for diseases may also affect a patient's gut microbiota, and the consequences of antibiotic use have been intensively investigated (22, 95).

The host genotype has been shown to influence the development of the gut microbiota, and the immune system has been identified as a contributing factor (188). Crosstalk between the microbiome and human immune system occurs in response to a number of environmental factors, such as diet, xenobiotics, and pathogens. Microbial host interactions occur in the gut, mainly in the epithelial cell layer, myeloid cells, and innate lymphoid cells, in which crosstalk and feedback loops contribute to the microbiome composition, host physiology, and disease susceptibility. These interactions contribute not only to the bacterial community along the GI tract, but also to the other microbiota (*Fungi*, *Archaea*, and *Viruses*). Our understanding of the immunology associated with *Fungi* (150) and *Archaea* is currently limited. Transkingdom commensal relationships among microbiota (including *Viruses*) are considered to form from infancy (29, 30, 106, 200) and several co-occurring relationships have been identified (35, 75, 76, 85, 214).

Bacteria. A more complete picture of human-associated bacterial communities obtained using molecular techniques has revealed that their diversity is greater than initially considered through cultivation (9, 20, 56, 90, 113). Using almost full-length 16S rRNA gene sequences, predicted taxa numbers range from 100–300 (20, 56), while pyrosequencing suggests there are 1000s of phylotypes (38, 49). Most of the

gut bacteria identified by 16S rRNA gene sequencing belong to the five phyla originally identified by cultivation, namely, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* (90), and, at lower proportions, *Fusobacteria*, *Tenericutes*, *Spirochaetes*, *Cyanobacteria*, and TM7 (189). At lower levels of the taxonomic classification, microbiome compositions vary with each individual. Attempts have been made to identify a single core microbiome of *Bacteria* in the GI tract. Although this has not been possible in the lower GI tract (mainly using fecal samples) based on taxonomy, it appears there are core microbial functions (152, 189, 191). It is possible to identify some core microbiota in the oral cavity, esophagus, and stomach (148). Although extensive efforts have been made to cultivate representative gut microbiota in an attempt to gain a better understanding of the relationship between taxa and function (156), there are still many undescribed taxa with unknown functional roles in the gut.

As the price of sequencing decreases, it is becoming more common to use a metagenomic approach that provides information on all microbiota and potential functions (3, 70, 167, 189). This provides a means to go beyond *Bacteria* and obtain information on eukaryotic microbes (mainly fungi) and viruses. Although *Fungi*, *Archaea*, and *Viruses* in the microbiome are a part of the 'rare biosphere' (organisms that comprise <0.1% of the microbiome) (173), they still have a significant impact on host health.

Fungi

Fungi are considered to comprise approximately 0.03% of the fecal microbiome (143); making them approximately 3,300-fold less abundant than *Bacteria*. Fungal diversity in the human gut is also lower than that of *Bacteria* (143, 166), although more taxa are being found as the number of individuals being studied using next generation sequencing is increasing (44, 126, 166, 182). In 2015, a review of 36 fungal gut microbiome studies revealed that there have been at least 267 distinct fungi identified in the human gut (181), while another study reported 221 (72). Despite the number of taxa that have been reported, most fungi are highly variable among individuals, with few appearing to be common to all.

Cultivation-based analyses have typically identified *Candida* as the most common fungal genus (166), and it is also frequently identified using non-cultivation-based methods, whereas the other taxa identified have been variable, which may be because of the analytical method used and/or subject variability. For example, 66 genera of fungi were found using pyrosequencing when 98 individuals were examined, with the genera *Saccharomyces*, *Candida*, and *Cladosporium* being the most prevalent (85). *Mucor* was common in Spanish individuals (126) and the most common fungi in 16 vegetarians were *Fusarium*, *Malassezia*, *Penicillium*, and *Aspergillus* (182). These studies suggested that some taxa, *e.g.*, *Penicillium* and *Aspergillus*, are not resident in the gut and enter through environmental sources, such as food and water, in which they are commonly found. This may account for some of the variability in taxa reported in various studies and for the increasing number of fungi being identified as more studies are being performed, even those based on cultivation (71). Under certain conditions, some fungi may flourish and become pathogenic including *Candida*, *Aspergillus*, *Fusarium*, and *Cryptococcus* (44, 84, 140, 143). More information on fungal interactions and diseases is available in a review by Wang *et al.* (204).

Despite their low abundance, fungi appear to have developed in mammalian guts along with the rest of the body from infancy (106, 169). Although there is no consensus of a core mycobiome, *Candida*, *Saccharomyces*, and *Malassezia* have been commonly reported (72). Most of the fungal species detected appear to be either transient or environmental fungi that cannot colonize the gut and are often found in a single study and/or one host only. A previous study indicated that the fungal community is unstable; only 20% of the initially identified fungi were detected again 4 months later (78). More studies on the stability of the mycobiome are needed in order to establish the ecological roles of the components of the mycobiome. Many non-bacterial organisms have been found in numerous mammalian systems, which indicates that they play an important role that has been largely overlooked and may lead to important discoveries and understanding in the coming years.

Archaea

The most commonly reported genus of *Archaea* that has been found in the GI tract is *Methanobrevibacter* (51, 55, 66, 85, 109). Other genera that have also been detected are *Methanosphaera* (51), *Nitrososphaera*, *Thermogynomonas*, and *Thermoplasma* (85) and the new candidate species, *Methanomethylophilus alvus* (27, 131). Although *Archaea* comprise a very small proportion of the microbiota, *Methanobrevibacter* species are important contributors to methanogenesis (66). Differences in *Archaea* in microbiome samples may be due to the method used (51) and/or complex relationships with other microbiota. For example, *Methanobrevibacter* and *Nitrososphaera* were previously shown to be mutually exclusive and potentially related to carbohydrate intake (85). More studies are needed in order to clarify the interaction between *Archaea* and other microbiota groups, which may contribute to our understanding of their fitness and function (beyond methanogenesis) in the microbiome.

Viruses

Viruses in the human microbiome have also been understudied and available information is limited (161); the majority of data are related primarily to disease and do not address the commensal virome (34, 40). The majority of viral reads in studies that have been performed cannot be assigned to a known group; this has contributed to the difficulties associated with assessing their roles in the GI tract (124, 160). A number of teams have made extensive efforts in order to advance human virome studies (157, 161). In the last ten years, the number of identified polyomaviruses has increased from 4 to 13 species (some that cause disease and some that do not) (47), and the accuracy of identification techniques has been improved to identify taxa at the genus level (199) and use metagenomic information for viral taxonomy (172). Viral communities are mainly comprised of bacteria-infecting phage families (~90%), while eukaryotic viruses (~10%) are

in lower abundance (157, 161). Metagenomic analyses have suggested that the new bacteriophage, crAssphage associated with *Bacteroides*, is potentially common in humans (53). The greatest diversity of phages is considered to occur in infants and decreases with age, in contrast to increases in bacterial diversity (116, 117, 162). With the availability of methods to enrich viruses in samples (41), and with more metagenomic sequences and bioinformatics tools to identify viral sequences (53, 139), more information will be obtained on viral diversity and associated physiological factors in humans.

Similar to the microbiota, considerable variability appears to exist in the viral taxa found among subjects (133). Limited information is currently available on the functional roles of most viruses in the human GI tract. However, some possible functions are: to increase bacterial fitness as sources of genetic information (*e.g.*, the source of antibiotic resistance genes), to increase the immunity of bacteria or the human host, and to protect against pathogens (40, 64, 157). The general consensus is that the presence of bacteria is beneficial for viruses that are increasingly trying to evade the immune system. This relationship may also be beneficial to bacteria as viruses may be sources of potentially advantageous genes (resistance or tolerance to stress environments). Researchers are now examining the ecological and evolutionary influences of phages on bacterial ecosystems (102), and the findings obtained may provide insights into the important roles played by phages in the gut microbiome.

The GI tract

Many challenges are associated with studying the microbial ecology of the GI tract because it is composed of chemically and physically diverse microhabitats stretching from the esophagus to the rectum, providing a surface area of 150–200 m^2 for colonization or transient occupation by microbes (16). The adult GI tract was initially estimated to harbor 10^{14} bacteria, 10 times more cells than the human body (16, 120); however, a more recent calculation estimates there to be 10^{13} bacteria, which is equivalent to the number of human cells (170). Lower bacterial numbers (10^3 to 10^4 bacteria mL^{-1} of intestinal content) are found in the upper end of the GI tract, stomach, and small intestine, in which pH is low and the transit time is short (16). The highest biodiversity (richness and evenness) of bacteria (10^{10}–10^{11} bacteria g^{-1} of intestinal content) is in the colon, in which cell turnover rate is low, redox potential is low, and the transit time is long. This section highlights the different functions and associated microbiota along the human GI tract starting from the oral cavity, then the esophagus, stomach, and intestines (Fig. 1).

The oral cavity. Activity in the mouth may have a large impact on the further digestion of food in the lower GI tract. Food is mechanically ground into small particles, typically 0.1 mm, which increases the surface area. The oral microbiome is composed of transient and commensal populations that often form biofilms on soft and hard surfaces in the mouth (8). The most up-to-date information on taxa of the oral microbiome may be found in the Human Oral Microbiome Database (HOMD, http://www.homd.org/) (50). Information in this database is limited to *Bacteria* and one *Archaea*. Cultivation-independent analyses indicate that the most common genus is *Streptococcus*, while other genera include *Neisseria*, *Gemella*, *Granulicatella*, and *Veillonella*, but not in all individuals examined (1, 91, 92, 107). The taxa present appear to be dependent on interactions between microbes within the community. For example, using a graph theory-based algorithm of an organism's nutritional profile, the species *Streptococcus oralis* and *S. gordonii* have low metabolic complementarity and high metabolic competition, indicating they are antagonistic to each other (110). In contrast, *Porphyromonas gingivalis* was shown to have high metabolic complementarity, indicating its ability to grow symbiotically with diverse oral microbiota taxa. This computational method was tested and confirmed with growth assays, making it a viable means to assess the ability of species to inhabit the same environment. This has also been shown using an *in situ* spectral analysis of microbiota in biofilm plaques. Biofilms were shown to be composed of a number of taxa with *Corynebacterium* at the foundation (209). The other taxa are considered to play complementary roles driven by the environmental and chemical gradients formed in biofilms that control nutrient availability. These findings indicate that, despite the large number of taxa identified in oral microbiome studies, the core taxa of all microbiota may be identified in the future based on spatial locations and functional roles (10).

