Comparative Genomics Guides Elucidation of Vitamin B₁₂ Biosynthesis in Novel Human-Associated Akkermansia Strains

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ABSTRACT Akkermansia muciniphila is a mucin-degrading bacterium found in the gut of most humans and is considered a “next-generation probiotic.” However, knowledge of the genomic and physiological diversity of human-associated Akkermansia sp. strains is limited. Here, we reconstructed 35 metagenome-assembled genomes and combined them with 40 publicly available genomes for comparative genomic analysis. We identified at least four species-level phylogroups (Aml to AmIV), with distinct functional potentials. Most notably, we identified genes for cobalamin (vitamin B₁₂) biosynthesis within the AmII and AmIII phylogroups. To verify these predictions, 10 Akkermansia strains were isolated from adults and screened for vitamin B₁₂ biosynthesis genes via PCR. Two AmII strains were positive for the presence of cobalamin biosynthesis genes, while all 9 Aml strains tested were negative. To demonstrate vitamin B₁₂ biosynthesis, we measured the production of acetate, succinate, and propionate in the presence and absence of vitamin supplementation in representative strains of the Aml and AmII phylogroups, since cobalamin is an essential cofactor in propionate metabolism. Results showed that the AmII strain produced acetate and propionate in the absence of supplementation, which is indicative of vitamin B₁₂ biosynthesis. In contrast, acetate and succinate were the main fermentation products for the Aml strains when vitamin B₁₂ was not supplied in the culture medium. Lastly, two bioassays were used to confirm vitamin B₁₂ production by the AmII phylogroup. This novel physiological trait of human-associated Akkermansia strains may affect how these bacteria interact with the human host and other members of the human gut microbiome.

IMPORTANCE There is significant interest in the therapeutic and probiotic potential of the common gut bacterium Akkermansia muciniphila. However, knowledge of both the genomic and physiological diversity of this bacterial lineage is limited. Using a combination of genomic, molecular biological, and traditional microbiological approaches, we identified at least four species-level phylogroups with differing functional potentials that affect how these bacteria interact with both their human host and other members of the human gut microbiome. Specifically, we identified and isolated Akkermansia strains that were able to synthesize vitamin B₁₂. The ability to synthesize this important cofactor broadens the physiological capabilities of human-associated Akkermansia strains, fundamentally altering our understanding of how this important bacterial lineage may affect human health.

KEYWORDS Akkermansia, human gut microbiome, intestinal bacteria, probiotics, vitamin B₁₂

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_Akkermansia muciniphila_ is a mucin-degrading, Gram-negative, intestinal bacterium that is widely present in the human population, typically at 1% to 4% relative abundance (1, 2). A number of studies in humans (3–5) and rodents (6–8) have found positive associations between its abundance and intestinal health, suggesting that _Akkermansia_ might be a beneficial member of the gut microbiome and could be used as a biomarker of a healthy gut (9–11). However, despite a diversity of phytypes being reported in previous sequence-based studies, _A. muciniphila_ MucT (ATCC BAA-835) represents the only described species of the _Verrucomicrobia_ phylum associated with humans (2, 12, 13). Therefore, before we can fully assess the health potential of human-associated _Akkermansia_ strains, a comprehensive understanding of the genomic and physiological diversity of this lineage is needed.

Recently, a pangenomic study that included 33 new isolates from adults and 6 from laboratory mice provided insights into the population structure and evolutionary history of the _Akkermansia_ lineage (14). Specifically, this study revealed an open pangenome with at least three species-level phylogroups (AmI, AmII, and AmIII), which appear to be evolving independently. Although genomic differences among phylogroups were noted, the physiological consequences were not explored.

To continue to expand our understanding of the genomic content and functional potential of human-associated _Akkermansia_ strains, we reconstructed 35 _Akkermansia_ genomes from children 2 to 9 years of age and combined our genomes with those reported by Guo et al. (14). With these genomes, we identified novel diversity and several putative functional differences among the _Akkermansia_ phylogroups. Most notably, we identified the presence of genes associated with _de novo_ cobalamin (vitamin B12) biosynthesis in selected phylogroups of _Akkermansia_. Furthermore, using isolates obtained from healthy adults, we tested these genomic predictions and confirmed vitamin B12 biosynthesis by select human-associated _Akkermansia_ strains. These results build on our understanding of the physiological capabilities of human-associated _Akkermansia_ strains and demonstrate an important biosynthetic activity for this bacterial lineage, which further expands its potential beneficial role in the intestinal environment.

