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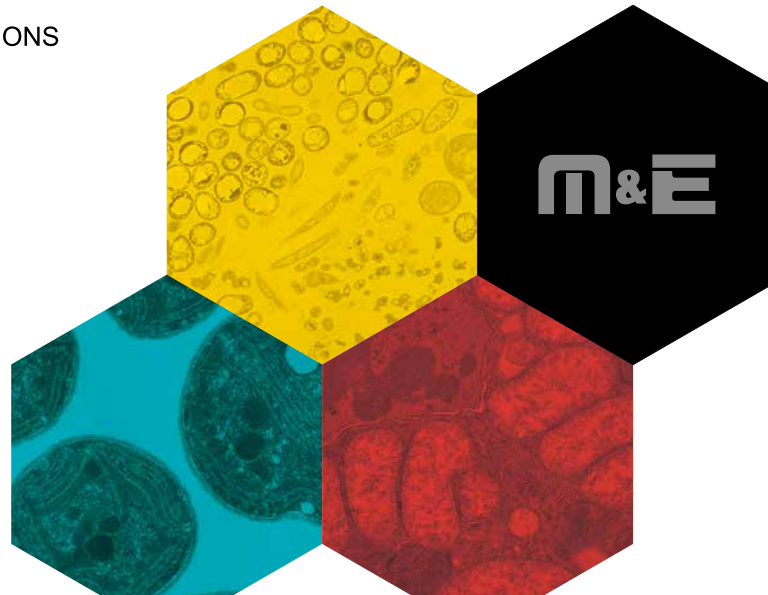

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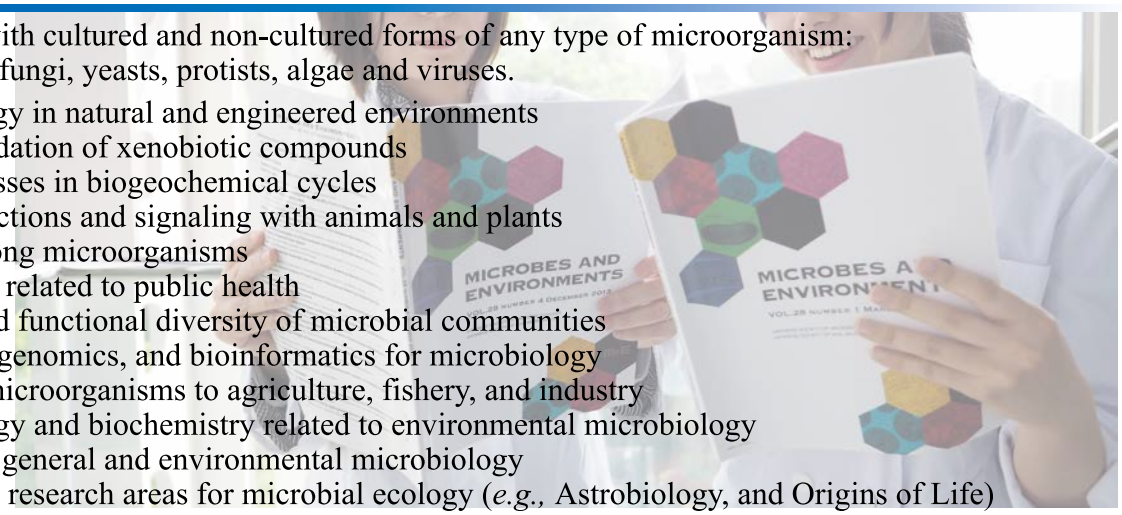

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MOST VALUABLE PAPER OF THE YEAR 2018

Short Communication

Nitric Oxide Production from Nitrite Reduction and Hydroxylamine Oxidation by Copper-containing Dissimilatory Nitrite Reductase (NirK) from the Aerobic Ammonia-oxidizing Archaeon, *Nitrososphaera viennensis*

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Aerobic ammonia-oxidizing archaea (AOA) play a crucial role in the global nitrogen cycle by oxidizing ammonia to nitrite, and nitric oxide (NO) is a key intermediate in AOA for sustaining aerobic ammonia oxidation activity. We herein heterologously expressed the NO-forming, copper-containing, dissimilatory nitrite reductase (NirK) from *Nitrososphaera viennensis* and investigated its enzymatic properties. The recombinant protein catalyzed the reduction of $^{15}NO_2^-$ to ^{15}NO, the oxidation of hydroxylamine ($^{15}NH_2OH$) to ^{15}NO, and the production of $^{14\text{-}15}N_2O$ from $^{15}NH_2OH$ and $^{14}NO_2^-$. To the best of our knowledge, the present study is the first to document the enzymatic properties of AOA NirK.

Key words: nitrite reduction, hydroxylamine oxidation, nitrous oxide production, ammonia oxidizing archaea, *Nitrososphaera viennensis*

Aerobic ammonia oxidation, a rate-limiting step of nitrification, drives the global nitrogen cycle (24, 40), which involves aerobic ammonia-oxidizing archaea and bacteria (AOA and AOB, respectively) and complete ammonia oxidizers (comammox) (9, 44). Of these, AOA primarily contribute to aerobic ammonia oxidation in natural environments including soil and open ocean (19, 31, 46). AOA are affiliated with the phylum *Thaumarchaeota*, which includes phylogenetically and physiologically diverse members (6) and the soil-inhabiting archaeon *Nitrososphaera viennensis* (41). The biochemistry of aerobic ammonia oxidation by AOA has received a great deal of interest because ammonia oxidation to nitrite (NO_2^-) proceeds in a different manner to that of AOB. AOA oxidize ammonia to hydroxylamine by ammonia monooxygenase (Amo) as well as AOB (43), while hydroxylamine is further oxidized to NO_2^- by an unidentified enzyme (17). All known AOA genomes lack the gene encoding hydroxylamine dehydrogenase (Hao), and the involvement of a copper-protein complex has been proposed (40, 45). In parallel with the oxidation of ammonia to NO_2^-, AOA produce nitric oxide (NO) (22). NO is a key intermediate in AOA cells because this highly reactive molecule is essential for sustaining aerobic ammonia oxidation activity (17, 33, 36, 47). To date, the following 2 pathways have been reported as a source of prokaryotic NO formation: NO_2^- reduction to NO by copper-containing and cytochrome cd_1-type dissimilatory nitrite reductases (NirK and NirS, respectively) (38) and NH_2OH oxidation to NO by hydroxylamine oxidoreductase (Hao) (4, 21). Although neither *nirS* nor *hao* are found in AOA genomes (6), AOA commonly possess *nirK*, which is transcribed and expressed during aerobic ammonia oxidation (8, 15, 20, 37). These findings suggest that NirK are involved in NO formation in AOA cells. However, NO_2^- reduction to NO by AOA NirK has never been demonstrated.

Bacterial NirK have been characterized as homotrimeric enzymes, and each subunit has 2 Cu-binding sites (Type 1 and 2 Cu-binding sites). Type 1 Cu-binding sites receive an electron from an electron donor, such as type 1 Cu proteins (single-domain cupredoxins) and/or cytochrome *c*, and the electron is then further transferred to a type 2 Cu-binding site that is the catalytic center of NirK (14, 25). Bacterial NirK have been classified into 2 phylogenetically distinct groups (class 1 and class 2 groups) based on sequence similarities, and the NirK of the class 1 group contains linker loop and tower loop regions in the amino acid sequence (3). AOA NirK, including *Ns. viennensis* NirK, are affiliated with a distinct clade of bacterial class 1 and 2 groups (Fig. 1A). Lund *et al.* (20) reported that AOA NirK may be further classified into several phylogenetic clades showing specific geographic distributions. *Ns. viennensis* NirK has amino acid residues consistent with those of type 1 and 2 Cu-binding sites (His_{106}, His_{140}, and His_{316} for type 1 Cu-binding sites and His_{101}, Cys_{141}, His_{152}, and Met_{157} for type 2 Cu-binding sites) as well as the linker and tower loop regions, whereas the C terminus has unusual extensions of ~26 residues (Fig. 1B). These phylogenetic affiliations of and structural variations in *Ns. viennensis* NirK raise concerns regarding its enzymatic properties, such as specific enzymatic activity, affinity for NO_2^-, and products of NO_2^- reduction.

Based on its unique sequence and lack of biochemical information, the purpose of the present study was to charac-

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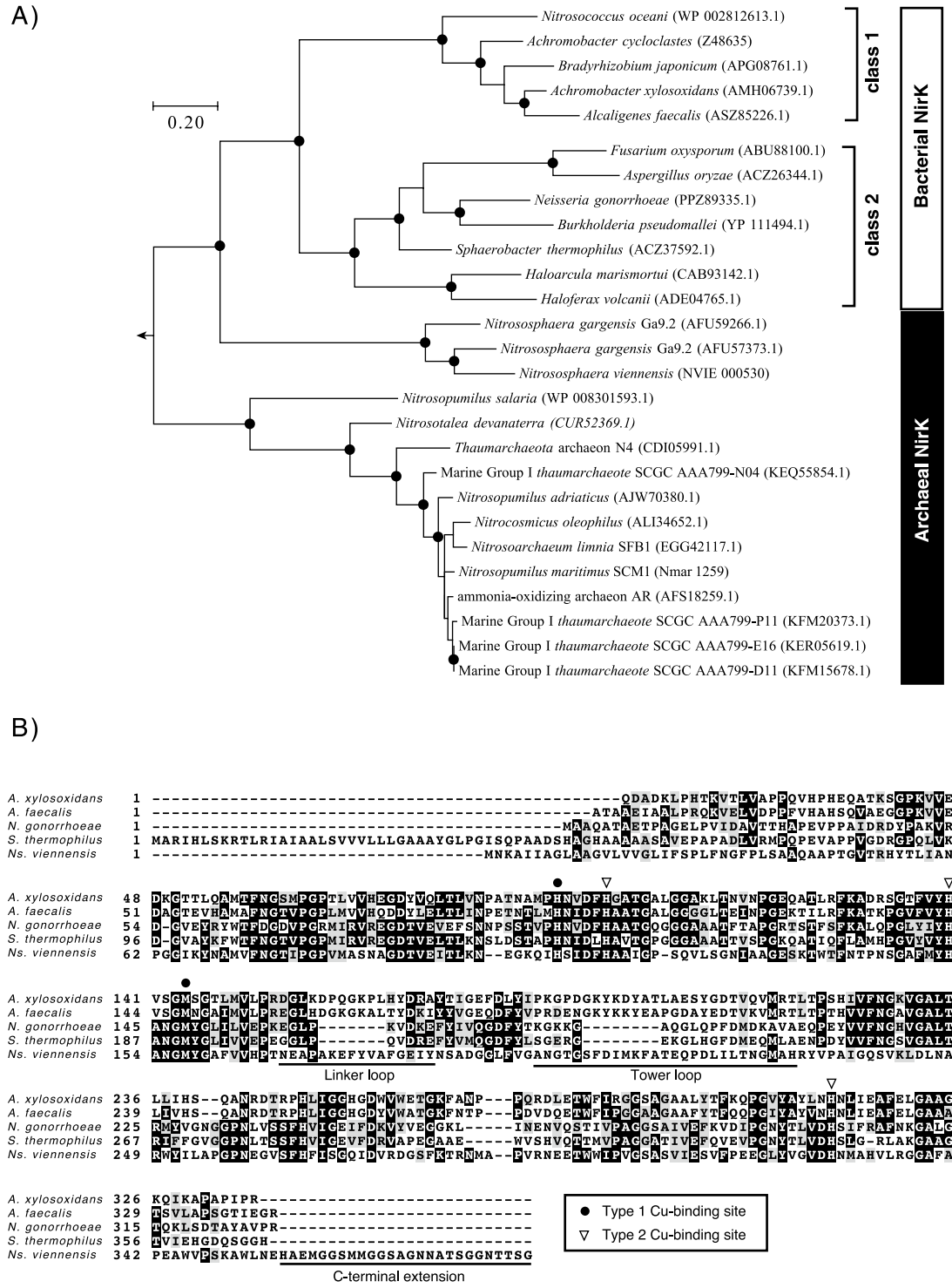