Similar to *Bacteria*, large variations have been noted in viruses found in the oral cavity among subjects (151). Most viruses are bacteriophages (approx. 99% of known sequences). Viral communities are reproducible across time points within a subject, suggesting that they are stable; however, the human and bacterial host significantly influence compositions (2, 151, 163). In addition to interactions among oral bacteria, many may associate with phages (57). Depending on the host range of the oral virome, this may make phages very common inhabitants of the oral cavity. Furthermore, in addition to survival within bacterial hosts, phages may also survive in the oral mucosa and contribute to host immunity (11). These are all new avenues of oral virome research that will likely be investigated in greater depth in the future.

In addition to the bacterial microbiome, two cultivation-independent studies have been conducted on oral fungi. Approximately 100 fungal species (20 genera) were detected in one study of the oral mycobiome of healthy individuals (68). Among the fungi detected, *Candida* species were the most common and abundant, while the other genera consisted of *Cladosporium*, *Aureobasidium*, *Saccharomycetales*, *Aspergillus*, *Fusarium*, and *Cryptococcus*. Most of these genera were also detected in a recent study on three subjects; however, *Malassezia*, a skin pathogen, accounted for the most sequence reads (52). Most of the other studies conducted on the oral mycobiome have focused on the role of fungi in disease (69, 136). Since the oral microbial community is directly exposed to the environment, the presence of a dynamic and transient community is expected, but warrants further study.

Esophagus. After swallowing, food is transported down the esophagus by peristalsis to the stomach. Limited information is available on microbes inhabiting the esophagus (5, 91, 147), and this may be due to the difficulties associated with obtaining samples because biopsies have typically been used. However, a less invasive method using an esophageal string has recently been demonstrated to be a feasible alternative

and yields similar findings to non-cultivation-based analyses of biopsies (60). Similar to the oral cavity, the most common genus found in the esophagus is *Streptococcus*; however, an overall comparison of the two communities has indicated that the number of taxa significantly differ between the two locations (15, 60). Among the few studies conducted on the viral and fungal microbiota of the esophagus, the focus has been on association with disease (204) and none of the pathogenic taxa inhabit healthy individuals.

Stomach. The stomach is the first digestive organ in the body (89). It holds food and mechanically mixes it with proteolytic enzymes and gastric acids that aid in the breakdown and subsequent absorption of nutrients. The growth of many common bacteria is inhibited by these acidic conditions (pH<4), making this a unique community with the lowest number of microbes, ranging between 10^1 and 10^3 CFU g^{-1}. In addition to digestion, the acidic conditions of the stomach are considered to have evolved as a means of protection from pathogens. This hypothesis is supported by the recent finding of a lower pH in the stomachs of scavengers and higher pH in herbivores, which are less likely to encounter pathogens in their food (13). Caution is needed when comparing the findings of various studies throughout the GI tract because gastric juice has a lower pH than the mucosal layer, resulting in differences in the microbiota present (89).

Despite the low pH, non-cultivation-based analyses on stomach biopsies revealed a more diverse microbiota than expected (5, 20, 115). Regardless of variations among subjects, there appears to be two major groups of individuals: those with and without *Helicobacter pylori* (20). There is a third subset in which *H. pylori* is present in lower proportions in some individuals that were negative using conventional testing. Microbiomes dominated by *H. pylori* had significantly greater proportions of the phylum *Proteobacteria*, of which it is a member, and lower alpha diversity (5, 20). Other common genera are *Streptococcus* and *Prevotella*, both of which are also found in the oral and esophageal communities; however, the communities at these locations appear to differ (5). Limited information is available on fungi analyzed in biopsy samples; although a cultivation study detected *Candida* species, this appeared to be associated more with disease (224). The major interaction currently studied in the stomach microbiota is with *Helicobacter* because of its association with gastritis, peptic ulcers, and gastric cancer. However, this taxon has been suggested to be beneficial for health, leading some to question whether the complete eradication of this microbe is the best option (67, 89).

In contrast, less information is available on the microbiome of stomach fluids; it appears to harbor fewer *Helicobacter* and an analysis of transcripts indicated that *Actinobacteria* are the most active phylum; however, the other major phyla, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*, are also present (197). In the same study, it also appeared to harbor novel fungi; 77.5% of the ITS reads were not identified at the phylum level or lower. *Candida* and *Phialemonium* were the only two identifiable fungal genera in all subjects tested, whereas an additional 66 genera were present in at least one of the nine subjects examined. Based on the infrequency and number of reads in this analysis, most of the taxa identified in stomach fluids appear to be transient, and those playing an active role are limited in this location.

Intestines. After mixing in the stomach, chime slowly passes through the pyloric sphincter and enters the intestines, in which the major digestion and absorption of nutrients begin (12). Humans have a small and large intestine. The small intestine, the main location in which food digestion and absorption occurs, is further divided into three parts, the duodenum, jejunum, and ileum. The duodenum, in which food chime enters from the stomach, is directly associated with digestion and is linked to the pancreas and gallbladder. Bile salts from the gallbladder and enzymes from the pancreas enter the duodenum and mix with stomach chime in order to start the digestion process. The epithelium in the jejunum and ileum is responsible for glucose absorption into the bloodstream via glucose transporters and sodium ions. The small intestine is followed by the large intestine (colon), which has a larger diameter, but shorter length and is divided into four sections: the ascending colon (cecum), transverse colon, descending colon, and sigmoid colon (123). Water and minerals are continuously absorbed along the colon before excretion. Furthermore, complex foods that cannot be digested by the host are used as growth substrates for the colonic microbiota (25, 178).

Spatial and temporal variabilities have been noted in the microbial composition among the different intestinal structures based on their functional roles and timing of food intake (18, 129, 186). Although spatial variability exists along the intestinal tract, the bacterial microbiome at the phylum level is considered to remain fairly stable over time (43, 155); however, many factors may affect its stability (119). Undigested food and most of the microbiota are found in the lumen, the central space surrounded by the mucosal layer of the tubular intestinal structure. The main absorption of growth substrates occurs through the epithelial cells of the mucosa, which also prevents the entry of the microbiota into host cells (174). A number of important host-microbe interactions occur within the mucosa. Energy from microbially produced metabolites, such as butyrate, contributes to epithelial metabolism (97). Most of the gut is anaerobic, but there is an oxygen gradient in the mucosa that provides a competitive advantage for facultative anaerobes (174). Recent studies have also shown the importance of metabolites produced by transkingdom microbiota to host physiology (185, 187, 188). Microbiota, such as *Akkermansia mucinophila*, are commonly found residing in the mucus layer and feed on mucin (39, 48). Therefore, the effects of host interactions with the gut microbiota, particularly those in the large intestine, have a prominent impact on overall human health, including energy reabsorption and immune system development.

Due to the difficulties associated with collecting multiple samples along a healthy human GI tract in order to capture the spatial heterogeneity of microbes in this environment, most studies use fecal samples as a surrogate. However, this limits the availability of regio-specific community information on the GI tract, resulting in portions, such as the small intestine, remaining poorly characterized. The few studies conducted on the small intestine have limited subject numbers because they used biopsy samples (4, 201, 203) or ileotomy patients (108, 195, 222). The bacterial genera most commonly found among these studies were *Clostridium*, *Streptococcus*, and *Bacteroides*. The number of studies that include fungi are

Table 1. Comparison of the anatomy of the intestinal tract in humans and animal models

	Human	Mouse	Rat	Pig
Stomach	Four regions: cardia, fundus, body, and pylorus	Three regions: forestomach, body, and pylorus	Three regions: forestomach, body, and pylorus	Four regions: esophagus, cardia, fundus, and pylorus
	pH 1.5 to 3.5	pH 3.0 to 4.0	pH 3.0 to 4.0	pH 1.5 to 2.5
Small intestine	5.5–6.4 m in length	350 mm in length	1,485 mm in length	1.2–2.1 m in length
	pH 6.4 to 7.3	pH 4.7 to 5.2	pH 5.0 to 6.1	pH 6.1 to 6.7
Cecum	Smaller than the colon	Larger than the colon	Larger than the colon	Smaller than the colon
	No fermentation	Main fermentation	Main fermentation	Some fermentation
	pH 5.7	pH 4.4 to 4.6	pH 5.9 to 6.6	pH 6.0 to 6.4
Appendix	Present	Absent	Absent	Absent
Colon	Divided into the ascending, transcending, and descending colon	Not divided	Not divided	Divided into the ascending, transcending, and descending colon
	Main fermentation	No fermentation	No fermentation	Main fermentation
	Thick mucosa	Thinner mucosa	Thinner mucosa	Thick mucosa
	pH 6.7	pH 4.4 to 5.0	pH 5.5 to 6.2	pH 6.1 to 6.6

Adapted from (59, 96, 128, 130, 137, 196)

even more limited, with the genera *Candida* and *Saccharomyces* being the most frequently detected (108, 114). Caution is also needed when extrapolating these findings to all individuals because the health of some subjects was compromised when samples were obtained.

Bacteria in the colon account for approximately 70% of all bacteria in the human body because it is the main site for the bacterial fermentation of non-digestible food components such as soluble fiber. The small number of studies that have examined microbial communities directly in the colon suggests that the bacterial composition is similar to that found in feces (86). However, fecal communities do not represent a single colonic environment, such as the mucosa (223), but a mixture of indigenous and transient microbes from the entire GI tract. In studies in which a global view of the GI tract microbial community is of interest, fecal material represents a good surrogate and is easily obtained, allowing for multiple samples to be obtained over short and long time periods from healthy individuals. The majority of microbiome reviews have extensively covered colonic communities using feces (74, 92, 121, 148, 189); therefore, we will not describe its composition in detail. However, later in this review, the impact of diet on the microbiome composition will be discussed. Furthermore, the above sections on fungi and viruses provide information on the taxa of these groups in the intestines.

Summary of the GI tract. The use of non-cultivation-based methods to investigate the microbiota in the GI tract has increased our knowledge of their diversity. One group that we neglected to mention in this review was *Protozoans*/*Protists*; however, recent reviews are available (79, 145). Despite representing a smaller biomass than fungi, they also appear to be important to the ecological structure of the gut microbiome. The predator-prey relationship they have with other microbiota (145) may, in some cases, lead to disease prevention (7). Difficulties are associated with elucidating the functional roles played by these various taxa at different points along the GI tract. Therefore, it is still important to obtain cultivated representatives to investigate their role and ecological significance along the GI tract. This consideration is important for all microbiota; however, it represents a larger issue for low diversity groups, such as fungi, which may not be numerically abundant, but still play a significant role (17).