**RESULTS**

**Comparative genomics.** A total of 334.9 Gbp of metagenomic sequence data were obtained from 70 children 2 to 9 years of age. Using SPAdes (15) to assemble contigs and MetaBAT (16) to bin contigs, we recovered 35 high-quality metagenome-assembled genomes (MAGs) of human-associated _Akkermansia_ strains from 35 of the 70 children (Table 1). The completeness of the MAGs was relatively high, ranging from 68.5% to 95.5%, with 31 of 35 MAGs being >90% complete. Similarly, contamination of the MAGs was low (<1% for all). On average, each MAG was 2.87 Mbp in length and contained approximately 2,420 protein-coding genes.

To explore the genomic diversity of human-associated _Akkermansia_ strains, we performed a pangenomic analysis using tools in anvi’o (17, 18). These analyses included the closed genome of the type strain (12) and 33 other human-associated and 6 mouse-associated _Akkermansia_ genomes (14). Previously, these 40 _Akkermansia_ genomes were used to define three species-level phylogroups, Aml, Amll, and AmIII (14). Merging our 35 MAGs with these 40 other genomes, we were able to regenerate the three original phylogroups and also observed a fourth phylogroup (AmIV), based on average nucleotide identity (ANI) values calculated using PyANI (19) (Fig. 1). Additional phylogenetic analyses of single-copy genes (20) also revealed at least four _Akkermansia_ phylogroups (see Fig. S1 in the supplemental material). Phylogroup Aml, which includes the type strain _A. muciniphila_ Muc^-^, contained the largest number of genomes (n = 40), followed by Amll (n = 26), AmIV (n = 7), and AmIII (n = 2). Phylogroup Amll was not observed in any of our 35 MAGs. Interestingly, both Aml and Amll included isolates obtained from mice. Within each phylogroup, ANI values ranged from 93.94% to 99.98% across >65% of each pair of genomes (Fig. S2). All ANI values for between-phylogroup comparisons were <92%. One genome in AmIV (CDI-148A-8) showed
lower similarity (on average, ~94%) to other genomes within this phylogroup, possibly indicating further species-level diversity among human-associated *Akkermansia* strains.

Across all phylogroups, we identified 6,557 gene clusters (GCs), with 1,021 being found in all 75 genomes and 1,240 being found in only 1 genome (Fig. 1). Functional genes within the core included the cytochrome *bd* genes (Amuc_1694 and Amuc_1695) (21) and type IV pilus genes (Amuc_1098 to Amuc_1102) (22–24) previously characterized from *A. muciniphila* MucT.

Next, we were interested in identifying functional gene predictions that differed among the phylogroups. Using Clusters of Orthologous Groups (COG) annotations of GCs implemented in anvi’o, we observed 7 GCs putatively involved in the corrin ring stage of cobalamin (vitamin B12) biosynthesis within the AmII (24/26 genomes) and AmIII (2/2 genomes) phylogroups (see Data Set S1 in the supplemental material). To investigate these genes in greater detail, we manually inspected the annotations of all 75 genomes using Integrated Microbial Genomes (IMG) (25) and Geneious 7.1.3 (Biomatters, Inc.). With this approach, we confirmed the COG annotations and identified a cluster of 8 genes that appeared to code for the corrin ring biosynthesis proteins in a subset of *Akkermansia* genomes (Fig. 2; also see Data Set S2). Included in this genomic region were genes *cbiK* (or *cbiX*), *cbiL*, *cbiC*, *cbiD*, *cbiET*, *cbiFGH*, and *cbiA*, which encode

### TABLE 1
Summary of 35 *Akkermansia* MAGs recovered from a diverse population of children 2 to 9 years of age, living in Los Angeles, California