Fig. 1. Phylogeny (A) and sequence alignments (B) of prokaryotic NirK. A) A phylogenetic tree of prokaryotic NirK was constructed by the maximum likelihood method with the Jones-Taylor-Thornton model using the protein sequence of multicopper oxidase type 3 of *Nitrososphaera viennensis* (accession number; AIC14243.1) as an outgroup. Branching points that support a probability >80% in bootstrap analyses (based on 500 replicates) are shown as filled circles. The scale bar represents 10% sequence divergence. Sequence accession numbers are indicated in parentheses. B) Protein sequence alignment of *nirK*. NirK sequences were aligned using ClustalW software. Circles and triangles correspond to the amino acid residues of type 1 and 2 Cu-binding sites, respectively. Linker, Tower loop (3), and C-terminal extension regions are underlined. Abbreviations of microorganisms are as follows: *Nitrosomonas europaea* is *N. europaea*, *A. xylosoxidans* is *Achromobacter xylosoxidans*, *A. faecalis* is *Alcaligenes faecalis*, *N. gonorrhoeae* is *Neisseria gonorrhoeae*, *S. thermophilus* is *Sphaerobacter thermophilus*, and *Ns. viennensis* is *Nitrososphaera viennensis*.

terize *Ns. viennensis* NirK. Prior to the present study, we aimed to isolate *Ns. viennensis* NirK from a batch culture of *Ns. viennensis* as a native enzyme. However, the activity of aerobic ammonia oxidation often disappeared when we scaled up the cultures (data not shown). Additionally, a slow growth rate (μ_{max} 0.024 h^{-1}) (41) and low biomass concentration in the culture (*ca.* $10^{7\sim8}$ cells mL^{-1}) further precluded the preparation of the biomass required for protein purification. Since

recombinant NirK proteins have been successfully used to previously examine several enzymatic properties (7, 16, 32), the authors decided to heterologously express *Ns. viennensis* NirK in *Escherichia coli*, and investigate its enzymatic properties. The *nirK* gene located in the *Ns. viennensis* genome (accession number; CP007536.1) was cloned into the expression vector pCold I (Takara Bio, Shiga, Japan) with the 6×His tag using the Mighty cloning reagent set (Takara Bio), and transformed into *E. coli* strain BL21(DE3) (Takara Bio). The N-terminal region of *Ns. viennensis* NirK was predicted to be the signal peptide sequence (Met_1 to Ala_{24}), and *nir*K without the signal peptide sequence was amplified by PCR using ExTaq polymerase (Takara Bio) and specific forward (5′-GGCATATGGCCCCGACTGGTGTCACTAGACACTAT-3′) and reverse (5′-GGAAGCTTAACCAGAGGTGGTGTTGC CACCGGAGG-3′) oligonucleotide primers. The restriction sites of NdeI and HindIII in the forward and reverse primers above are underlined. Genomic DNA extracted from *Ns. viennensis* cells (JCM19564) was used as the DNA template for PCR. The constructed plasmid was subjected to Sanger sequencing, and no mutations were found in the sequence. Regarding the expression of the recombinant protein in *E. coli* cells, the expression culture was aerobically cultivated at 37°C in Luria-Bertani media containing 100 ng μL^{-1} ampicillin. When the OD_{600} of the culture increased to 0.4, the culture was transferred to 15°C and held for 30 min, and protein expression was then induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM. After being incubated at 15°C for 24 h, cells were harvested by centrifugation at 8,500×*g* at 4°C for 10 min. The harvested cells were suspended in buffer containing 20 mM Tris HCl (pH 8), 200 mM NaCl, and 10% glycerol. The cells were disrupted using a sonifier 250 (Branson) (output 20, duty 20% for 60 s, 6 cycles), and centrifuged at 13,000×*g* at 4°C for 1 h. The supernatant was recovered as a soluble protein fraction, and the recombinant protein was purified using His-tag affinity chromatography. The recombinant protein was bound to His60 Ni Superflow resin (Takara Bio), and washed with washing buffer containing 20 mM Tris HCl (pH 8), 200 mM NaCl, 10% glycerol, and 20 mM imidazole. The bound recombinant protein was eluted with elution buffer containing 20 mM Tris HCl (pH 8), 200 mM NaCl, 10% glycerol, and 300 mM imidazole. Protein concentrations were measured using the DC-protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as previously described (26), and purity was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel as previously described (28). As shown in Fig. 2A, a single protein band appeared at a molecular mass of 40 kDa, which closely matched the molecular mass deduced from amino acid sequences of the recombinant protein (*i.e.*, 39.7 kDa). The protein band was excised from the gel, and subjected to a matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis after in-gel tryptic digestion for protein identification (The detailed methodology is described in the Supplementary text). The MALDI-TOF MS analysis confirmed that the protein band corresponded to *Ns. viennensis* NirK (Fig. S1). Regarding the reconstitution of Cu-binding sites of the recombinant protein, the purified recombinant protein was dialyzed against buffer containing 20 mM Tris HCl (pH 8), 300 mM NaCl, and 0.5 mM $CuSO_4$ at 4°C for 57 h. The protein solution was dialyzed again using the above Tris buffer without $CuSO_4$ at 4°C for 6 h. The dialyzed recombinant protein was concentrated using a Vivaspin column (MWCO; 30 kDa) (GE Healthcare Japan, Tokyo, Japan). The recombinant protein was loaded onto a gel-filtration HiLoad 16/600 Superdex 200 pg column (GE Healthcare) to assess the molecular mass of the recombinant protein, which was 105±1.3 kDa (Fig. 2B). Since the deduced molecular mass of *Ns. viennensis* NirK was 39.7 kDa, the molecular mass obtained by gel filtration indicated that the recombinant protein forms a homotrimeric structure, similar to canonical NirK.

NirK have been characterized as metalloproteins showing a blue or green color spectrum, and exhibit absorption peaks at approximately 450 and/or 600 nm (3). Bacterial NirK, which belong to the class 1 group, often show a maximum absorption peak at approximately 450 nm, although an exception (*Achromobacter xylosoxidans* NirK) that shows a peak at 593 nm has been previously reported (16). The purified recombinant protein was pale blue in color, and showed an absorption peak at 590 nm (Fig. 2C). This feature indicated that *Ns. viennensis* NirK is affiliated with the subgroup of NirK showing a blue color spectrum. The blue or green color spectrum of NirK is derived from a copper atom in the type 1 Cu-binding site (14), while the type 2 Cu-binding site does not contribute to the UV or visible spectrum. The type 2 Cu-binding site shows a characteristic electron spin resonance (ESR) spectrum (7, 16); therefore, an ESR analysis was performed using a JES-FA200 spectrometer (JEOL, Tokyo, Japan) to test for the presence of the type 2 Cu-binding site in the recombinant protein. An axial type 2 Cu signal ($g_{//}$=2.24, $A_{//}$=18.31 mT, and $g_{\perp}$=2.06) was found in the ESR measurement (Fig. 2D), indicating that the recombinant protein has a type 2 Cu-binding site coordinating with a copper atom. Additionally, we assessed the copper content of the recombinant protein by inductively coupled plasma mass spectrometry (ICP-MS). The copper content was found to be 2.9 atoms per subunit of the recombinant protein, indicating that Cu was fully incorporated into the recombinant protein. Overall, the recombinant protein shared the structural and spectroscopic features of class 1 and 2 bacterial NirK, which is consistent with sequencing information.

The kinetics of NO_2^- reduction were examined by anoxically incubating the recombinant protein at 25°C and pH 6.5 with $^{15}NO_2^-$ and artificial electron donors as previously described (7). All of the buffers and stock solutions were prepared anoxically as previously described (27). Two milliliters of reaction buffer (20 mM phosphate buffer, 0.1 to 1.6 mM $Na^{15}NO_2^-$, 0.5 mM benzyl viologen (BV), and 0.24 mM sodium dithionite) was dispensed into a 1-cm sealable quartz cuvette and placed in an anaerobic chamber in which the O_2 concentration was maintained at lower than 1 ppm. BV was used as an artificial electron donor because it has been employed to examine the kinetics of the NO_2^- reduction of bacterial NirK (7, 13). The cuvette was set in a UV-VIS spectrometer UV-2700 (Shimadzu, Kyoto, Japan), and the initial absorbance of the prepared reaction mixture at a wavelength of 550 nm was approximately 2.0. The reaction was initiated by adding the recombinant protein (50 μL containing 250 μg of protein) using a gastight syringe, and the oxidation rate of reduced BV (molecular extinction coefficient, 10.4 mM^{-1} cm^{-1}) (13) was monitored at 550 nm.

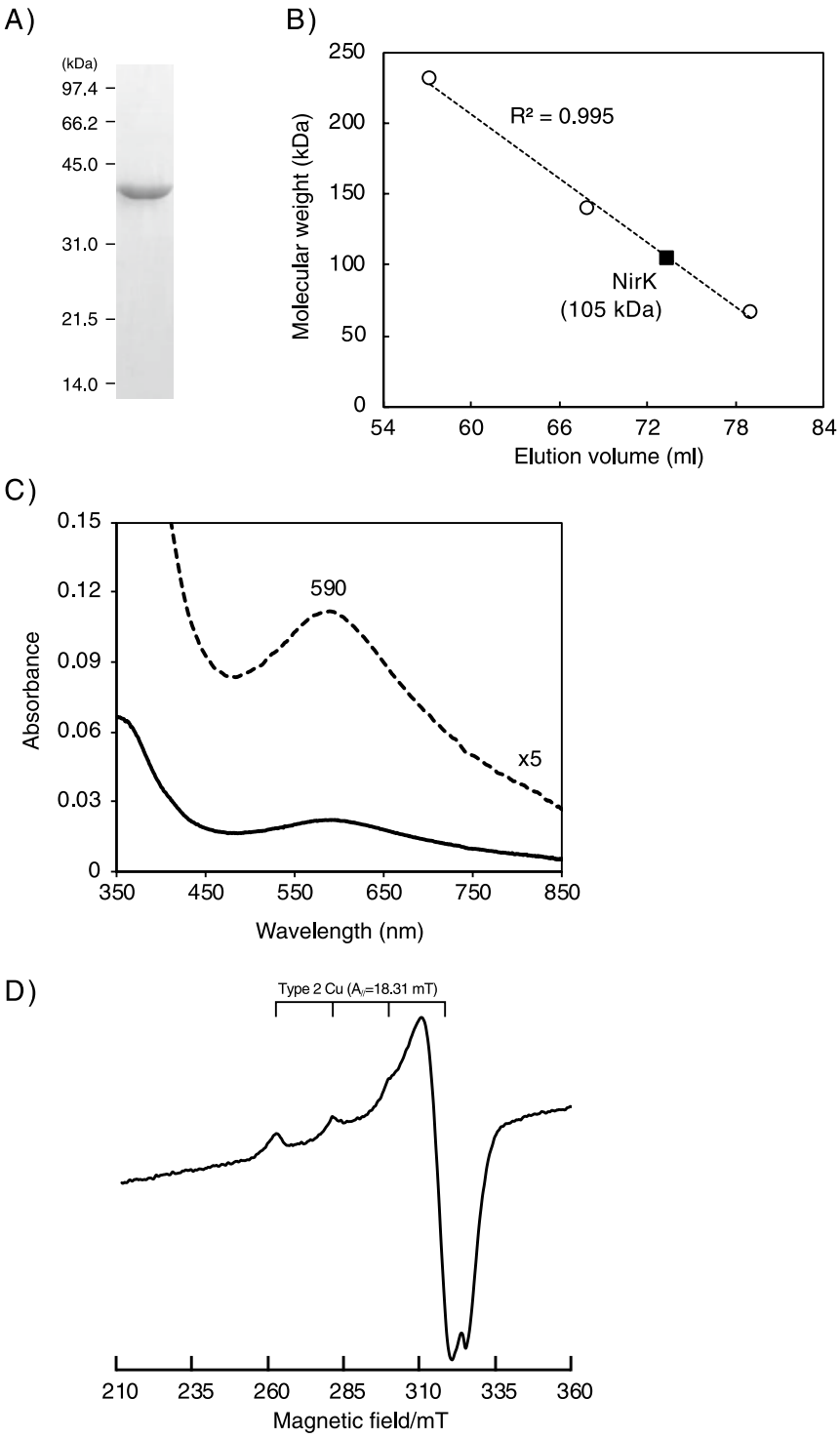

Fig. 2. Characterization of recombinant *Nitrososphaera viennensis* NirK. A) SDS-PAGE of the recombinant protein purified by His-tag affinity chromatography. B) Assessment of the molecular mass of the recombinant protein by gel filtration chromatography. Catalase from bovine liver (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (66 kDa) were used to prepare a standard calibration curve. C) UV-VIS absorption spectra. The measurement was performed in a 20 mM Tris buffer (pH 8) containing 300 mM NaCl at 25°C. The solid line indicates the recombinant protein (1 mL mL^{-1}) oxidized with air. A 5×enlarged spectrum is also shown as a dashed line. D) ESR spectra. The measurement was performed using the recombinant protein (4.9 mg mL^{-1}) at –253°C.