Use of animal models

Animal models have been widely adopted in human gut microbiome research (28, 98, 220) to reduce confounding experimental factors such as genetics, age, and diet, which may be more easily controlled in laboratory animals. Additionally, animal models with modified genetic backgrounds are available for investigating potential mechanisms (137). Ideally, animal models with relatively similar genetic information (217), gut structures, metabolism (142), and diets and behavior patterns (202) to humans need to be selected. Comprehensive comparisons of mice (137) and pigs (217) to humans were recently conducted in order to aid in translating information from animal models to humans. In this section, we will highlight some of their findings and compare GI tract structures and microbial community compositions. Furthermore, some advantages and limitations associated with the use of animal models in human microbiome research will be discussed.

Similarities exist in the anatomy of the GI tract between humans and most animal models (Table 1). However, differences in anatomical structures and pH at different locations along the GI tract may contribute to differences in the microbiota found in humans versus animal models (26). The human colon also has a thicker mucosal layer than those of mice and rats (137), which may have an effect on the diversity of the microbiota colonizing the colon. Human gut bacteria are dominated by two phyla: *Firmicutes* and *Bacteroidetes* (189), which also dominate the GI tract of commonly used model animals (112). However, at lower taxonomic levels, some differences have been reported in microbiome compositions in the gut between humans and animal models (Table 2). The dominant taxa reported have varied as the number of comparisons performed has increased (137, 152); therefore, the findings shown in Table 2 need to be used cautiously.

A pig gut gene catalogue of metabolic function was recently developed and compared to catalogues available for humans and mice (217). They found that 96% of the KEGG orthologs in humans were also present in pigs, whereas the overlap at the gene level was markedly lower (9.46%). However, there was a greater overlap between humans and pigs than between humans and mice. Microbial activity also differs along the GI tract, with the most relevant being fermentation occurring in the ceca of most animal models, but

Table 2. Major taxa of the gut microbiota in humans and animal models

	Human	Mouse	Rat	Pig
Bacteria	*Firmicutes* *Bacteroidetes* *Actinobacteria* *Proteobacteria*	*Firmicutes* *Bacteroidetes*	*Firmicutes* *Bacteroidetes*	*Firmicutes* *Bacteroidetes*
Archaea	*Methanobrevibacter* *Nitrososphaera*	*Methanobrevibacter*	*Methanobrevibacter*	*Methanomicrobia*, *Methanosphaera*
Viruses	*Herpesviridae* *Papillomaviridae* *Polyomaviridae* *Adenoviridae*	Variable	Variable	*Picornaviridae* *Astroviridae* *Coronaviridae* *Caliciviridae*
Eukarya	*Candida* *Malassezia* *Saccharomyces* *Cladosporium*	*Ascomycota* *Basidiomycota* *Chytridiomycota* *Zygomycota*	*Ascomycota* *Basidiomycota* *Chytridiomycota* *Zygomycota*	*Kazachstania* *Candida* *Galactomyces* *Issatchenkia*

Adapted from (85, 103, 105, 112, 125, 137, 153, 154, 171, 179, 193, 194, 215, 216, 221)

not in humans (137). Strengths and weaknesses are associated with the major animal models being used, and these need to be taken into consideration when conducting translational research.

Rats. The use of rats as lab animals dates back to the 1850s. They were considered to be a good candidate for human microbiome research because the rat contains the same four dominant bacteria phyla in the GI tract (31), with *Firmicutes* (74%) and *Bacteroidetes* (23%) representing the largest proportions (221). The advantages of using rats in human microbiome research include quick reproduction, a fully sequenced genome, and easy handling and maintenance due to their relatively small size. The limitation of this model is that the diet used in rats differs from that for humans, and their behavior and living environment are also different, which will affect the gut microbiota. The diet used in rat studies is normal chow that is rich in fiber (205), and diet may rapidly alter gut microbiota diversity (46). Although most studies emphasize the impact of diet on the microbiota in the cecum and/or colon (feces), the oral cavity of rats has been used to clarify the impact of diet on the microbiome (93).

Mice. Many of the strengths and weaknesses associated with using rats are also applicable to mice. Similar to humans, the microbiota in the GI tract of mice is dominated by *Firmicutes* (74%) and *Bacteroidetes* (23%) at the phylum level (217). However, there are differences at the genus level, and this has led to the use of "humanized" mice. This is achieved by inoculating human gut microbiota into germ-free (GF) mice (192) or mice treated with antibiotics to eliminate their gut microbiome (83). The microbiome of these mice after fecal transplants may have a composition at the phyla level that is 100% similar to humans and 88% at the genus level (137). A recent study (175) used humanized mice to test microbiome diversity after feeding with poorly accessible carbohydrates, and found a similar reduction in OTU numbers to a human study (219). However, there are also some limitations to using these animals, including the diet and environmental living conditions. Furthermore, gnotobiotic mice may not reflect the human-microbe relationship due to their weaker immune system (6).

Approximately 10 years ago, Scupham (168) showed that all four major fungal phyla, *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Zygomycota*, were present in the murine gut. Additionally, many genera were identified, including *Acremonium*, *Monilinia*, *Fusarium*, *Cryptococcus*, *Filobasidium*, *Scleroderma*, *Catenomyces*, *Spizellomyces*, *Neocallimastix*, *Powellomyces*, *Entophlyctis*, *Mortierella*, and *Smittium*. When comparing these studies to the human gut, it is important to note that this study indicated a more diverse fungal community than those found in humans; the eukaryotic diversity of the human gut is low (143).

Pigs. Pigs have been used as surrogates for human microbiome research due to their highly similar genetics, physiological structures, behavior, metabolism, and immune functions to those of humans (81, 202). The greater similarities in the omnivorous diet and GI tract structure between pigs and humans are more advantageous than the murine model. The microbiome of pigs is dominated by two phyla: *Firmicutes* and *Bacteroidetes* (104); however, there are some notable differences at the genus level. The genus *Prevotella* was found to be common in two pig metagenomic studies (104, 118). Since the number of pigs used in most studies is less than humans, the pig core microbiome at the genus level may change as more pigs are studied. Another contributing factor to shaping the microbiome composition is diet. Most studies have found that the number of *Bifidobacteria* in pigs, even those on high fiber diets, is lower than that in humans (132, 218), while that of *Lactobacillus* is higher (149). In nutrition studies, humans and pigs are both dependent on the quality of the nutrient load; however, the pig cecum has a larger capacity to ferment indigestible compounds than the human cecum (54). The microbiota composition in pigs may differ from that in humans due in part to differences in diet (81). Similar to mice, humanized GF pigs have been developed and the microbiome after human fecal transplantation more closely resembles that of the donor than conventional pigs (144). However, the same disadvantages associated with using GF mice are also true for GF pigs.

The genome of pigs may be mutated to study human diseases; this is typically performed using miniature pigs such as those from the Ossabaw and Gottingen islands (146). Genetic mutations for metabolic syndrome and insulin resistance have successfully been performed using Ossabaw pigs to study human diseases such as type 2 diabetes (14, 177) and obesity (101). The ratio of *Firmicutes* to *Bacteroidetes* is higher in obese Ossabaw pigs than in lean pigs (146), similar to some

obese humans (111, 190). This finding suggests that Ossabaw pigs are a good model for researching the role of the microbiota in human obesity. However, disadvantages are associated with using miniature pigs, mainly the higher cost for maintenance and longer reproductive period than rodents (146).

Although more extensive efforts have been made to investigate fungi in pigs than in other animal models, many of these studies were cultivation-based or for use as probiotics. Fungi in pigs have been recently studied using a non-cultivation approach and up to 17 species of yeast (belonging to the genera *Kazachstania*, *Galactomyces*, *Candida*, *Issatchenkia*, *Pichia*, *Rhodotorula*, and *Trichosporon*) were common in the gut (194). The number of studies on viruses is limited, but the composition appears to be highly variable among samples (164, 171) and affected by disease (24). These groups need to be examined in more detail in order to establish whether pigs are good models for use in understanding fungi and viruses in humans.

Animal model summary. The convenience and cost of using animal models for human research are appealing. However, researchers need be very careful when selecting animal models appropriate for their objectives, particularly when the objective is to directly extrapolate findings from animals to humans, due to the significant differences in GI tract physiology and microbiome composition (65, 137, 217).

Diet in health

Many studies have found that diet is one of the main factors shaping the composition of gut microbial populations. Dietary approaches, such as the ingestion of non-digestible carbohydrates (prebiotics) and fermented food products containing live cultures (probiotics), have been suggested to confer health benefits by enhancing the growth of beneficial intestinal bacteria (100, 158). As described earlier, the microbiota may break down food components, such as non-digestible carbohydrates, which are indigestible by the host in order to aid in maximizing available nutrients (9) and produce metabolites that contribute to host health. Probiotics have been used as a means to replenish the gut with "beneficial" microbiota after antibiotic treatments or to treat diseases (82, 159). This section will highlight some studies that demonstrated the health benefits of prebiotics and probiotics and possible roles played by the microbiota.

Dietary prebiotics and probiotics. Non-digestible and fermentable food components are often consumed as prebiotics to selectively stimulate the growth and/or activity of endogenous colonic bacteria that may be beneficial to host health. The increased consumption of prebiotics often correlates with enhancements in certain bacterial genera (a common example is *Bifidobacterium* sp.); however, the reason they are beneficial remains unclear (208). Challenges are associated with elucidating the role being played by specific bacterial phylotypes because many of their processes are interactive (207). For example, SCFA produced by bacterial fermentation may lower intestinal pH, thereby increasing the solubility of essential minerals, such as calcium, iron, and magnesium, and consequently enhancing their absorption and improving health. Metabolites produced by microbes may also play an important role in cellular differentiation and proliferation in the colonic mucosa by inducing apoptosis and may confer protection against colitis and colorectal cancer by modulating oncogene expression. These functions do not appear to be performed by a single species; a number of different species may be acting independently or in combination. Research is leading to an understanding of microbial community structure and composition dynamics with respect to diet aids in establishing testable hypotheses for future research in health and beneficial microbes (32). Most research has been performed on the influence of beneficial intestinal bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. on host health monitored using a cultivation approach. Cultivation-independent approaches have now become more popular, leading to the identification of new beneficial microbiota taxa and their potential functional roles in the gut as they relate to diet.

Dietary fibers and oligosaccharides are carbohydrate ingredients that vary in composition and structure, but are considered to be non-digestible because of the lack of appropriate intestinal enzymes to hydrolyze them or structural hindrances that prevent enzyme access in the gut. Although bacteria in the lower gut may ferment these carbohydrates, the rate and degree of fermentation vary with the polysaccharide (80). The range of fermentation in the colon for various fibers is broad, from approximately 5% for cellulose to nearly 100% for pectin (42). The resulting SCFA, including butyrate and propionate, are considered to reduce pH and solubilize minerals, thereby improving their absorption and subsequent utilization. Inulin, a long chain fructooligosaccharide (FOS) often obtained from chicory root, and FOS from other sources are the fibers that have been studied in the most detail (206). Several novel fibers have been tested in an *in vitro* large intestine model for their effects on the microbial stimulation and production of SCFA (122). All these novel fibers stimulated the growth of beneficial *Bifidobacteria* and some *Lactobacillus* species along with increases in SCFA production. Only a few studies have examined the effects of fibers and resistant starches on the human microbiome (87, 127, 198, 210, 211). A soluble corn fiber product has been demonstrated to increase Ca absorption in a number of different studies (210, 211). More benefits to human health may be attributed to the consumption of prebiotics and fermentation by the gut microbiome.

The number of studies that include diet effects on *Archaea*, *Fungi*, and/or *Viruses* are limited; however, some examples are included herein. Examinations of *Archaea*, *Fungi*, and *Bacteria* correlations in response to diet revealed a syntrophic model involving *Candida*, *Prevotella*, *Ruminococcus*, and *Methanobrevibacter* (85). *Candida* was considered to break down carbohydrates into metabolites used by *Prevotella* and *Ruminococcus* that produce CO_2 for *Methanobrevibacter* (85). However, shifts in carbon sources or breaking down starches via amylases from the human mouth may alter this relationship because *Prevotella* may no longer be dependent on *Candida*. This is a good example of how *Archaea*, which represent a very small portion of the microbiome, are a key contributor to methanogenesis and waste decomposition. The absence of *Archaea* may have severe effects on the surrounding community as hydrogen, glucose metabolites, and other carbon sources accumulate. Other organisms will eventually fill this niche, but may diminish or accumulate new metabolites that

ultimately shift the surrounding community based on their fitness for using these substrates.

A recent study investigated rapid changes in the microbiome composition when diets were either high in animal-based or plant-based fat and protein (46). The fungus *Candida* was found to increase in subjects placed on a plant-based diet, whereas *Penicillium* increased on animal-based diets. The most commonly found fungi in vegetarians were *Fusarium*, *Malassezia*, *Penicillium*, *Aspergillus*, and *Candida* (182). Caution is needed when interpreting findings because some of these fungi may be found on food prior to ingestion (46, 78, 182)

Phages assembled in the gut may also be modified by diet. A recent study examined changes in the fecal viral community over an 8-d period in six subjects supplied different diets (134). Shotgun sequencing of virus-like particles revealed that interpersonal differences in the virome were the largest source of variations in this study. However, the virome of subjects whose diets were changed differed more than in those who maintained their normal diet. Although this is only one study with a few human subjects, studies using a mouse model and different dietary fats support these findings (88, 99). Collectively, these findings indicate that diet plays a key role in shaping the gut virome, and further research is needed in order to investigate interactions between diet and the virome.

Summary

Advances have been made in the last decade in our understanding of the role of the GI tract microbiome in human health. This review has highlighted changes and differences in the microbiome along the GI tract that are due to changes in physical, chemical, and biological interactions. Although extensive research has been conducted on *Bacteria* in fecal samples, the main kingdom inhabiting the gut, our knowledge is still insufficient, particularly in other regions of the GI tract. Furthermore, other groups (*Archaea*, *Fungi*, and *Viruses*) have not yet been investigated in adequate detail, demonstrating a real void in knowledge. This highlights that the basic ecology of microbiomes is important for gaining a greater understanding to improve human health and decrease disease. In order to achieve this goal, it is important to include all microbiota in studies and remain cognizant of the limitations associated with understanding the entire GI tract of humans despite challenges in sampling and cultivation. Furthermore, the use of appropriate animal models in mechanistic studies requires careful consideration.

References

1. Aas, J.A., B.J. Paster, L.N. Stokes, I. Olsen, and F.E. Dewhirst. 2005. Defining the normal bacterial flora of the oral cavity. J. Clin. Microbiol. 43:5721–5732.
2. Abeles, S.R., R. Robles-Sikisaka, M. Ly, A.G. Lum, J. Salzman, T.K. Boehm, and D.T. Pride. 2014. Human oral viruses are personal, persistent and gender-consistent. ISME J. 8:1753–1767.
3. Abubucker, S., N. Segata, J. Goll, *et al.* 2012. Metabolic reconstruction for metagenomic data and Its application to the human microbiome. PLoS Comput. Biol. 8:e1002358.
4. Ahmed, S., G.T. Macfarlane, A. Fite, A.J. McBain, P. Gilbert, and S. Macfarlane. 2007. Mucosa-associated bacterial diversity in relation to human terminal ileum and colonic biopsy samples. Appl. Environ. Microbiol. 73:7435–7442.
5. Andersson, A.F., M. Lindberg, H. Jakobsson, F. Bäckhed, P. Nyrén, and L. Engstrand. 2008. Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLoS One 3:e2836.
6. Atarashi, K., T. Tanoue, K. Oshima, *et al.* 2013. T-reg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature 500:232–236.
7. Audebert, C., G. Even, A. Cian, A. Loywick, S. Merlin, E. Viscogliosi, and M. Chabe. 2016. Colonization with the enteric protozoa *Blastocystis* is associated with increased diversity of human gut bacterial microbiota. Sci. Rep. 6:25255.
8. Avila, M., D.M. Ojcius, and Ö. Yilmaz. 2009. The oral microbiota: Living with a permanent guest. DNA Cell Biol. 28:405–411.
9. Backhed, F., R.E. Ley, J.L. Sonnenburg, D.A. Peterson, and J.I. Gordon. 2005. Host-bacterial mutualism in the human intestine. Science 307:1915–1920.
10. Baker, J.L., B. Bor, M. Agnello, W.Y. Shi, and X.S. He. 2017. Ecology of the oral microbiome: Beyond bacteria. Trends Microbiol. 25:362–374.
11. Barr, J.J., R. Auro, M. Furlan, *et al.* 2013. Bacteriophage adhering to mucus provide a non-host-derived immunity. Proc. Natl. Acad. Sci. U.S.A. 110:10771–10776.
12. Barrett, K.E. 2014. Gastrointestinal Physiology, 2nd ed. Lange Medical Books/McGraw-Hill.
13. Beasley, D.E., A.M. Koltz, J.E. Lambert, N. Fierer, and R.R. Dunn. 2015. The evolution of stomach acidity and is relevance to the human microbiome. PLoS One 10:e.0134116.
14. Bellinger, D.A., E.P. Merricks, and T.C. Nichols. 2006. Swine models of type 2 diabetes mellitus: Insulin resistance, glucose tolerance, and cardiovascular complications. ILAR J. 47:243–258.
15. Benitez, A.J., C. Hoffmann, A.B. Muir, K.K. Dods, J.M. Spergel, F.D. Bushman, and M.-L. Wang. 2015. Inflammation-associated microbiota in pediatric eosinophilic esophagitis. Microbiome 3:23.
16. Berg, R.D. 1996. The indigenous gastrointestinal microflora. Trends Microbiol. 4:430–435.
17. Berrilli, F., D. Di Cave, S. Cavallero, and S. D'Amelio. 2012. Interactions between parasites and microbial communities in the human gut. Front. Cell. Infect. Microbiol. 2:141.
18. Biggs, M.B., G.L. Medlock, T.J. Moutinho, H.J. Lees, J.R. Swann, G.L. Kolling, and J.A. Papin. 2017. Systems-level metabolism of the altered Schaedler flora, a complete gut microbiota. ISME J. 11:426–438.
19. Bik, E.M. 2009. Composition and function of the human-associated microbiota. Nutr. Rev. 67:S164–S171.
20. Bik, E.M., P.B. Eckburg, S.R. Gill, K.E. Nelson, E.A. Purdom, F. Francois, G. Perez-Perez, M.J. Blaser, and D.A. Relman. 2006. Molecular analysis of the bacterial microbiota in the human stomach. Proc. Natl. Acad. Sci. U.S.A. 103:732–737.
21. Bik, E.M., C.D. Long, G.C. Armitage, *et al.* 2010. Bacterial diversity in the oral cavity of 10 healthy individuals. ISME J. 4:962–974.
22. Blaser, M.J. 2016. Antibiotic use and its consequences for the normal microbiome. Science 352:544–545.
23. Blaut, M., M.D. Collins, G.W. Welling, J. Dore, J. van Loo, and W. de Vos. 2002. Molecular biological methods for studying the gut microbiota: the EU human gut flora project. Brit. J. Nutr. 87:S203–S211.
24. Blomstrom, A.L., C. Fossum, P. Wallgren, and M. Berg. 2016. Viral metagenomic analysis displays the co-infection situation in healthy and PMWS affected pigs. PLoS One 11:e0166863.
25. Bolam, D.N., and J.L. Sonnenburg. 2011. Mechanistic insight into polysaccharide use within the intestinal microbiota. Gut microbes 2:86–90.
26. Booijink, C., E.G. Zoetendal, M. Kleerebezem, and W.M. de Vos. 2007. Microbial communities in the human small intestine: coupling diversity to metagenomics. Fut. Microbiol. 2:285–295.
27. Borrel, G., H.M. Harris, W. Tottey, *et al.* 2012. Genome sequence of "Candidatus *Methanomethylophilus alvus*" Mx1201, a methanogenic archaeon from the human gut belonging to a seventh order of methanogens. J. Bacteriol. 194:6944–6945.
28. Bowey, E., H. Adlercreutz, and I. Rowland. 2003. Metabolism of isoflavones and lignans by the gut microflora: a study in germ-free and human flora associated rats. Food Chem. Toxicol. 41:631–636.
29. Breitbart, M., I. Hewson, B. Felts, J.M. Mahaffy, J. Nulton, P. Salamon, and F. Rohwer. 2003. Metagenomic analyses of an uncultured viral community from human feces. J. Bacteriol. 185:6220–6223.