The recombinant protein reduced NO_2^- by oxidizing BV, whereas no significant BV oxidation was found in the cuvette without the recombinant protein. The turnover number and K_m value for NO_2^- reduction by the recombinant protein were 3.1 s^{-1} and 287 μM, respectively (Table 1), and the turnover number and affinity constant were markedly lower and higher, respectively, than those of other canonical NirK proteins, including those from AOB. The product of NO_2^- reduction by the recombinant protein was examined using phenazine methosulfate (PMS) as the electron donor instead of BV.

Table 1. Enzymatic properties of archaeal and bacterial copper-containing nitrite reductase (NirK). ND; not determined.

Organisms	MW* (kDa)	Cu content† (atom per subunit)	Absorption (nm)	Activity‡ Turnover (s^{-1})	K_m (μM)	Reference
Archaeal NirK						
Nitrososphaera viennensis	105±1.5	2.9	590			
NO_2^- reduction				3.1	287	This study
NH_2OH oxidation				0.039	97	This study
Bacterial NirK (NO_2^- reduction)						
Nitrosomonas europaea	96	ND	450, 597	288	ND	18
Nitrosococcus oceani	114	1.67	455, 575	1,600	52	16
Achromobacter xylosoxidans	110	1.99	595	172	35	14, 32
Candidatus Jettenia caeni	101	ND	449, 598	319	250	7

* Molecular weight (MW) of a trimeric NirK. The MW of *Ca.* Jettenia caeni NirK was calculated from amino acid sequences without a signal peptide sequence. † Copper contents previously assessed by chemical analyses were shown. ‡ The following electron donors were used to evaluate the turnover number of NO_2^- reduction; methyl viologen for *N. europaea* and *Nc. oceani*, pseudoazurine for *A. xylosoxidans*, and benzyl viologen for *Ns. viennensis* and *Ca.* Jettenia caeni NirK.

When BV was used as the electron donor, NO_2^- was reduced to NO, and further reduced to ammonia (approx. 60% of consumed $^{15}NO_2^-$) as observed in a previous study in which the NO_2^- reduction activity of *A. xylosoxidans* NirK was examined using methyl viologen (MV) as the electron donor (1). BV and MV have low redox potentials (–350 and –440 mV, respectively) (23), resulting in the reduction of NO to NH_3; therefore, PMS with a higher redox potential (+80 mV) was used in the present study. The recombinant protein was incubated as described above in a 1.8-mL gas-tight vial with the addition of 0.5 mM PMS and 5 mM ascorbic acid instead of BV and dithionite, and the production of ^{15}N-labeled gaseous compounds (*i.e.*, N_2, NO, and N_2O) in the headspace was examined by gas chromatography mass spectrometry (GC/MS) as previously described (27). The diluted gases of $^{15\text{-}15}N_2$ (Cambridge Isotope Laboratories, Tewksbury, MA, USA), ^{14}NO, and $^{14\text{-}14}N_2O$ (GL Science, Tokyo, Japan) were also analyzed to prepare standard curves for quantification. The recombinant protein reduced $^{15}NO_2^-$ with the oxidation of PMS, and 38 and 48% of consumed $^{15}NO_2^-$ were converted to ^{15}NO and $^{15\text{-}15}N_2O$, respectively. This is direct evidence to show that the recombinant protein is a NO-forming nitrite reductase. We found that the production of $^{15\text{-}15}N_2O$ was equal to the production of ^{15}NO, which likely results from the reduction of $^{15}NO_2^-$ to $H^{15}NO$ (*i.e.*, $NO_2^- + 2e^- + 3H^+ \rightarrow HNO + H_2O$) and the chemical formation of $^{15\text{-}15}N_2O$ from the formed $H^{15}NO$ (*i.e.*, $2HNO \rightarrow N_2O + H_2O$) (35), as previously observed for a sulfide-linked nitrite reductase (34).

Aside from NO_2^- reduction, NH_2OH oxidation was also investigated using the recombinant protein because NH_2OH is produced as an intermediate during aerobic ammonia oxidation by AOA. The kinetics of NH_2OH oxidation were examined by aerobically incubating the recombinant protein (245 μg mL^{-1}) at 30°C and pH 7.5 with 0.5 mM NH_2OH, with dissolved oxygen being available as an oxidant. The reaction was initiated by the addition of NH_2OH solution, and the concentration of NH_2OH was assessed colorimetrically (5). The concentration of H_2O_2, which may be produced by the oxidase activity of NirK (12), was also evaluated colorimetrically using horseradish peroxidase (Wako, Osaka, Japan) and 3,3′,5,5′-tetramethylbenzidine (TMBZ) (Dojindo, Kumamoto, Japan) (2). As shown in Fig. S2, the recombinant protein oxidized NH_2OH with the production of H_2O_2. No NH_2OH oxidation or H_2O_2 production was observed when the incubation was repeated without the addition of the recombinant protein.

The values for the turnover number and affinity constant for NH_2OH oxidation were 0.039 s^{-1} and 97 μM (Table 1), respectively, and the value for the turnover number was two orders of magnitude lower than that observed for NO_2^- reduction; therefore, the recombinant protein catalyzed NO_2^- reduction more efficiently. The addition of cytochrome *c* from equine heart (1 mg mL^{-1}) or BV (0.5 mM) did not result in an increase in the reaction rate or affinity for NH_2OH oxidation. The product of NH_2OH oxidation by the recombinant protein was examined in a $^{15}NH_2OH$ tracer experiment (29). The recombinant protein was incubated in a 1.8-mL gas-tight vial with the addition of 0.5 mM $^{15}NH_2OH$ (Cambridge Isotope Laboratories) instead of $^{14}NH_2OH$. After a 2-h incubation, the concentrations of the ^{15}N-labeled gaseous products were assessed by GC/MS. The recombinant protein oxidized $^{15}NH_2OH$ and produced ^{15}NO, $^{15\text{-}15}N_2O$, and $^{15\text{-}15}N_2$ gases quantitatively (Fig. 3), whereas the production of NO_2^- and NH_3 was not detectable (detection limits: 50 and 100 μM, respectively). The oxidation of NH_2OH to NO has been

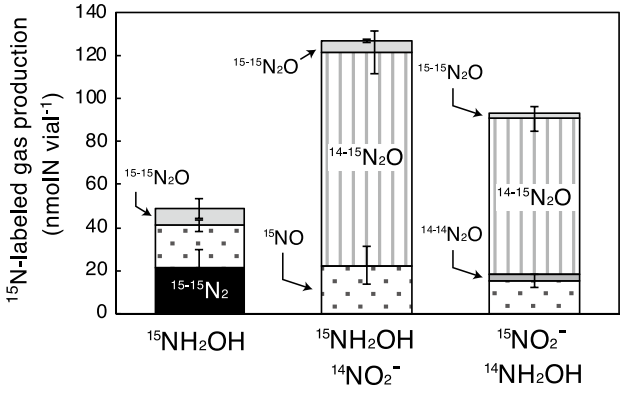

Fig. 3. NH_2OH oxidation by recombinant *Nitrososphaera viennensis* NirK. The recombinant protein was incubated at 30°C and pH 7.5 in 1.8-mL vials (volume of the headspace: 1.5 mL), with i) 0.5 mM $^{15}NH_2OH$, ii) $^{15}NH_2OH$ and $^{14}NO_2^-$ (each 0.5 mM), or iii) $^{14}NH_2OH$ and $^{15}NO_2^-$. The production of N_2, NO, and N_2O in the headspace was examined by gas chromatography mass spectrometry (GC/MS). NH_3 and NO_2^- concentrations were also measured; however, they were not detectable during the incubation. During a 2-h incubation, i) 63±35 (mean±SD), ii) 149±1, and iii) 120±1 nmol N of NH_2OH were consumed in the liquid phase, resulting in 75–137% of the ^{15}N-labeled nitrogen mass balance in the vials. Error bars represent the SD derived from triplicate incubations, and the graph bars represent the mean values. NH_2OH oxidation was not found in the vials without the addition of the recombinant protein.

described in bacterial Hao (21); however, to the best of our knowledge, this is the first description of NH_2OH oxidation by NirK. We also observed $^{15\text{-}15}N_2O$ production from $^{15}NH_2OH$ oxidation, which likely resulted from the oxidation of $^{15}NH_2OH$ to $H^{15}NO$ and abiotic coupling of $H^{15}NO$, as previously described. Notably, $^{15\text{-}15}N_2$ was the major product of $^{15}NH_2OH$ oxidation by the recombinant protein. Hydroxylamine disproportionation (30) may not be responsible for $^{15\text{-}15}N_2$ production because NH_3 production was not detectable in the liquid phase. The molecular mechanisms underlying the oxidation of $^{15}NH_2OH$ to $^{15\text{-}15}N_2$ by the recombinant protein warrant further studies.

We repeated the above incubation with the addition of NH_2OH and NO_2^- because both compounds are available in AOA cells during aerobic ammonia oxidation. Therefore, the above incubation was repeated with the addition of $^{15}NH_2OH$ and $^{14}NO_2^-$ (each 0.5 mM) or $^{14}NH_2OH$ and $^{15}NO_2^-$ (Cambridge Isotope Laboratories) (each 0.5 mM). In both cases, $^{14\text{-}15}N_2O$ was the major product (Fig. 3), indicating that the recombinant protein produces N_2O by oxidizing NH_2OH using NO_2^- as an electron acceptor. N_2O production by the denitrifier NirK from NH_2OH and NO_2^- has been previously described (10), and the N-nitrosation reaction is involved in N_2O production (39). Notably, *Ns. viennensis* cells produce N_2O when they are incubated aerobically with NH_3 and NO_2^- (42), although the *Ns. viennensis* genome lacks the gene encoding nitric oxide reductase (*nor*) that is involved in N_2O production from nitrifier-denitrification. Stieglmeier *et al.* (42) suggested the involvement of *Ns. viennensis* NirK in the production of N_2O in an *Ns. viennensis* culture, and our results support this hypothesis. Although the catalytic efficiency of *Ns. viennensis* NirK for NH_2OH oxidation was markedly lower than that of NO_2^- reduction (Table 1), *Ns. viennensis* NirK may act as an NH_2OH oxidase in *Ns. viennensis* cells and produce N_2O under oxic growth conditions. Aside from $^{14\text{-}15}N_2O$ production, the production of ^{15}NO and $^{15\text{-}15}N_2O$ was also observed when the recombinant protein was incubated with $^{14}NH_2OH$ and $^{15}NO_2^-$ (Fig. 3).