30. Breitbart, M., M. Haynes, S. Kelley, *et al.* 2008. Viral diversity and dynamics in an infant gut. Res. Microbiol. 159:367–373.
31. Brooks, S.P.J., M. McAllister, M. Sandoz, and M.L. Kalmokoff. 2003. Culture-independent phylogenetic analysis of the faecal flora of the rat. Can. J. Microbiol. 49:589–601.
32. Brownawell, A.M., W. Caers, G.R. Gibson, C.W.C. Kendall, K.D. Lewis, Y. Ringel, and J.L. Slavin. 2012. Prebiotics and the health benefits of fiber: Current regulatory status, future research, and goals. J. Nutr. 142:962–974.
33. Cadwell, K. 2015. Expanding the role of the virome: Commensalism in the gut. J. Virol. 89:1951–1953.
34. Cadwell, K. 2015. The virome in host health and disease. Immunity 42:805–813.
35. Cavalcanti, I.M.G., A.H. Nobbs, A.P. Ricomini, H.F. Jenkinson, and A.A.D. Cury. 2016. Interkingdom cooperation between *Candida albicans*, *Streptococcus oralis* and *Actinomyces oris* modulates early biofilm development on denture material. Pathog. Dis. 74:ftw002.
36. Charbonneau, M.R., L.V. Blanton, D.B. DiGiulio, D.A. Relman, C.B. Lebrilla, D.A. Mills, and J.I. Gordon. 2016. A microbial perspective of human developmental biology. Nature 535:48–55.
37. Cho, I., and M.J. Blaser. 2012. The human microbiome: at the interface of health and disease. Nat. Rev. Genet. 13.
38. Claesson, M.J., O. O'Sullivan, Q. Wang, J. Nikkilä, J.R. Marchesi, H. Smidt, W.M. de Vos, R.P. Ross, and P.W. O'Toole. 2009. Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS ONE 4:e6669.
39. Collado, M.C., M. Derrien, E. Isolauri, W.M. de Vos, and S. Salminen. 2007. Intestinal Integrity and *Akkermansia muciniphila*, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. Appl. Environ. Microbiol. 73:7767–7770.
40. Columpsi, P., P. Sacchi, V. Zuccaro, S. Cima, C. Sarda, M. Mariani, A. Gori, and R. Bruno. 2016. Beyond the gut bacterial microbiota: The gut virome. J. Med. Virol. 88:1467–1472.
41. Conceição-Neto, N., M. Zeller, H. Lefrère, *et al.* 2015. Modular approach to customise sample preparation procedures for viral metagenomics: a reproducible protocol for virome analysis. Sci. Rep. 5:16532.
42. Cook, S.I., and J.H. Sellin. 1998. Review article: short chain fatty acids in health and disease. Ailment Pharmacol. Ther. 12:499–507.
43. Costello, E.K., C.L. Lauber, M. Hamady, N. Fierer, J.I. Gordon, and R. Knight. 2009. Bacterial community variation in human body habitats across space and time. Science 326:1694–1697.
44. Cui, L., A. Morris, and E. Ghedin. 2013. The human mycobiome in health and disease. Genome Med. 5:63.
45. Dalmasso, M., C. Hill, and R.P. Ross. 2014. Exploiting gut bacteriophages for human health. Trends Microbiol. 22:399–405.
46. David, L.A., C.F. Maurice, R.N. Carmody, *et al.* 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature 505:559–563.
47. DeCaprio, J.A., and R.L. Garcea. 2013. A cornucopia of human polyomaviruses. Nat. Rev. Microbiol. 11:264–276.
48. Derrien, M., E.E. Vaughan, C.M. Plugge, and W.M. de Vos. 2004. *Akkermansia muciniphila* gen. nov., sp nov., a human intestinal mucin-degrading bacterium. Internat. J. Syst. Evol. Microbiol. 54:1469–1476.
49. Dethlefsen, L., S. Huse, M.L. Sogin, and D.A. Relman. 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol. 6:e280.
50. Dewhirst, F.E., T. Chen, J. Izard, B.J. Paster, A.C.R. Tanner, W.-H. Yu, A. Lakshmanan, and W.G. Wade. 2010. The human oral microbiome. J. Bacteriol. 192:5002–5017.
51. Dridi, B., D. Raoult, and M. Drancourt. 2011. Archaea as emerging organisms in complex human microbiomes. Anaerobe 17:56–63.
52. Dupuy, A.K., M.S. David, L. Li, T.N. Heider, J.D. Peterson, E.A. Montano, A. Dongari-Bagtzoglou, P.I. Diaz, and L.D. Strausbaugh. 2014. Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: Discovery of *Malassezia* as a prominent commensal. PLoS One 9:e90899.
53. Dutilh, B.E., C.W. Noriko, K. McNair, *et al.* 2014. A highly abundant bacteriophage discovered in the unknown sequences of human faecal metagenomes. Nat. Commun. 5:4498.
54. Eberhard, M., U. Hennig, S. Kuhla, R.M. Brunner, B. Kleessen, and C.C. Metges. 2007. Effect of inulin supplementation on selected gastric, duodenal, and caecal microbiota and short chain fatty acid pattern in growing piglets. Arch. Animal Nutr. 61:235–246.
55. Eckburg, P.B., P.W. Lepp, and D.A. Relman. 2003. Archaea and their potential role in human disease. Infect. Immun. 71:591–596.
56. Eckburg, P.B., E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S.R. Gill, K.E. Nelson, and D.A. Relman. 2005. Diversity of the human intestinal microbial flora. Science 308:1635–1638.
57. Edlund, A., T.M. Santiago-Rodriguez, T.K. Boehm, and D.T. Pride. 2015. Bacteriophage and their potential roles in the human oral cavity. J. Oral Microbiol. 7:27423.
58. Egert, M., A.A. de Graaf, H. Smidt, W.M. de Vos, and K. Venema. 2006. Beyond diversity: functional microbiomics of the human colon. Trends Microbiol. 14:86.
59. Fallingborg, J., L.A. Christensen, M. Ingeman-Nielsen, B.A. Jacobsen, K. Abildgaard, and H.H. Rasmussen. 1989. pH-profile and regional transit times of the normal gut measured by a radiotelemetry device. Aliment Pharmacol. Ther. 3:605–613.
60. Fillon, S.A., J.K. Harris, B.D. Wagner, *et al.* 2012. Novel device to sample the esophageal microbiome—The esophageal string test. PLoS One 7:e42938.
61. Fleet, J.C. 2006. Molecular regulation of calcium metabolism, p. 163–189. *In* C.M. Weaver and R.P. Heaney (ed.), Calcium in Human Health. Humana Press Inc., Totowa, NJ.
62. Fleet, J.C., and R.D. Schoch. 2010. Molecular mechanisms for regulation of intestinal calcium absorption by vitamin D and other factors. Crit. Rev. Clin.l Lab. Sci. 47:181–195.
63. Flint, H.J., S.H. Duncan, K.P. Scott, and P. Louis. 2007. Interactions and competition within the microbial community of the human colon: links between diet and health. Environ. Microbiol. 9:1101–1111.
64. Foca, A., M.C. Liberto, A. Quirino, N. Marascio, E. Zicca, and G. Pavia. 2015. Gut inflammation and immunity: What is the role of the human gut virome? Mediat. Inflamm. Article ID 326032.
65. Fritz, J.V., M.S. Desai, P. Shah, J.G. Schneider, and P. Wilmes. 2013. From meta-omics to causality: experimental models for human microbiome research. Microbiome 1:14.
66. Gaci, N., G. Borrel, W. Tottey, P.W. O'Toole, and J.-F. Brugère. 2014. Archaea and the human gut: New beginning of an old story. World J. Gastroenterol. 20:16062–16078.
67. Gagnaire, A., B. Nadel, D. Raoult, J. Neefjes, and J.-P. Gorvel. 2017. Collateral damage: insights into bacterial mechanisms that predispose host cells to cancer. Nat. Rev. Microbiol. 15:109–128.
68. Ghannoum, M.A., R.J. Jurevic, P.K. Mukherjee, F. Cui, M. Sikaroodi, A. Naqvi, and P.M. Gillevet. 2010. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. PLoS Pathog 6:e1000713.
69. Gholizadeh, P., H. Eslami, M. Yousefi, M. Asgharzadeh, M. Aghazadeh, and H.S. Kafil. 2016. Role of oral microbiome on oral cancers, a review. Biomed. Pharmacother. 84:552–558.
70. Gill, S.R., M. Pop, R.T. DeBoy, *et al.* 2006. Metagenomic analysis of the human distal gut microbiome. Science 312:1355–1359.
71. Gouba, N., D. Raoult, and M. Drancourt. 2014. Eukaryote culturomics of the gut reveals new species. PLoS One 9:e106994.
72. Gouba, N., and M. Drancourt. 2015. Digestive tract mycobiota: A source of infection. Med. Mal. Infect. 45:9–16.
73. Greenhalgh, K., K.M. Meyer, K.M. Aagaard, and P. Wilmes. 2016. The human gut microbiome in health: establishment and resilience of microbiota over a lifetime. Environ. Microbiol. 18:2103–2116.
74. Grice, E.A., and J.A. Segre. 2012. The human microbiome: Our second genome, p. 151–170. *In* A. Chakravarti and E. Green (ed.), Ann. Rev. Genom. Hum. Genet., vol. 13.
75. Grimaudo, N.J., W.E. Nesbitt, and W.B. Clark. 1996. Coaggregation of *Candida albicans* with oral Actinomyces species. Oral Microbiol. Immunol. 11:59–61.
76. Grimaudo, N.J., and W.E. Nesbitt. 1997. Coaggregation of *Candida albicans* with oral Fusobacterium species. Oral Microbiol. Immunol. 12:168–173.
77. Guarner, F., and J.-R. Malagelada. 2003. Gut flora in health and disease. The Lancet 361:512–519.
78. Hallen-Adams, H.E., S.D. Kachman, J. Kim, R.M. Legge, and I. Martínez. 2015. Fungi inhabiting the healthy human gastrointestinal tract: A diverse and dynamic community. Fung. Ecol. 15:9–17.