Although the recombinant protein catalyzes NO_2^- reduction and NH_2OH oxidation, the catalytic efficiency of both reactions was low, as shown in Table 1. AOA *nirK* transcripts are abundant in the transcriptome (8, 11, 20, 37), suggesting the strong expression of AOA NirK in cells. NirK was the 225th most abundant protein of the 1,503 proteins detected in the proteome of the late exponential phase of *Ns. viennensis* cells aerobically oxidizing ammonia (15). The strong expression of NirK appears to support the activity of NO_2^- reduction to NO as well as NH_2OH oxidation to NO by the low efficiency catalytic enzyme. *Ns. viennensis* NirK may function as a bifunctional enzyme that supplies NO molecules from 2 different sources (*i.e.*, NH_2OH and NO_2^-), which provides *Ns. viennensis* cells with a competitive advantage. In the present study, the enzymatic kinetics of recombinant *Ns. viennensis* NirK for NO_2^- reduction were examined using artificial electron donors; further studies are needed to identify physiological electron donors in *Ns. viennensis* cells. Bacterial NirK may accept electrons supplied from single-domain cupredoxin and cytochrome *c* (14, 25). A number of genes encoding single-domain cupredoxin were found in the *Ns. viennensis* genome (Table S1), whereas the ortholog of the gene encoding cytochrome *c* was not. To date, the biochemistry of AOA cupredoxin has not been investigated using natural enzymes and recombinant proteins, and our study provides basic information that furthers our understanding of the biochemistry of AOA.

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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

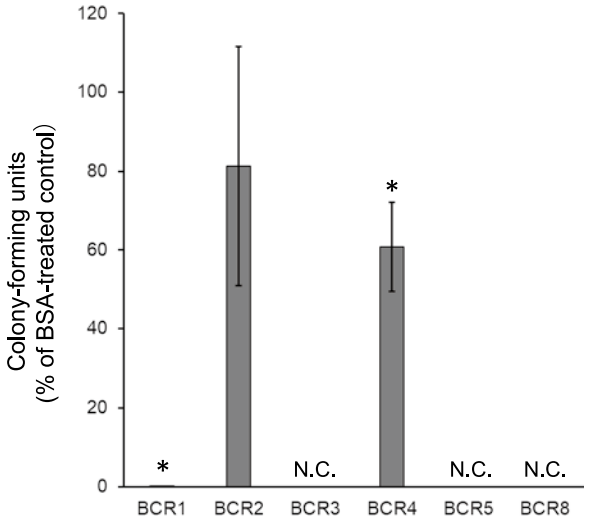

Fig. 1. Antimicrobial activities of BCR peptides. *E. coli* MG1655 was treated with 5 μM BCR peptides for 3 h and colony-forming units were estimated relative to the BSA control. Each value is the mean±SE of three independent experiments. Asterisks indicate a significant difference between BCR4 and the 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

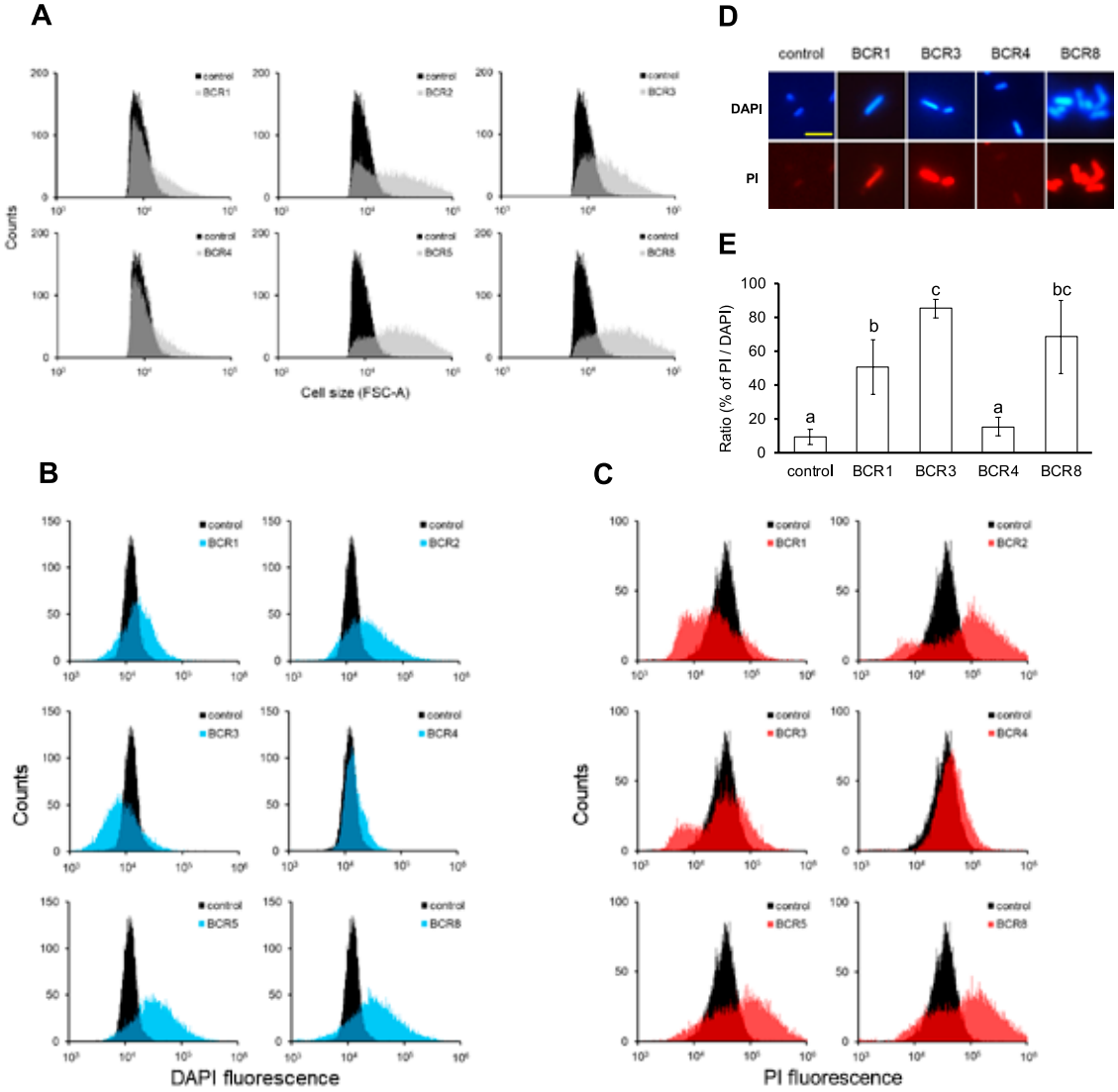

Fig. 2. Effects of BCR peptides on *E. coli* cells. *E. coli* MG1655 cells were treated for 3 h with 5 μM of the BCR peptide or BSA as the control. Cells were stained with DAPI and PI and analyzed by a cell sorter. (A) Forward scatter (FSC), (B) DAPI, (C) PI. (D) Fluorescent microscopy of MG1655 cells 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).

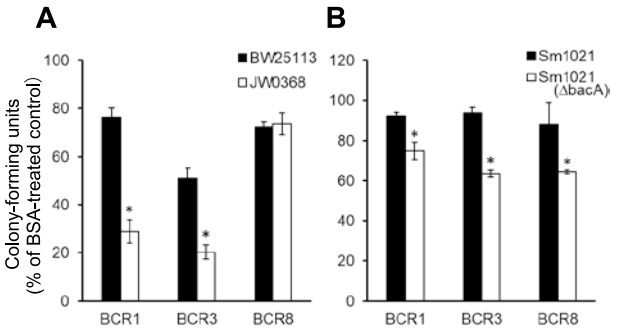

Fig. 3. Sensitivity of the *E. coli sbmA* mutant and *S. meliloti* Δ*bacA* mutant to BCR peptides. After a treatment with 3 μM BCRs for 3 h, the colony-forming units of each strain were estimated relative to the BSA control. The dataset shown is representative of three independent experiments. (A) *E. 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

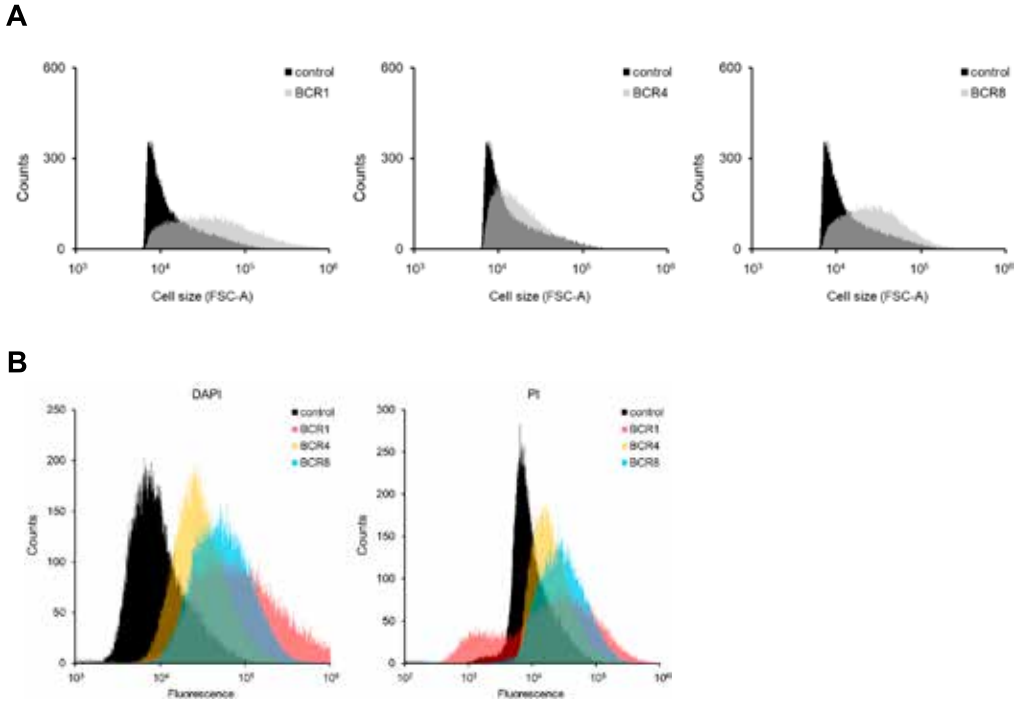

Fig. 4. Effects of BCR peptides on *S. meliloti*. *S. meliloti* 1021 cells were treated for 3 h with 5 μM BCR peptides or 5 μM BSA. Cells stained with DAPI and PI were analyzed by flow cytometry. (A) Forward scatter (FSC), (B) DAPI and PI. Data are representative of at least three independent experiments.

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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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<1×10^{-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<1×10^{-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).

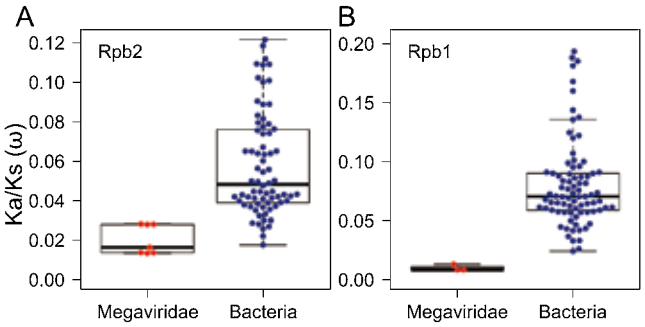

Fig. 1. Functional constraints on Megaviridae Rpb2 and Rpb1. Non-synonymous (Ka) and synonymous (Ks) substitution rate ratios (ω=Ka/Ks) are plotted for Megaviridae and bacterial Rpb2 (A) and Rpb1 (B). We selected pairs of orthologs from Megaviridae (shown in red dots) based on the following criteria: 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

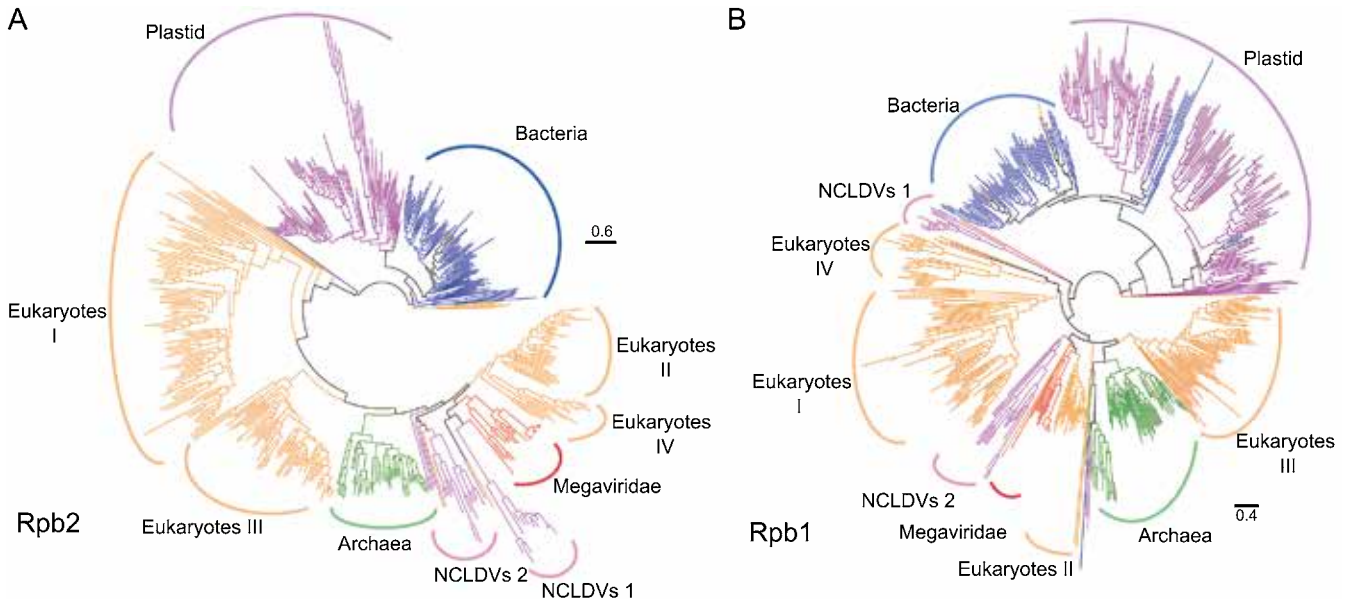

Fig. 2. Maximum likelihood phylogenetic trees of Rpb2 and Rpb1. The Rpb2 tree (RTree-Rpb2) was constructed using 511 representative sequences (A), and the Rpb1 tree (RTree-Rpb1) with 575 representative sequences (B). Branches are colored as follows: Eukaryotes I-IV (orange), Bacteria (blue), Archaea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

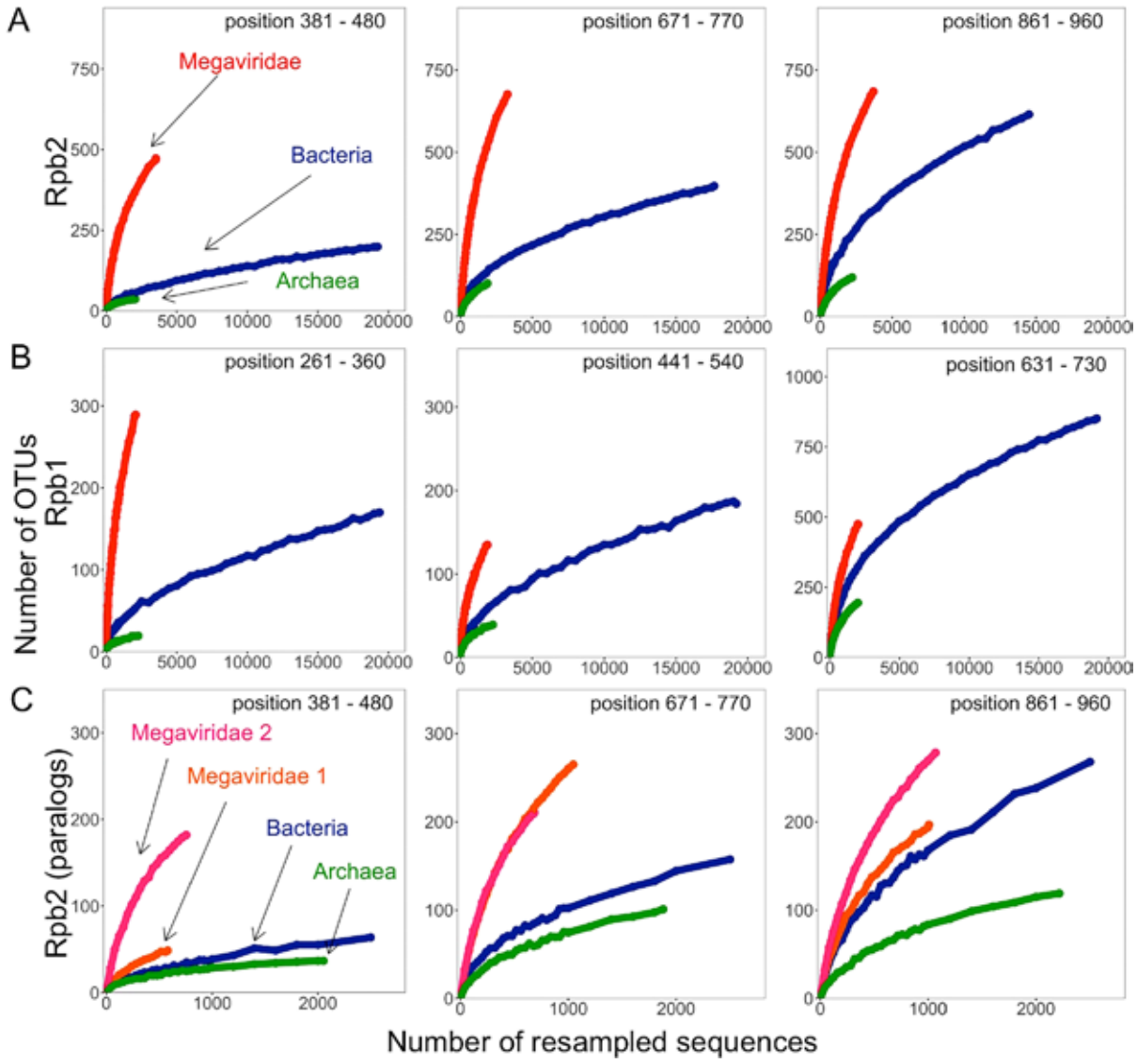

Fig. 3. Rarefaction curves of richness for metagenomic Rpb2/Rpb1 sequences. The X-axis indicates the numbers of resampled sequences for each organism group and the Y-axis indicates the average number of OTUs over 10 resamplings. Sequence clustering was performed using an 80% amino acid sequence identity 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.

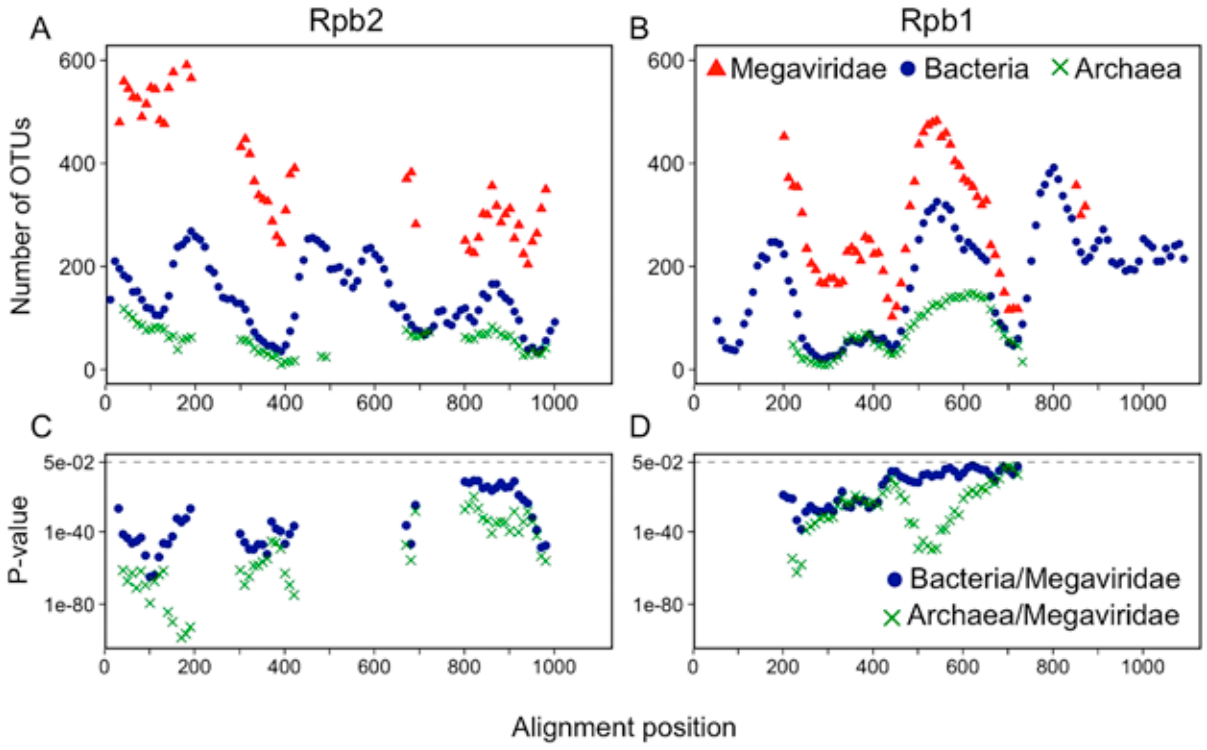

Fig. 4. Richness for 1,000 metagenomic Rpb2/Rpb1 sequences along the length of reference alignments. The numbers of OTUs after the resampling of 1,000 metagenomic sequences were plotted at each sequence region of Rpb2 (A) and Rpb1 (B). The significance (p-value) of differences between Megaviridae and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

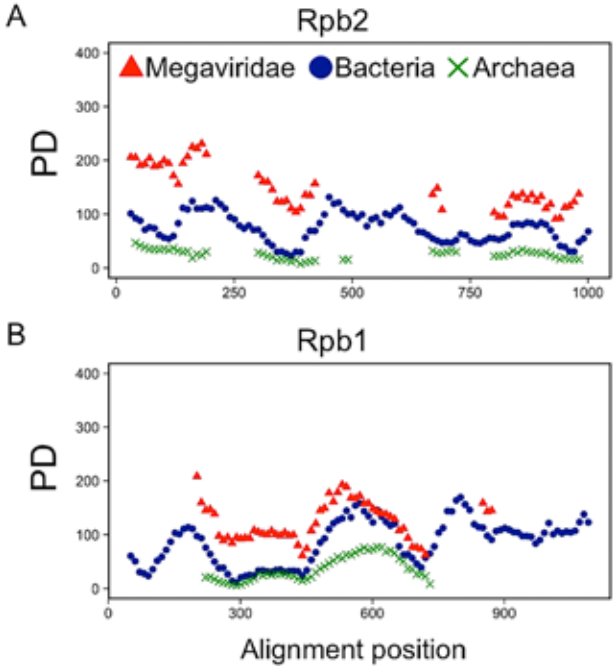

Fig. 5. Phylogenetic diversity of metagenomic Rpb2/Rpb1 sequences along the length of reference alignments. Phylogenetic diversity (PD) scores were computed with phylogenetic trees constructed using 1,000 metagenomic sequences at each sequence region of Rpb2 (A) and Rpb1 (B).