79. Hamad, I., D. Raoult, and F. Bittar. 2016. Repertory of eukaryotes (eukaryome) in the human gastrointestinal tract: Taxonomy and detection methods. Parasite Immunol. 38:12–36.
80. Hamaker, B.R., and Y.E. Tuncil. 2014. A perspective on the complexity of dietary fiber structures and their potential effect on the gut microbiota. J. Mol. Biol. 426:3838–3850.
81. Heinritz, S.N., R. Mosenthin, and E. Weiss. 2013. Use of pigs as a potential model for research into dietary modulation of the human gut microbiota. Nutr. Res. Rev. 26:191–209.
82. Hemarajata, P., and J. Versalovic. 2013. Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. Therapeu. Adv. Gastroenterol. 6:39–51.
83. Hintze, K.J., J.E. Cox, G. Rompato, A.D. Benninghoff, R.E. Ward, J. Broadbent, and M. Lefevre. 2014. Broad scope method for creating humanized animal models for animal health and disease research through antibiotic treatment and human fecal transfer. Gut Microbes 5:183–191.
84. Hoarau, G., P.K. Mukherjee, C. Gower-Rousseau, *et al.* 2016. Bacteriome and mycobiome interactions underscore microbial dysbiosis in familial Crohn's disease. mBio 7:e01250-16.
85. Hoffmann, C., S. Dollive, S. Grunberg, J. Chen, H. Li, G.D. Wu, J.D. Lewis, and F.D. Bushman. 2013. Archaea and fungi of the human gut microbiome: Correlations with diet and bacterial residents. PLoS One 8:e66019.
86. Hold, G.L., S.E. Pryde, V.J. Russell, E. Furrie, and H.J. Flint. 2002. Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. FEMS Microbiol. Ecol. 39:33–39.
87. Hooda, S., B.M.V. Boler, M.C.R. Serao, J.M. Brulc, M.A. Staeger, T.W. Boileau, S.E. Dowd, G.C. Fahey, and K.S. Swanson. 2012. 454 Pyrosequencing reveals a shift in fecal microbiota of healthy adult men consuming polydextrose or soluble corn fiber. The J. Nutr. 142:1259–1265.
88. Howe, A., D.L. Ringus, R.J. Williams, Z.N. Choo, S.M. Greenwald, S.M. Owens, M.L. Coleman, F. Meyer, and E.B. Chang. 2016. Divergent responses of viral and bacterial communities in the gut microbiome to dietary disturbances in mice. ISME J. 10:1217–1227.
89. Hunt, R.H., M. Camilleri, S.E. Crowe, *et al.* 2015. The stomach in health and disease. Gut 64:1650–1668.
90. Huse, S.M., L. Dethlefsen, J.A. Huber, D. Mark Welch, D.A. Relman, and M.L. Sogin. 2008. Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing. PLoS Genet. 4:e1000255.
91. Huse, S.M., Y. Ye, Y. Zhou, and A.A. Fodor. 2012. A core human microbiome as viewed through 16S rRNA sequence clusters. PLoS ONE 7:e34242.
92. Huttenhower, C., D. Gevers, R. Knight, *et al.* 2012. Structure, function and diversity of the healthy human microbiome. Nature 486:207–214.
93. Hyde, E.R., B. Luk, S. Cron, *et al.* 2014. Characterization of the rat oral microbiome and the effects of dietary nitrate. Free Radic. Biol. Med. 77:249–257.
94. Iliev, I.D., V.A. Funari, K.D. Taylor, *et al.* 2012. Interactions between commensal fungi and the C-type lectin receptor dectin-1 Influence colitis. Science 336:1314–1317.
95. Jernberg, C., S. Lofmark, C. Edlund, and J.K. Jansson. 2010. Long-term impacts of antibiotic exposure on the human intestinal microbiota. Microbiol. 156:3216–3223.
96. Kararli, T.T. 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. Biopharm. Drug Dispos. 16:351–380.
97. Kelly, C.J., L. Zheng, E.L. Campbell, *et al.* 2015. Crosstalk between microbiota-derived short-chain fatty acids and intestinal epithelial HIF augments tissue barrier function. Cell Host Microbe 17:662–671.
98. Kibe, R., M. Sakamoto, H. Yokota, H. Ishikawa, Y. Aiba, Y. Koga, and Y. Benno. 2005. Movement and fixation of intestinal microbiota after administration of human feces to germfree mice. Appl. Environ. Microbiol. 71:3171–3178.
99. Kim, M.-S., and J.-W. Bae. 2016. Spatial disturbances in altered mucosal and luminal gut viromes of diet-induced obese mice. Environ. Microbiol. 18:1498–1510.
100. Kleerebezem, M., and E.E. Vaughan. 2009. Probiotic and gut Lactobacilli and Bifidobacteria: Molecular approaches to study diversity and activity. Ann. Rev. Microbiol. 63:269–290.
101. Koopmans, S.J., and T. Schuurman. 2015. Considerations on pig models for appetite, metabolic syndrome and obese type 2 diabetes: From food intake to metabolic disease. Eur. J. Pharmacol. 759:231–239.
102. Koskella, B., and M.A. Brockhurst. 2014. Bacteria–phage coevolution as a driver of ecological and evolutionary processes in microbial communities. FEMS Microbiol. Rev. 38:916–931.
103. Lam, V., J.D. Su, S. Koprowski, A.N. Hsu, J.S. Tweddell, P. Rafiee, G.J. Gross, N.H. Salzman, and J.E. Baker. 2012. Intestinal microbiota determine severity of myocardial infarction in rats. FASEB J. 26:1727–1735.
104. Lamendella, R., J.W.S. Domingo, S. Ghosh, J. Martinson, and D.B. Oerther. 2011. Comparative fecal metagenomics unveils unique functional capacity of the swine gut. BMC Microbiol. 11:103.
105. Lamendella, R., J.W. Santo Domingo, S. Ghosh, J. Martinson, and D.B. Oerther. 2011. Comparative fecal metagenomics unveils unique functional capacity of the swine gut. BMC Microbiol 11:103.
106. LaTuga, M.S., J.C. Ellis, C.M. Cotton, R.N. Goldberg, J.L. Wynn, R.B. Jackson, and P.C. Seed. 2011. Beyond bacteria: A study of the enteric microbial consortium in extremely low birth weight infants. PLoS ONE 6:e27858.
107. Lazarevic, V., K. Whiteson, S. Huse, D. Hernandez, L. Farinelli, M. Osteras, J. Schrenzel, and P. Francois. 2009. Metagenomic study of the oral microbiota by Illumina high-throughput sequencing. J. Microbiol. Meth. 79:266–271.
108. Leimena, M.M., J. Ramiro-Garcia, M. Davids, *et al.* 2013. A comprehensive metatranscriptome analysis pipeline and its validation using human small intestine microbiota datasets. BMC Genom. 14:530.
109. Lepp, P.W., M.M. Brinig, C.C. Ouverney, K. Palm, G.C. Armitage, and D.A. Relman. 2004. Methanogenic Archaea and human periodontal disease. Proc. Natl. Acad. Sci. U.S.A. 101:6176–6181.
110. Levy, R., and E. Borenstein. 2013. Metabolic modeling of species interaction in the human microbiome elucidates community-level assembly rules. Proc. Natl. Acad. Sci. U.S.A. 110:12804–12809.
111. Ley, R.E., P.J. Turnbaugh, S. Klein, and J.I. Gordon. 2006. Microbial ecology—Human gut microbes associated with obesity. Nature 444:1022–1023.
112. Ley, R.E., M. Hamady, C. Lozupone, *et al.* 2008. Evolution of mammals and their gut microbes. Science 320:1647–1651.
113. Li, M., B. Wang, M. Zhang, *et al.* 2008. Symbiotic gut microbes modulate human metabolic phenotypes. Proc. Natl. Acad. Sci. U.S.A. 105:2117–2122.
114. Li, Q., C. Wang, Q. Zhang, C. Tang, N. Li, B. Ruan, and J. Li. 2012. Use of 18S ribosomal DNA polymerase chain reaction–denaturing gradient gel electrophoresis to study composition of fungal community in 2 patients with intestinal transplants. Hum. Pathol. 43:1273–1281.
115. Li, X.-X., G.L.-H. Wong, K.-F. To, V.W.-S. Wong, L.H. Lai, D.K.-L. Chow, J.Y.-W. Lau, J.J.-Y. Sung, and C. Ding. 2009. Bacterial microbiota profiling in gastritis without *Helicobacter pylori* infection or non-steroidal anti-inflammatory drug use. PLoS One 4:e7985.
116. Lim, E.S., Y.J. Zhou, G.Y. Zhao, *et al.* 2015. Early life dynamics of the human gut virome and bacterial microbiome in infants. Nat. Med. 21:1228–1234.
117. Lim, E.S., D. Wang, and L.R. Holtz. 2016. The bacterial microbiome and virome milestones of infant development. Trends Microbiol. 24:801–810.
118. Looft, T., T.A. Johnson, H.K. Allen, *et al.* 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proc. Natl. Acad. Sci. U.S.A. 109:1691–1696.
119. Lozupone, C.A., J.I. Stombaugh, J.I. Gordon, J.K. Jansson, and R. Knight. 2012. Diversity, stability and resilience of the human gut microbiota. Nature 489:220–230.
120. Luckey, T.D. 1972. Introduction to intestinal microecology Amer. J. Clin. Nutr. 25:1292–1294.
121. Lynch, S.V., and O. Pedersen. 2016. The human intestinal microbiome in health and disease. New Engl. J. Med. 375:2369–2379.
122. Maathuis, A., A. Hoffman, A. Evans, L. Sanders, and K. Venema. 2009. The effect of the undigested fraction of maize products on the activity and composition of the microbiota determined in a dynamic *in vitro* model of the human proximal large intestine. J. Am. Coll. Nutr. 28:657–666.