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

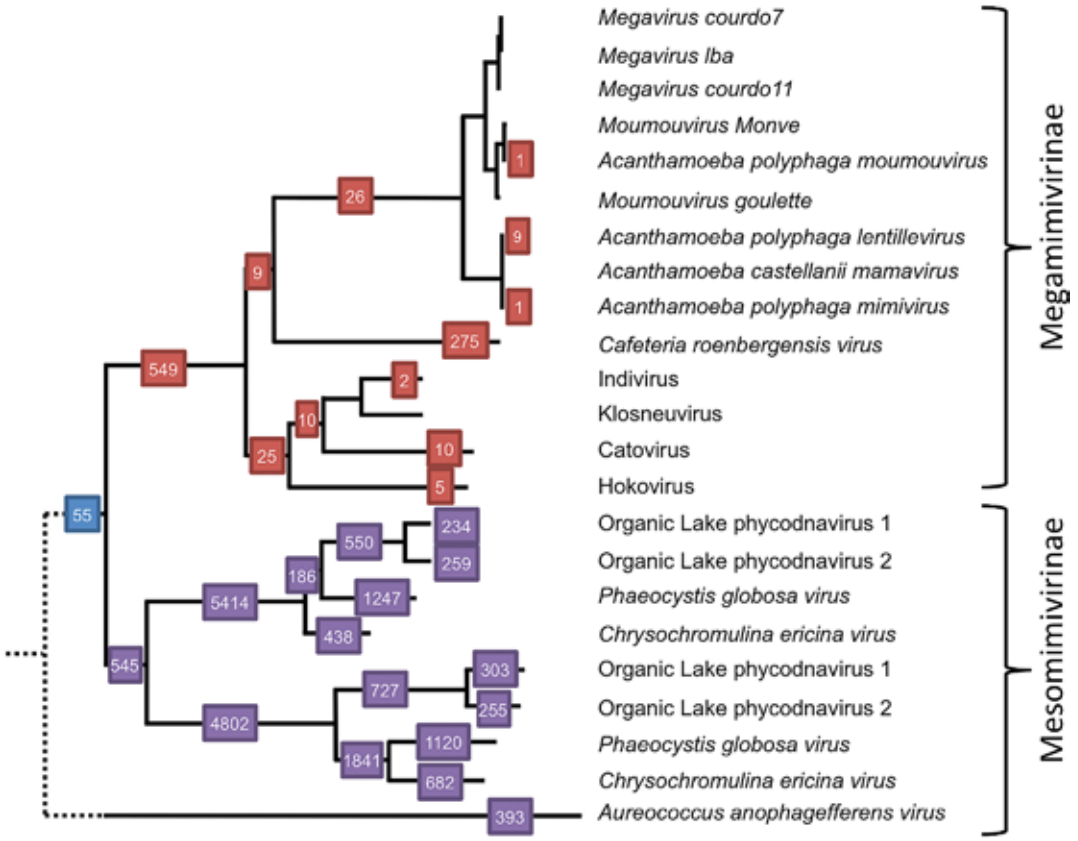

Fig. 6. Numbers of metagenomic sequences assigned to branches of Megaviridae Rbp2. This phylogenetic tree is part of the full phylogenetic tree in Fig. 2A. The numbers in red squares are the numbers of sequences in the Megamimivirinae subfamily and those in purple are the numbers of the Mesomimivirinae 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).

Acknowledgements

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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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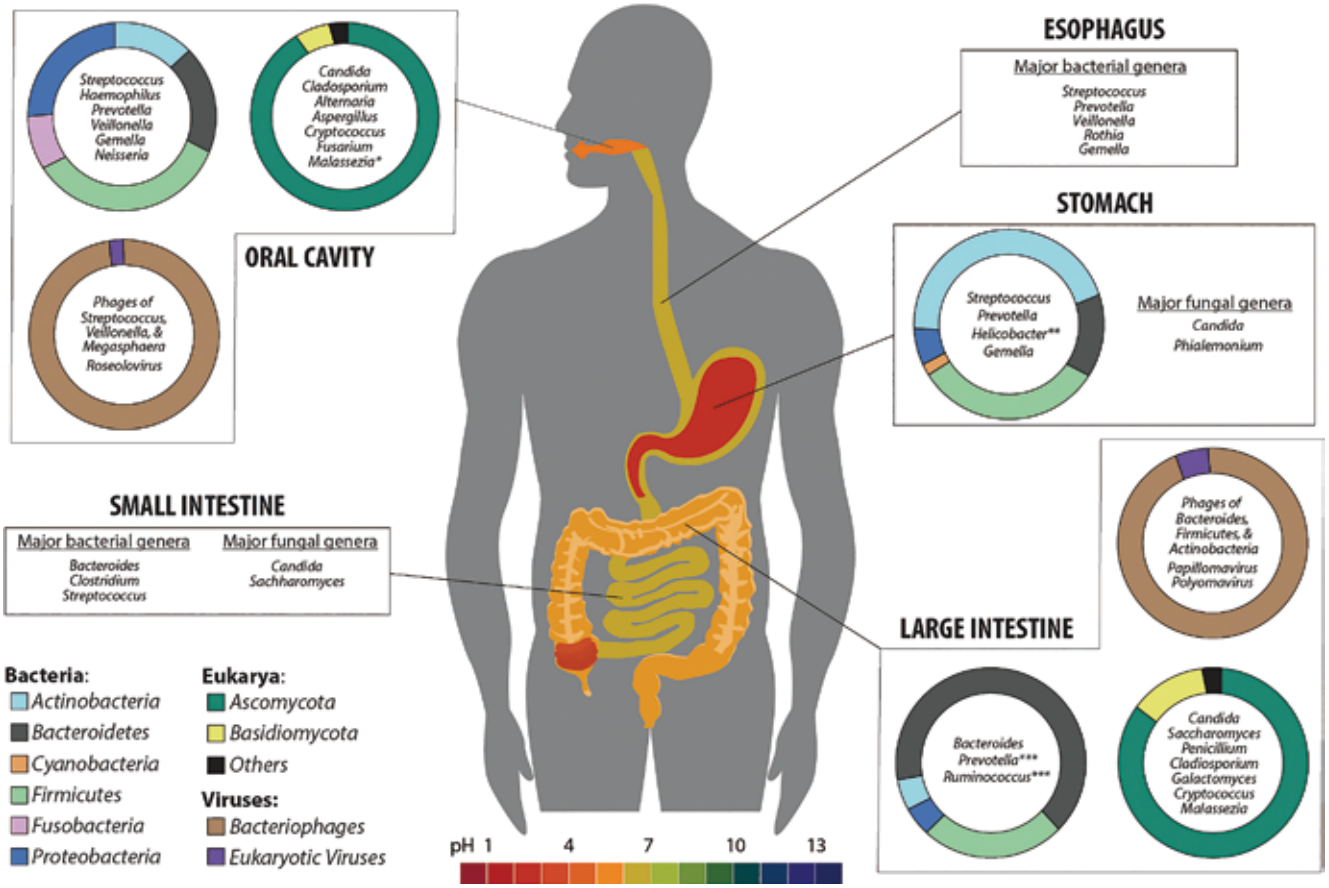

Fig. 1. Microbiome composition of *Bacteria* (1, 5, 20, 21, 43, 147, 156, 223), *Eukarya* (52, 85, 114, 126, 182, 197), and *Viruses* (45, 134, 151, 215) among the physiological niches of the human gastrointestinal (GI) tract. Phylum level compositional data are presented where available along with the 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.

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Candidatus Methanogranum caenicola: a Novel Methanogen from the Anaerobic Digested Sludge, and Proposal of *Methanomassiliicoccaceae* fam. nov. and *Methanomassiliicoccales* ord. nov., for a Methanogenic Lineage of the Class *Thermoplasmata*

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The class *Thermoplasmata* harbors huge uncultured archaeal lineages at the order level, so-called Groups E2 and E3. A novel archaeon Kjm51a affiliated with Group E2 was enriched from anaerobic sludge in the present study. Clone library analysis of the archaeal 16S rRNA and *mcrA* genes confirmed a unique archaeal population in the enrichment culture. The 16S rRNA gene-based phylogeny revealed that the enriched archaeon Kjm51a formed a distinct cluster within Group E2 in the class *Thermoplasmata* together with *Methanomassiliicoccus luminyensis* B10^T and environmental clone sequences derived from anaerobic digesters, bovine rumen, and landfill leachate. Archaeon Kjm51a showed 87.7% 16S rRNA gene sequence identity to the closest cultured species, *M. luminyensis* B10^T, indicating that archaeon Kjm51a might be phylogenetically novel at least at the genus level. In fluorescence *in situ* hybridization analysis, archaeon Kjm51a was observed as coccoid cells completely corresponding to the archaeal cells detected, although bacterial rod cells still coexisted. The growth of archaeon Kjm51a was dependent on the presence of methanol and yeast extract, and hydrogen and methane were produced in the enrichment culture. The addition of 2-bromo ethanesulfonate to the enrichment culture completely inhibited methane production and increased hydrogen concentration, which suggested that archaeon Kjm51a is a methanol-reducing hydrogenotrophic methanogen. Taken together, we propose the provisional taxonomic assignment, named *Candidatus* Methanogranum caenicola, for the enriched archaeon Kjm51a belonging to Group E2. We also propose to place the methanogenic lineage of the class *Thermoplasmata* in a novel order, *Methanomassiliicoccales* ord. nov.

Key words: Methanogranum caenicola, methanogen, *Thermoplasmata*, rice cluster III, anaerobic digested sludge

Until recently, the class *Thermoplasmata* had consisted of mainly acidophilic, aerobic, mesophilic to thermophilic, and sulfur-reducing archaea such as genera *Acidiplasma* (15), *Ferroplasma* (14), *Picrophilus* (47), *Thermoplasma* (4), *Thermogymnomonas* (28), and *Candidatus* Aciduliprofundum boonei (50). Archaeal members of those genera mainly inhabit extreme environments such as acidic and solfataric fields. On the other hand, culture-independent approaches have retrieved a diverse array of environmental clones belonging to the class *Thermoplasmata* from ordinary environments, and many of these clones form huge uncultured archaeal lineages at the order level, so-called Groups E2 and E3 (6, 33, 39). Groups E2 and E3 consist of sublineages such as Marine group II, deep-sea hydrothermal vent Euryarchaeotic group 1 and 2 (DHVE1 and DHVE2), and rice cluster III (RC-III), which is derived from the alimentary canal (12, 19, 54), anaerobic digester (13), contaminated aquifer (8), deep-sea hydrothermal vent (40, 55), marine plankton (5, 7), and rice field soil (3, 17, 30). More recently, a uniformly shaped pure culture B10^T, given the name *Methanomassiliicoccus luminyensis*, was isolated from human feces, and revealed to be a methanol-reducing, mesophilic, slightly alkaliphilic methanogen belonging to the class *Thermoplasmata* (9). These findings suggest that the class *Thermoplasmata* is a phenotypically versatile taxon; however, very little is known about the phylogenetic diversity and ecological distribution of methanogens in the class *Thermoplasmata*.

In our previous study, members of RC-III within Group E2 in the *Thermoplasmata* as well as *Methanoculleus*, *Methanosarcina*, and *Methanothermobacter* species have been detected from methanogenic bioreactors (1, 21, 43, 44, 46). To obtain cultures of those methanogens, we conducted

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enrichment cultures from methanogenic digester sludge and eventually succeeded in enriching a novel methanogen belonging to Group E2 in class *Thermoplasmata*. Thus, this paper deals with the phylogenetic characterization of the enriched methanogen in *Thermoplasmata* and the provisional characterization of the phenotypes.

Materials and Methods

Sampling

The anaerobic sludge was collected from a methanogenic packed-bed reactor at Kajima Technical Research Institute on 16th December 2004. The reactor, which was packed with carbon fiber textile as supporting media (43–45), had been properly operated at 55°C and was stably producing methane gas from garbage slurry as feedstock. The garbage slurry was prepared from kitchen waste from the company cafeteria. It was diluted with an equal amount of water after removing non-biodegradable materials and then pulverized using a homogenizer. The physicochemical properties of the slurry were as follows: pH 5.2; chemical oxygen demand (COD), approx. 203 g L^{-1}; and volatile suspended solids (VSS), approx. 104 g L^{-1}.

Enrichment from the sludge

The basal medium was used with or without 0.01% (w/v) yeast extract (Becton Dickinson, Franklin Lakes, NJ, USA), designated YB and B media, respectively, in this study. Basal medium was composed of (L^{-1}): 0.54 g NH_4Cl, 0.14 g KH_2PO_4, 0.20 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 2.5 g $NaHCO_3$, and 1.0 mL trace element solution (58) containing 4.0 mg $Na_2WO_4 \cdot H_2O$ and eliminating NaCl. Prior to inoculation, the pH of the medium was adjusted to 7.0 with 6 N HCl, dissolved oxygen was removed by flushing with N_2:CO_2 (4:1, v/v), and 10 mL vitamin solution (L^{-1}) (60) and 10 mL sterile stock solution of Na_2S/cysteine-HCl solution (each 50.0 g L^{-1}) (26) were added. H_2:CO_2 (4:1, v/v; approx. 150 kPa), formate, acetate, or methanol (all at 10 mM) was added to the basal medium as the sole substrate. For enrichment, 0.5 mL anaerobic sludge was inoculated into 20 mL of each medium and incubated at 30°C for a week. A stable enrichment culture was obtained after three cultivations in MYB medium, YB medium supplied with methanol. The enrichment culture was maintained in MYB medium by consecutive transfer monthly.

Preparation of DNA, PCR amplification, and DNA sequencing

The genomic DNA was extracted from the enrichment culture and purified as described previously (49). The archaeal and bacterial 16S rRNA genes were amplified by PCR using the following primers: A10F (5'-TCYGGTTGATCCYGCCRG-3') and A1400R (5'-ACGGGCGGTGTGTGCAAG-3') for the domain *Archaea*, U27F and U1492R (25) for the domain *Bacteria*. The PCR mixture (50 μL) contained 1×PCR buffer, 3.5 mM $MgCl_2$, 10 mM deoxynucleoside triphosphates (dNTPs), 1.25 U AmpliTaq Gold (each from Applied Biosystems, Foster City, CA, USA), and 0.4 μM of each forward and reverse primer. Approximately 100 ng genomic DNA was used as a template under the following cycling conditions: initial AmpliTaq Gold activation at 95°C for 9 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1.5 min, and a final extension step at 72°C for 5 min. The *mcrA* gene encoding the alpha-subunit of methyl-coenzyme M reductase was also partially amplified by PCR with primers MR1mod and ME2mod (35) under almost the same PCR conditions except for its cycle number (40 cycles) and time of extension step (1 min) in the cycle. The PCR product was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced using the BigDye terminator v3.1 cycle sequencing kit with a 3130*xl* genetic analyzer (both from Applied Biosystems).

Clone library

The purified archaeal 16S rRNA and *mcrA* genes were cloned with a pT7Blue T-vector kit (Novagen, Madison, WI, USA). The clonal DNAs were amplified from randomly selected recombinants by direct PCR with M13 primers, and then used as templates for sequencing. A universal primer 907r (56) and T7 promoter primers were used for sequencing the cloned 16S rRNA and *mcrA* genes, respectively. The obtained sequences of all the 16S rRNA gene clones (~690 bp) and the *mcrA* gene clones (~475 bp) were compared with those in the GenBank database using the BLAST program (NCBI-BLAST, www.ncbi.nlm.nih.gov/BLAST), and aligned using the CLUSTAL_X program. Sequence identity of 99% was used as the cut-off value for grouping the sequences into different operational taxonomic units (OTUs).

Phylogenetic analyses

Almost full-length 16S rRNA gene and partial *mcrA* gene sequences were determined for phylogenetic analysis. The following primers were used for sequencing the PCR product of the archaeal 16S rRNA gene: A10F, Kjm700F (5'-TGGGGTAGGGGTAAA ATCCT-3'), Kjm1000F (5'-ACTCACCAGGGGAGACTGTT-3'), A500R (5'-GTGTTACCGCGGCKGCTGG-3'), Kjm700R (5'-GTG GTCCTTCTAGGATTACA-3'), and A1400R, that of the bacterial 16S rRNA gene: U520F (5'-GTGCCAGCAGCCGCGG-3') and U1492R, and that of *mcrA* gene: MR1mod and ME2mod. Sequences were compared using the BLAST program with those available in the DDBJ/EMBL/GenBank databases. Phylogenetic analyses were carried out using the 16S rRNA gene sequence and deduced amino acid sequence of the *mcrA* gene. The 16S rRNA gene sequences were aligned with an ARB data set using ARB software (32). According to the previously described method (27), thirty-five reference sequences of the phylogenetically related archaea and environmental clones were selected as authentic sequences located in the class *Thermoplasmata*. The data set of deduced McrA amino acid sequences was aligned using the CLUSTAL_X program. Phylogenetic trees were constructed by the neighbor-joining (NJ) method with the CLUSTAL_X program (42, 57) and the maximum-likelihood (ML) method with MORPHY software version 2.3b3 (10, 20). In addition, the posterior probabilities of branching points were estimated by Bayesian inference using MrBayes 3.1 (23, 41).

Fluorescence in situ *hybridization*

The enriched archaeon Kjm51a grew on the aforementioned MYB medium for 8 d. The harvested cells were fixed in 4% paraformaldehyde at 4°C for 2 h and stored in 99% ethanol–phosphate-buffered saline (1:1). The fixed cells were incubated in hybridization buffer (0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl, pH 7.2, and an appropriate amount of formamide) containing fluorescently labeled probes (0.5 pmol μL^{-1}). After incubation at 46°C for 10 h, the buffer was replaced with washing solution (378 mM NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl, pH 7.2, and 5 mM EDTA). The sample was incubated at 46°C for 20 min. and then stained with 1 μg mL^{-1} of 4',6-diamidino-2-phenylindole (DAPI). The sample obtained was observed under a confocal laser scanning microscope (LSM710; Carl Zeiss Microscopy, Tokyo, Japan). A specific oligonucleotide probe targeting the 16S rRNA gene of the enriched archaeon Kjm51a (RC281r2, 5'-AAGGCCCATACCCGTCATC-3') was designed using the Probe Design tool of the ARB software package (32). The overall Gibbs free energy of this probe and target sequence calculated with the mathFISH web server was –9.2 kcal mol^{-1} (62). The probes were labeled with fluorescent dye, Alexa Fluor 555 (Japan Bio Services, Saitama, Japan). Two domain-specific probes were also used: EUB338 labeled with Alexa Fluor 647 for detection of almost all bacteria (2), and ARC915 labeled with Alexa Fluor 488 for detection of almost all archaea (53). The stringency of hybridization was adjusted by adding formamide to the hybridization buffer (15% [v/v] for all the probes used in this study). More than 8,000 DAPI-stained cells were counted to determine the ratio of ARC915-hybridized cells to EUB338-hybridized cells.

Physiological characteristics

Growth conditions were determined using MYB medium. Aerobic and microaerobic conditions were prepared by the substitution of air and the addition of 2% (v/v) oxygen, respectively, with filtration through a 0.2 μm-pore membrane filter. Prior to inoculation, acetate, lactate, or pyruvate (all at 10 mM) were added as carbon sources instead of yeast extract. 2-Bromo ethanesulfonate (BES, final concentration 20 mM) was added as the inhibitor of methane production. Then, 0.2 mL of the preculture of the enrichment was inoculated into 20 mL fresh medium containing each substrate. The culture was incubated at 30°C for two weeks. After the transfer twice, hydrogen and methane concentrations in the headspaces of serum bottles were determined with a gas chromatograph (GC-14A; Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector and a porapack Type Q 80–100, mesh 80–100 (Waters, Tokyo, Japan). The analysis conditions were as follows; column temperature, 60°C; injector temperature, 80°C; and detector temperature, 100°C; current, 80 mA; carrier gas, N_2.

Accession numbers

The 16S rRNA gene and *mcrA* gene sequences of the enriched archaeon Kjm51a have been deposited in the DDBJ/EMBL/NCBI, and GenBank nucleotide sequence databases under accession numbers AB749767 and AB749768, respectively.

Results

Methanogenic enrichment cultures from anaerobic sludge

A methanogenic enrichment culture was obtained from anaerobic digester using MYB medium containing methanol and yeast extract. The archaeal population in the culture was analyzed using archaeal 16S rRNA gene- and *mcrA* gene-specific primers. A total of 113 and 61 clones were obtained, respectively. A sole phylotype was obtained in both clone libraries, *i.e.*, all the cloned 16S rRNA gene and *mcrA* gene sequences in the two libraries were almost identical to the sequence identities of 99.4–100% and 99.5–100%, respectively.

An almost full-length 16S rRNA gene sequence (1,309 bp) was determined for a novel archaeon, designated phylotype Kjm51a, in the enrichment culture. In the phylogenetic trees of the 16S rRNA gene sequences constructed using NJ, ML, and Bayesian methods, the enriched archaeon Kjm51a was placed into an uncultured archaeal lineage, Group E2, in the class *Thermoplasmata* (6) (Fig. 1). The topologies of the trees generated by the three phylogenetic analysis methods were almost identical, and were supported by high bootstrap values (99–100%). Archaeon Kjm51a was a member of RC-III, a sublineage within Group E2, and showed the highest sequence similarities (91.3–96.2%) to the environmental clones derived from anaerobic digesters, bovine rumen, and landfill leachate (13, 22, 54, 61). The nearest cultivated neighbor of archaeon Kjm51a was *Methanomassiliicoccus luminyensis* B10^T with 87.7% sequence identity. A partial *mcrA* gene sequence (1,109 bases) was also determined for the enriched archaeon Kjm51a. The NJ tree constructed using the McrA amino acid sequence deduced from the *mcrA* gene sequence demonstrated that archaeon Kjm51a formed a monophyletic cluster together with *M. luminyensis* B10^T and environmental clone sequences derived from anaerobic bioreactor and bovine rumen (Fig. 2), and that the cluster was apparently distinct from the four known methanogenic lineages, the classes *Methanobacteria*, '*Methanomicrobia*',

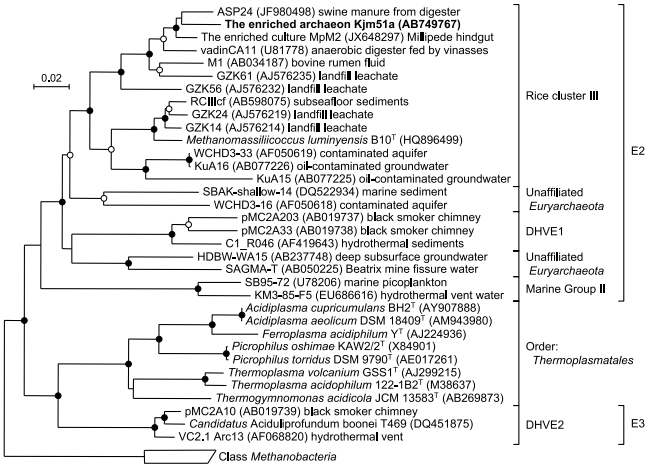

Fig. 1. Phylogenetic affiliation of the enriched archaeon Kjm51a within Group E2 in the class *Thermoplasmata* on the basis of the 16S rRNA gene sequences. The tree was constructed using the neighbor-joining method. Solid circles at branching nodes indicate supporting probabilities above 95% by all the phylogenetic analysis methods (NJ, ML, and Bayesian), and open circles indicate probabilities above 85% by two or more analyses. Bar, 0.02 substitutions per nucleotide position.

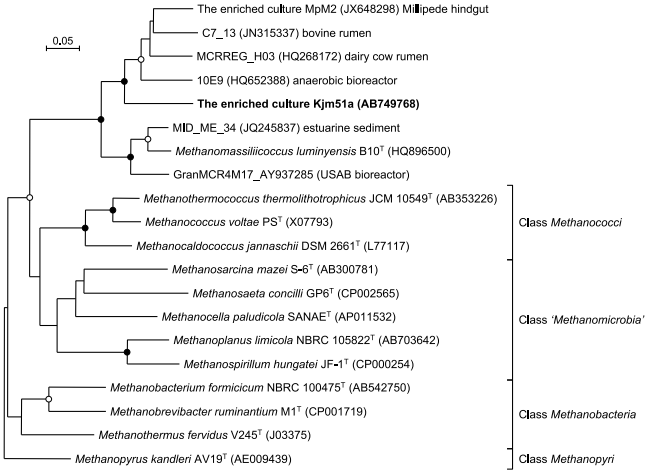

Fig. 2. Deduced McrA amino acid sequence-based phylogeny showing the phylogenetic relationships among the enriched archaeon Kjm51a, its related archaeon and environmental clones, and other known methanogens. Solid circles at branching nodes indicate supporting probabilities above 95%, and open circles indicate probabilities above 80%. Bar, 0.02 substitutions per nucleotide position.