123. Macfarlane, G.T., and J.H. Cummings. 1991. The colonic flora, fermentation and large bowel digestive function. Raven Press, New York, N.Y.
124. Manrique, P., B. Bolduc, S.T. Walk, J. van der Oost, W.M. de Vos, and M.J. Young. 2016. Healthy human gut phageome. Proc. Natl. Acad. Sci. U.S.A. 113:10400–10405.
125. Mao, S.Y., C.F. Yang, and W.Y. Zhu. 2011. Phylogenetic analysis of methanogens in the pig feces. Curr. Microbiol. 62:1386–1389.
126. Mar Rodríguez, M., D. Pérez, F. Javier Chaves, *et al.* 2015. Obesity changes the human gut mycobiome. Sci. Rep. 5:14600.
127. Martinez, I., J. Kim, P.R. Duffy, V.L. Schlegel, and J. Walter. 2010. Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects. PLoS One 5:e15046.
128. McConnell, E.L., A.W. Basit, and S. Murdan. 2008. Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. J. Pharm. Pharmacol. 60:63–70.
129. McHardy, I.H., M. Goudarzi, M. Tong, *et al.* 2013. Integrative analysis of the microbiome and metabolome of the human intestinal mucosal surface reveals exquisite inter-relationships. Microbiome 1:17.
130. Merchant, H.A., E.L. McConnell, F. Liu, C. Ramaswamy, R.P. Kulkarni, A.W. Basit, and S. Murdan. 2011. Assessment of gastrointestinal pH, fluid and lymphoid tissue in the guinea pig, rabbit and pig, and implications for their use in drug development. Eur. J. Pharm. Sci. 42:3–10.
131. Mihajlovski, A., M. Alric, and J.-F. BrugËre. 2008. A putative new order of methanogenic Archaea inhabiting the human gut, as revealed by molecular analyses of the mcrA gene. Res. Microbiol. 159:516–521.
132. Mikkelsen, L.L., C. Bendixen, M. Jakobsen, and B.B. Jensen. 2003. Enumeration of bifidobacteria in gastrointestinal samples from piglets. Appl. Environ. Microbiol. 69:654–658.
133. Mills, S., F. Shanahan, C. Stanton, C. Hill, A. Coffey, and R.P. Ross. 2013. Movers and shakers: Influence of bacteriophages in shaping the mammalian gut microbiota. Gut Microbes 4:4–16.
134. Minot, S., R. Sinha, J. Chen, H.Z. Li, S.A. Keilbaugh, G.D. Wu, J.D. Lewis, and F.D. Bushman. 2011. The human gut virome: Inter-individual variation and dynamic response to diet. Genome Res. 21:1616–1625.
135. Mitsuoka, T. 1992. Intestinal flora and aging. Nutr. Rev. 50:438–446.
136. Mukherjee, P.K., J. Chandra, M. Retuerto, *et al.* 2014. Oral mycobiome analysis of HIV-infected patients: Identification of *Pichia* as an antagonist of opportunistic fungi. PLoS Pathog. 10:e1003996.
137. Nguyen, T.L.A., S. Vieira-Silva, A. Liston, and J. Raes. 2015. How informative is the mouse for human gut microbiota research? Dis. Mod. Mech. 8:1–16.
138. Nicholson, J.K., E. Holmes, and I.D. Wilson. 2005. Gut microorganisms, mammalian metabolism and personalized health care. Nat. Rev. Microbiol. 3:431–438.
139. Nielsen, H.B., M. Almeida, A.S. Juncker, *et al.* 2014. Identification and assembly of genomes and genetic elements in complex metagenomic samples without using reference genomes. Nat. Biotechnol. 32:822–828.
140. Noble, S.M., B.A. Gianetti, and J.N. Witchley. 2017. Candida albicans cell-type switching and functional plasticity in the mammalian host. Nat. Rev. Microbiol. 15:96–108.
141. O'Hara, A.M., and F. Shanahan. 2006. The gut flora as a forgotten organ. EMBO Rep. 7:688–693.
142. Odle, J., X. Lin, S.K. Jacobi, S.W. Kim, and C.H. Stahl. 2014. The suckling piglet as an agrimedical model for the study of pediatric nutrition and metabolism. Ann. Rev. Anim. Biosci. 2:419–444.
143. Ott, S.J., T. Kuhbacher, M. Musfeldt, *et al.* 2008. Fungi and inflammatory bowel diseases: Alterations of composition and diversity. Scand. J. Gastroenterol. 43:831–841.
144. Pang, X.Y., X.G. Hua, Q. Yang, D.H. Ding, C.Y. Che, L. Cui, W. Jia, P. Bucheli, and L.P. Zhao. 2007. Inter-species transplantation of gut microbiota from human to pigs. ISME J. 1:156–162.
145. Parfrey, L.W., W.A. Walters, and R. Knight. 2011. Microbial eukaryotes in the human microbiome: ecology, evolution, and future directions. Front. Microbiol. 2:153.
146. Pedersen, R., H.C. Ingerslev, M. Sturek, M. Alloosh, S. Cirera, B.O. Christoffersen, S.G. Moesgaard, N. Larsen, and M. Boye. 2013. Characterisation of gut microbiota in Ossabaw and Gottingen minipigs as models of obesity and metabolic syndrome. PLoS One 8:e 0056612.
147. Pei, Z.H., E.J. Bini, L.Y. Yang, M.S. Zhou, F. Francois, and M.J. Blaser. 2004. Bacterial biota in the human distal esophagus. Proc. Natl. Acad. Sci. U.S.A. 101:4250–4255.
148. Pflughoeft, K.J., and J. Versalovic. 2012. Human Microbiome in Health and Disease. Ann. Revi. Pathol. 7:99–122.
149. Pieper, R., P. Janczyk, A. Zeyner, H. Smidt, V. Guiard, and W.B. Souffrant. 2008. Ecophysiology of the developing total bacterial and *Lactobacillus* communities in the terminal small intestine of weaning piglets. Microb. Ecol. 56:474–483.
150. Pothoulakis, C. 2009. Review article: anti-inflammatory mechanisms of action of *Saccharomyces boulardii*. Alimen. Pharmacol. Therapeut. 30:826–833.
151. Pride, D.T., J. Salzman, M. Haynes, F. Rohwer, C. Davis-Long, R.A. White, III, P. Loomer, G.C. Armitage, and D.A. Relman. 2012. Evidence of a robust resident bacteriophage population revealed through analysis of the human salivary virome. ISME J. 6:915–926.
152. Qin, J., R. Li, J. Raes, *et al.* 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464:59–65.
153. Qiu, X.Y., F. Zhang, X. Yang, N. Wu, W.W. Jiang, X. Li, X.X. Li, and Y.L. Liu. 2015. Changes in the composition of intestinal fungi and their role in mice with dextran sulfate sodium-induced colitis. Sci. Rep. 5:10416.
154. Queipo-Ortuno, M.I., L.M. Seoane, M. Murri, M. Pardo, J.M. Gomez-Zumaquero, F. Cardona, F. Casanueva, and F.J. Tinahones. 2013. Gut microbiota composition in male rat models under different nutritional status and physical activity and its association with serum leptin and ghrelin levels. PLoS One 8:e65465.
155. Rajilic-Stojanovic, M., H.G.H.J. Heilig, S. Tims, E.G. Zoetendal, and W.M. de Vos. 2013. Long-term monitoring of the human intestinal microbiota composition. Environ. Microbiol. 15:1146–1159.
156. Rajilic-Stojanovic, M., and W.M. de Vos. 2014. The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol. Rev. 38:996–1047.
157. Rascovan, N., R. Duraisamy, and C. Desnues. 2016. Metagenomics and the human virome in asymptomatic individuals, p. 125–141. *In* S. Gottesman (ed.), Ann. Rev. Microbiol. 70:125–141.
158. Rastall, R.A., G.R. Gibson, H.S. Gill, F. Guarner, T.R. Klaenhammer, B. Pot, G. Reid, I.R. Rowland, and M.E. Sanders. 2005. Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: An overview of enabling science and potential applications. FEMS Microbiol. Ecol. 52:145.
159. Rauch, M., and S.V. Lynch. 2012. The potential for probiotic manipulation of the gastrointestinal microbiome. Curr. Opin. Biotechnol. 23:192–201.
160. Reyes, A., M. Haynes, N. Hanson, F.E. Angly, A.C. Heath, F. Rohwer, and J.I. Gordon. 2010. Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature 466:334–338.
161. Reyes, A., N.P. Semenkovich, K. Whiteson, F. Rohwer, and J.I. Gordon. 2012. Going viral: next-generation sequencing applied to phage populations in the human gut. Nat. Rev. Microbiol. 10:607–617.
162. Reyes, A., L.V. Blanton, S. Cao, *et al.* 2015. Gut DNA viromes of Malawian twins discordant for severe acute malnutrition. Proc. Natl. Acad. Sci. U.S.A. 112:11941–11946.
163. Robles-Sikisaka, R., M. Ly, T. Boehm, M. Naidu, J. Salzman, and D.T. Pride. 2013. Association between living environment and human oral viral ecology. ISME J. 7:1710–1724.
164. Sachsenroder, J., S.O. Twardziok, M. Scheuch, and R. Johne. 2014. The general composition of the faecal virome of pigs depends on age, but not on feeding with a probiotic bacterium. PLoS One 9:e88888.
165. Savage, D.C. 1977. Microbial ecology of gastrointestinal-tract. Ann. Revi. Microbiol. 31:107–133.
166. Scanlan, P.D., F. Shanahan, C. O'Mahony, and J.R. Marchesi. 2006. Culture-independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's Disease. J. Clin. Microbiol. 44:3980–3988.
167. Schwartz, S., I. Friedberg, I.V. Ivanov, *et al.* 2012. A metagenomic study of diet-dependent interaction between gut microbiota and host in infants reveals differences in immune response. Genome Biol. 13:r32.
168. Scupham, A.J., L.L. Presley, B. Wei, *et al.* 2006. Abundant and diverse fungal microbiota in the murine intestine. Appl. Environ. Microbiol. 72:793–801.
169. Seed, P.C. 2015. The Human Mycobiome. Cold Spring Harbor Perspectives in Medicine 5.

170. Sender, R., S. Fuchs, and R. Milo. 2016. Revised estimates for the number of human and bacteria cells in the body. PLoS Biol. 14:e1002533.
171. Shan, T.L., L.L. Li, P. Simmonds, C.L. Wang, A. Moeser, and E. Delwart. 2011. The fecal virome of pigs on a high-density farm. J. Virol. 85:11697–11708.
172. Simmonds, P., M.J. Adams, M. Benko, *et al.* 2017. Virus taxonomy in the age of metagenomics. Nat. Rev. Microbiol. 15:161–168.
173. Sogin, M.L., H.G. Morrison, J.A. Huber, D.M. Welch, S.M. Huse, P.R. Neal, J.M. Arrieta, and G.J. Herndl. 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc. Natl. Acad. Sci. U.S.A. 103:12115–12120.
174. Sommer, F., and F. Bäckhed. 2016. Know your neighbor: Microbiota and host epithelial cells interact locally to control intestinal function and physiology. BioEssays 38:455–464.
175. Sonnenburg, E.D., S.A. Smits, M. Tikhonov, S.K. Higginbottom, N.S. Wingreen, and J.L. Sonnenburg. 2016. Diet-induced extinctions in the gut microbiota compound over generations. Nature 529:212–215.
176. Sonnenburg, J.L., and F. Backhed. 2016. Diet-microbiota interactions as moderators of human metabolism. Nature 535:56–64.
177. Spurlock, M.E., and N.K. Gabler. 2008. The development of porcine models of obesity and the metabolic syndrome. J. Nutr. 138:397–402.
178. Stephen, A.M., and J.H. Cummings. 1980. Mechanism of action of dietary fibre in the human colon. Nature 284:283–284.
179. Su, Y., G.R. Bian, Z.G. Zhu, H. Smidt, and W.Y. Zhu. 2014. Early methanogenic colonisation in the faeces of Meishan and Yorkshire piglets as determined by pyrosequencing analysis. Archaea 2014:547908.
180. Suau, A., R. Bonnet, M. Sutren, J.J. Godon, G.R. Gibson, M.D. Collins, and J. Dore. 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. 65:4799–4807.
181. Suhr, M.J., and H.E. Hallen-Adams. 2015. The human gut mycobiome: pitfalls and potentials—a mycologist's perspective. Mycologia 107:1057–1073.
182. Suhr, M.J., N. Banjara, and H.E. Hallen-Adams. 2016. Sequence-based methods for detecting and evaluating the human gut mycobiome. Lett. Appl. Microbiol. 62:209–215.
183. Tannock, G.W. 2002. Exploring the relationships between intestinal microflora and inflammatory conditions of the human bowel and spine. Anton. Leeuw. Int. J. G. 81:529–535.
184. Tannock, G.W. 2008. Molecular analysis of the intestinal microflora in IBD. Mucosal Immunol. 1:S15–S18.
185. Thaiss, C.A., D. Zeevi, M. Levy, *et al.* 2014. Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. Cell 159:514–529.
186. Thaiss, C.A., D. Zeevi, M. Levy, E. Segal, and E. Elinav. 2015. A day in the life of the meta-organism: diurnal rhythms of the intestinal microbiome and its host. Gut Microbes 6:137–142.
187. Thaiss, C.A., M. Levy, T. Korem, *et al.* 2016. Microbiota diurnal rhythmicity programs host transcriptome oscillations. Cell 167: 1495–1510.e12.
188. Thaiss, C.A., N. Zmora, M. Levy, and E. Elinav. 2016. The microbiome and innate immunity. Nature 535:65–74.
189. The Human Microbiome Project Consortium. 2012. Structure, function and diversity of the healthy human microbiome. Nature 486:207–214.
190. Turnbaugh, P., R. Ley, M.A. Mahowald, V. Magrini, E.R. Mardis, and J. Gordon. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444:1027–1131.
191. Turnbaugh, P.J., and J.I. Gordon. 2009. The core gut microbiome, energy balance and obesity. J. Physiol. 587:4153–4158.
192. Turnbaugh, P.J., V.K. Ridaura, J.J. Faith, F.E. Rey, R. Knight, and J.I. Gordon. 2009. The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. Sci. Trans. Med. 1:6ra14–6ra14.
193. Underhill, D.M., and L.D. Lliev. 2014. The mycobiota: interactions between commensal fungi and the host immune system. Nat. Rev. Immunol. 14:405–416.
194. Urubschurov, V., P. Janczyk, W.B. Souffrant, G. Freyer, and A. Zeyner. 2011. Establishment of intestinal microbiota with focus on yeasts of unweaned and weaned piglets kept under different farm conditions. FEMS Microbiol. Ecol. 77:493–502.
195. van den Bogert, B., O. Erkus, J. Boekhorst, M. de Goffau, E.J. Smid, E.G. Zoetendal, and M. Kleerebezem. 2013. Diversity of human small intestinal *Streptococcus* and *Veillonella* populations. FEMS Microbiol. Ecol. 85:376–388.
196. Vdoviaková, K., E. Petrovová, M. Maloveská, L. Krešáková, J. Teleky, M.Z.J. Elias, and D. Petrášová. 2016. Surgical anatomy of the gastrointestinal tract and its vasculature in the laboratory rat. Gastroenterol. Res. Pract. 2016, Article ID 2632368.
197. von Rosenvinge, E.C., Y. Song, J.R. White, C. Maddox, T. Blanchard, and W.F. Fricke. 2013. Immune status, antibiotic medication and pH are associated with changes in the stomach fluid microbiota. ISME J. 7:1354–1366.
198. Walker, A.W., J. Ince, S.H. Duncan, *et al.* 2011. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J. 5:220–230.
199. Waller, A.S., T. Yamada, D.M. Kristensen, J.R. Kultima, S. Sunagawa, E.V. Koonin, and P. Bork. 2014. Classification and quantification of bacteriophage taxa in human gut metagenomes. ISME J. 8:1391–1402.
200. Wampach, L., A. Heintz-Buschart, A. Hogan, *et al.* 2017. Colonization and succession within the human gut microbiome by Archaea, Bacteria, and Microeukaryotes during the first year of life. Front. Microbiol. 8:738.
201. Wang, M., S. Ahrn, B. Jeppsson, and G. Molin. 2005. Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. FEMS Microbiol. Ecol. 54:219.
202. Wang, M., and S.M. Donovan. 2015. Human microbiota-associated swine: Current progress and future opportunities. ILAR J. 56:63–73.
203. Wang, X., S.P. Heazlewood, D.O. Krause, and T.H.J. Florin. 2003. Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J. Appl. Microbiol. 95:508–520.
204. Wang, Z.K., Y.S. Yang, A.T. Stefka, G. Sun, and L.H. Peng. 2014. Review article: fungal microbiota and digestive diseases. Alimen. Pharmacol. Therapeu. 39:751–766.
205. Warden, C.H., and J.S. Fisler. 2008. Comparisons of diets used in animal models of high fat feeding. Cell Metabol. 7:277.
206. Weaver, C.M., L.D. McCabe, G.M. McCabe, R. Novotny, M.D. Van Loan, S.B. Going, C. Boushey, D.A. Savaiano, and V. Matkovic. 2005. Bone mineral and predictors of whole body, total hip, and lumbar spine for 740 early pubertal white, Hispanic, and Asian girls. J. Bone Miner. Res. 20:S314–S314.
207. Weaver, C.M., B.R. Martin, J.A. Story, I. Hutchinson, and L. Sanders. 2010. Novel fibers Increase bone calcium content and strength beyond efficiency of large intestine fermentation. J. Ag. Food Chem. 58:8952–8957.
208. Weaver, C.M., B.R. Martin, C.H. Nakatsu, *et al.* 2011. Galactooligosaccharides improve mineral absorption and bone properties in growing rats through gut fermentation. J. Ag. Food Chem. 59:6501–6510.
209. Welch, J.L.M., B.J. Rossetti, C.W. Rieken, F.E. Dewhirst, and G.G. Borisy. 2016. Biogeography of a human oral microbiome at the micron scale. Proc. Natl. Acad. Sci. U.S.A. 113:E791–E800.
210. Whisner, C.M., B.R. Martin, C.H. Nakatsu, G.P. McCabe, L.D. McCabe, M. Peacock, and C.M. Weaver. 2014. Soluble maize fibre affects short-term calcium absorption in adolescent boys and girls: a randomised controlled trial using dual stable isotopic tracers. Brit. J. Nutr. 112:446–456.
211. Whisner, C.M., B.R. Martin, C.H. Nakatsu, J.A. Story, C.J. MacDonald-Clarke, L.D. McCabe, G.P. McCabe, and C.M. Weaver. 2016. Soluble corn fiber increases calcium absorption associated with shifts in the gut microbiome: A randomized dose-response trial in free-living pubertal girls. Nutr. J. 146:1298–1306
212. Wilson, K., and R. Blitchington. 1996. Human colonic biota studied by ribosomal DNA sequence analysis. Appl. Environ. Microbiol. 62:2273–2278.
213. Woodmansey, E.J. 2007. Intestinal bacteria and ageing. J. Appl. Microbiol. 102:1178–1186.
214. Wright, C.J., L.H. Burns, A.A. Jack, C.R. Back, L.C. Dutton, A.H. Nobbs, R.J. Lamont, and H.F. Jenkinson. 2013. Microbial interactions in building of communities. Mol. Oral Microbiol. 28:83–101.
215. Wylie, K.M., K.A. Mihindukulasuriya, Y. Zhou, E. Sodergren, G.A. Storch, and G.M. Weinstock. 2014. Metagenomic analysis of double-stranded DNA viruses in healthy adults. BMC Biol. 12:71.
216. Xiao, L., Q. Feng, S. Liang, *et al.* 2015. A catalog of the mouse gut metagenome. Nat Biotechnol. 33:1103–1108.

217. Xiao, L., J. Estellé, P. Kiilerich, *et al.* 2016. A reference gene catalogue of the pig gut microbiome. Nat. Microbiol. 1:16161.
218. Yan, H., R. Potu, H. Lu, *et al.* 2013. Dietary fat content and fiber type modulate hind gut microbial community and metabolic markers in the pig. PLoS One 8:e59581.
219. Yatsunenko, T., F.E. Rey, M.J. Manary, *et al.* 2012. Human gut microbiome viewed across age and geography. Nature 486:222–227.
220. Zhang, Q., G. Widmer, and S. Tzipori. 2013. A pig model of the human gastrointestinal tract. Gut Microbes 4:193–200.
221. Zhu, Y.Y., H. Li, X.L. Xu, C.B. Li, and G.H. Zhou. 2016. The gut microbiota in young and middle-aged rats showed different responses to chicken protein in their diet. BMC Microbiol. 16:281.
222. Zoetendal, E.G., A. von Wright, T. Vilpponen-Salmela, K. Ben-Amor, A.D.L. Akkermans, and W.M. de Vos. 2002. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl. Environ. Microbiol. 68:3401–3407.
223. Zoetendal, E.G., J. Raes, B. van den Bogert, *et al.* 2012. The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. ISME J. 6:1415–1426.
224. Zwolinska-Wcislo, M., A. Budak, D. Trojanowska, J. Bogdal, and J. Stachura. 1998. Fungal colonization of the stomach and its clinical relevance. Mycoses 41:327–334.

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