Methanococci, and *Methanopyri*. The McrA amino acid sequence of the enriched archaeon Kjm51a showed 76.0% identity with that of the closest species, *M. luminyensis* B10^T.

Coccoid- and rod-shaped cells were observed under the microscope. The cocci and rods were identified as archaeal and bacterial cells, respectively, by fluorescence *in situ* hybridization with archaeal and bacterial probes (Fig. 3A and B). A ratio of archaeal cells to total cells was at least 3.5±1.4% in the enrichment culture. Cocci were hybridized with a Kjm51a-specific probe, but rods were not (Fig. 3C). Cells hybridized with the Kjm51a-specific probe completely corresponded to those with the archaeal probe (Fig. 3B and D). The bacterial rods in the enrichment culture were provisionally identified as *Clostridium celerecrescens* (sequence

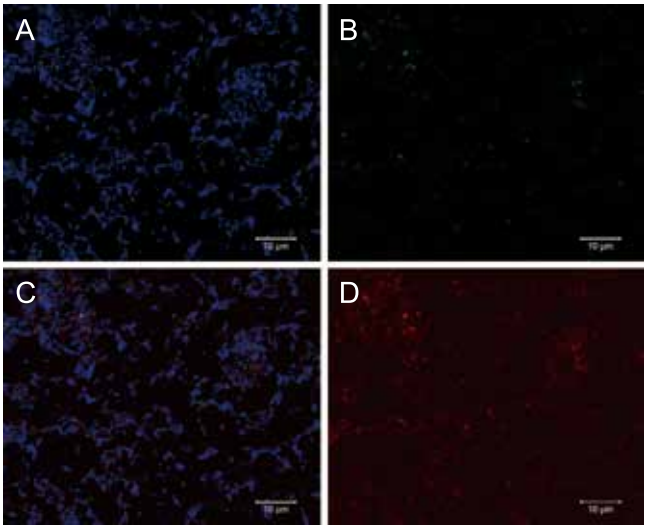

Fig. 3. Epifluorescence micrographs of *in situ* hybridization of the enrichment archaeon Kjm51a grown on MYB medium for a week. The same microscopic field is shown after hybridization with a Kjm51a-specific probe (red), an archaeal probe ARC915 (green), and a bacterial probe EUB338 (blue). A, blue color; B, green color; C, merge of red, green and blue colors; D, red color. Bars, 10 μm.

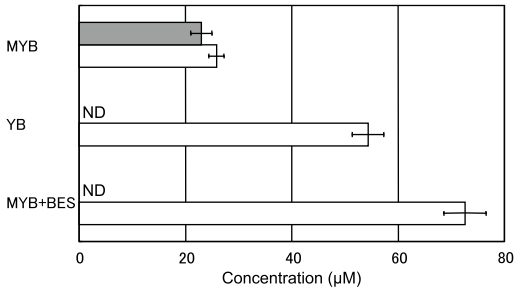

Fig. 4. Methane and hydrogen production in the enrichment culture obtained from anaerobic sludge. Filled bars, methane; open squares, hydrogen. Data points and bars are the means and standard deviations, respectively (n=3). Abbreviations: MYB, MYB medium supplied with methanol in YB medium; YB, YB medium; MYB+BES, MYB medium supplied with BES; ND, not detected.

identity: 99.9%, X71848) by bacterial 16S rRNA gene sequence analysis.

Physiological property of the enriched archaeon Kjm51a

The enriched archaeon Kjm51a was strictly anaerobic and was capable of growing in MYB medium under a N_2/CO_2 (4:1 [v/v]) atmosphere, but could not grow under microaerobic or aerobic conditions. Both methanol and yeast extract were required for the growth of archaeon Kjm51a. Acetate, lactate, and pyruvate were not utilized as carbon sources instead of yeast extract. Metabolic products of the enrichment culture with methanol and yeast extract were hydrogen and methane, and that with yeast extract and without methanol was hydrogen (Fig. 4). Growth of the enriched archaeon Kjm51a in the presence of methanol and its methane production were completely inhibited by the addition of BES. The amount of hydrogen in the presence of BES was approximately three times higher than that in the absence of BES.

Discussion

The novel archaeon Kjm51a was successfully enriched from anaerobic sludge using MYB medium containing methanol and yeast extract. The archaeon was not yet purified in this study because *C. celerecrescens* was dominantly isolated in the presence of yeast extract, although we made a great effort to isolate the archaeon using agar plate culture and dilution-extinction culture. Clone library analysis demonstrated that cloned 16S rRNA and *mcrA* gene sequences obtained from the enrichment culture were almost identical in each. Furthermore, FISH analysis also showed that coccal cells hybridized with a Kjm51a-specific probe completely corresponded to those with an archaeal probe. These findings strongly support the archaeal purity of the enriched archaeon Kjm51a in MYB medium, although bacterial cells still coexisted.

The enriched archaeon Kjm51a was a strictly anaerobic and chemoheterotrophic cocci showing growth and methane production in the presence of methanol, the inhibition of methane production by BES, a well-known inhibitor of methanogenesis (18). Hydrogen production was also observed in the absence of methanol, which indicated that the coexisting bacterium, *C. celerecrescens*, produced hydrogen by its fermentation (38). Inhibition of methane production by BES resulted in increased hydrogen production. These physiological properties suggest that the enriched archaeon Kjm51a might be a methanol-reducing hydrogenotrophic methanogen.

As reported for *Methanosphaera stadtmanae* (34), *Methanomicrococcus blatticola* (51), and *Methanosarcina barkeri* strain Fusaro (36), the enriched archaeon Kjm51a is likely to produce methane by the hydrogen-dependent reduction of methanol through the following reaction: $H_2+CH_3OH \rightarrow CH_4+H_2O$ (11, 29, 52, 59). *Methanomassiliicoccus luminyensis*, a recently isolated methanogen from human feces, belonging to Group E2, also produced methane from methanol in the presence of hydrogen (9). The genome of *M. luminyensis* likely encodes only a partial methanogenesis pathway (16). Most recently, archaeon MpT1 in Group E2 was enriched from termite guts as a methanogen, which converted methanol to methane (39). Methanol may be a common substrate for methanogenesis in Group E2. Schink and Zeikus reported that heterotrophic microbes anaerobically produced methanol as a major end product from pectin, which is a component of plant tissue (48). Biodegradation of plants occurs in a wide variety of environments, such as the rumen, rice field soil and anaerobic digester treating garbage, and Group E2 methanogens may contribute to carbon flux.

The enriched archaeon Kjm51a is the first culture representative derived from an anaerobic methanogenic digester in Group E2. In the phylogenetic trees constructed using 16S rRNA gene sequences, the archaeon Kjm51a and *M. luminyensis* $B10^T$ were completely separated in Group E2 with their low sequence identity (87.7%), which was sufficiently low to classify them into different genera. The enriched archaeon Kjm51a and *M. luminyensis* $B10^T$ were affiliated with RC-III, one of the sublineages in Group E2. RC-III was clearly and completely separated from the validly described order *Thermoplasmatales*. Its monophyletic lineage

was strongly supported by the probability scores (>99%) calculated using all the phylogenetic analysis methods. The 16S rRNA gene sequence of the enriched archaeon Kjm51a and *M. luminyensis* B10^T had similarities of only 77.1 to 80.3% with those of the known archaeal species in the order *Thermoplasmatales*. These similarities are lower than the 85% similarity that is generally used as a cut-off value for distinguishing lineages at the phylum, as suggested by Hugenholtz *et al.* (24). Therefore, RC-III composed of the enriched culture Kjm51a and *M. luminyensis* B10^T is a distinct order level lineage in the class *Thermoplasmata*. Previously, Kemnitz *et al.* (30) reported that the RC-III archaea might heterotrophically grow using peptides, based on their enrichment culture experiment; however, taken together with the recent study (6), our findings clearly indicated that RC-III is a novel methanogenic lineage.

In conclusion, an archaeal representative enriched from the anaerobic methanogenic digester is a novel methanogen belonging to RC-III within Group E2 in the class *Thermoplasmata*. According to the recommendations of Murray and Stackebrandt (37), we propose the provisional taxonomic assignment of *Candidatus* Methanogranum caenicola for the enriched archaeon Kjm51a. Most recently, the order *Methanoplasmatales* was provisionally proposed for the deep-branching lineage accommodating *M. luminyensis* and the enriched archaea MpT1 and MpM2 (39); however, this lineage should be proposed as the *Methanomassiliicoccales* on the basis of Rule 47a of the Bacteriological Code to avoid bacteriological confusion (31). Consequently, we propose to rename the order '*Methanoplasmatales*' as *Methanomassiliicoccales* for the sublineage accommodating *M. luminyensis* B10^T and the enriched archaeon Kjm51a as described below. The proposal of this novel order follows the description of the new family *Methanomassiliicoccaceae*. To purify the enriched archaeon Kjm51a and understand its ecological role in the methanogenic environment, further study via enrichment culture will be necessary.

Description of *Candidatus* Methanogranum caenicola

Methanogranum caenicola (Me.tha.no.gra'num. cae.ni. co'la. N.L. n. methanum [from French n. méth(yle) and chemical suffix -ane], methane; N.L. pref. methano-, pertaining to methane; L. neut. n. granum, grain, kernel; N.L. neut. n. Methanogranum, a methane-producing grain: L. n. caenum, mud, sludge; L. suff. -cola [from L. n. incola], inhabitant, dweller; N.L. n. caenicola, an inhabitant of sludge).

Strictly anaerobic, chemoheterotrophic. Cells form cocci occurring as single cells. Produce methane dependent on hydrogen and methanol. Represent a distinct phylogenetic lineage in the class *Thermoplasmata* based on 16S rRNA gene sequence analysis. Enriched from an anaerobic sludge in a methanogenic digester.

Description of *Methanomassiliicoccaceae* fam. nov.

Methanomassiliicoccaceae (Me.tha.no.mas.si.li.i.coc.ca'ce.ae. N.L. neut. n. *Methanomassiliicoccus* type genus of the family; *-aceae* ending to denote a family; N.L. fem. pl. n. *Methanomassiliicoccaceae* family of the genus *Methanomassiliicoccus*).

The family *Methanomassiliicoccaceae* is defined on the basis of a phylogenetic tree constructed by phylogenetic analysis of the 16S rRNA gene sequence of a single cultivated representative, of the enriched culture, and of environmental clone sequences derived mainly from the alimentary canal, anaerobic digester, landfill leachate, and rice field soil. The type genus is *Methanomassiliicoccus*.

Description of *Methanomassiliicoccales* ord. nov.

Methanomassiliicoccales (Me.tha.no.mas.si.li.i.coc.cal'es. N.L. neut. n. *Methanomassiliicoccus* type genus of the order; *-ales* ending to denote an order; N.L. fem. pl. n. *Methanomassiliicoccales* order of the genus *Methanomassiliicoccus*).

The description is the same as that for the family *Methanomassiliicoccaceae*. The type genus is *Methanomassiliicoccus*.

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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).

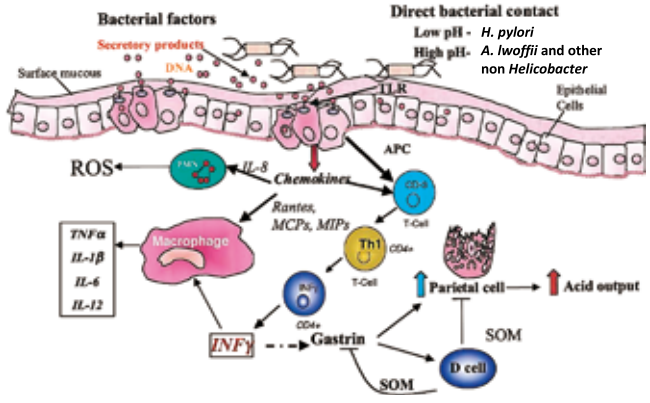

Fig. 1. Schematic model of bacterial colonization of the gastric mucosa, activation of antigen presenting cells (APC), release of pro-inflammatory cytokines and alteration in the number of gastric epithelial cells involved in acid secretion. MCP, macrophage chemotactic protein; MIP, macrophage inflammatory 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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