| Genome name | Phylogroup | % completeness | % contamination | No. of predicted proteins | % coding density | Assembly properties | GC content |
|-------------|------------|----------------|-----------------|--------------------------|-----------------|---------------------|-----------|
| *A. muciniphila* MucT | AmI | 100 | 0.0 | 2,238 | 88.6 | 1 | 2.66 | 55.8 |
| CDI-75C-7 | AmI | 95.5 | 0.0 | 2,433 | 88.4 | 26 | 2.82 | 55.5 |
| CDI-92A-19 | AmI | 68.5 | 0.0 | 2,117 | 89.0 | 317 | 2.27 | 55.8 |
| CDI-93C-15 | AmI | 91.0 | 0.0 | 2,345 | 88.8 | 231 | 2.59 | 55.8 |
| CDI-16B-22 | AmI | 94.6 | 0.0 | 2,291 | 88.5 | 79 | 2.65 | 55.6 |
| CDI-15B8-12 | AmI | 95.5 | 0.0 | 2,302 | 88.2 | 42 | 2.70 | 55.3 |
| CDI-50B-13 | AmI | 95.5 | 0.0 | 2,293 | 88.4 | 22 | 2.72 | 55.6 |
| CDI-51A-11 | AmI | 95.5 | 0.0 | 2,295 | 88.4 | 22 | 2.72 | 55.6 |
| CDI-28A-8 | AmI | 95.5 | 0.0 | 2,301 | 88.2 | 27 | 2.71 | 55.4 |
| CDI-85A-12 | AmI | 95.5 | 0.0 | 2,225 | 88.4 | 19 | 2.66 | 55.4 |
| CDI-30A-11 | AmI | 95.5 | 0.0 | 2,272 | 88.4 | 22 | 2.68 | 55.5 |
| CDI-42C-15 | AmI | 95.5 | 0.0 | 2,340 | 88.4 | 25 | 2.74 | 55.2 |
| CDI-151B-10 | AmI | 94.6 | 0.0 | 2,383 | 88.3 | 64 | 2.77 | 55.4 |
| CDI-193A-6 | AmI | 95.5 | 0.0 | 2,416 | 88.3 | 32 | 2.83 | 55.4 |
| CDI-143C-7 | AmII | 81.1 | 0.9 | 2,301 | 88.4 | 206 | 2.67 | 58.7 |
| CDI-10B-12 | AmII | 94.6 | 0.0 | 2,439 | 88.1 | 32 | 2.98 | 58.3 |
| CDI-128B-11 | AmII | 92.8 | 0.0 | 2,428 | 88.1 | 22 | 2.96 | 58.3 |
| CDI-129B-12 | AmII | 88.3 | 0.0 | 2,375 | 88.1 | 229 | 2.70 | 58.5 |
| CDI-77C-9 | AmII | 95.5 | 0.0 | 2,435 | 88.0 | 25 | 2.99 | 58.2 |
| CDI-24B-9 | AmII | 94.6 | 0.0 | 2,450 | 88.2 | 31 | 3.02 | 58.1 |
| CDI-182B-6 | AmII | 95.5 | 0.0 | 2,478 | 88.1 | 22 | 3.02 | 58.3 |
| CDI-198C-9 | AmII | 95.5 | 0.0 | 2,512 | 88.3 | 51 | 3.00 | 58.2 |
| CDI-69C-9 | AmII | 95.5 | 0.0 | 2,421 | 88.2 | 24 | 2.96 | 58.2 |
| CDI-138A-11 | AmII | 95.5 | 0.0 | 2,483 | 88.0 | 25 | 3.01 | 58.2 |
| CDI-70C-8 | AmII | 95.5 | 0.0 | 2,481 | 88.0 | 32 | 3.01 | 58.2 |
| CDI-26A-8 | AmII | 92.8 | 0.9 | 2,610 | 88.1 | 251 | 2.95 | 58.0 |
| CDI-34A-8 | AmII | 95.5 | 0.0 | 2,558 | 87.2 | 29 | 3.09 | 57.8 |
| CDI-65B-6 | AmII | 87.4 | 0.0 | 2,538 | 87.4 | 55 | 3.04 | 57.8 |
| CDI-203B-7 | AmII | 94.6 | 0.9 | 2,479 | 87.8 | 25 | 2.99 | 58.1 |
| CDI-150B-9 | AmIII | 95.5 | 0.0 | 2,457 | 87.7 | 29 | 2.99 | 57.2 |
| CDI-12C-16 | AmIII | 95.5 | 0.0 | 2,461 | 87.8 | 32 | 2.99 | 57.2 |
| CDI-156A-7 | AmIII | 95.5 | 0.9 | 2,532 | 87.5 | 56 | 3.05 | 56.7 |
| CDI-74B-7 | AmIV | 94.6 | 0.9 | 2,502 | 87.4 | 48 | 3.01 | 56.7 |
| CDI-188-8 | AmIV | 94.6 | 0.0 | 2,509 | 88.0 | 124 | 2.95 | 56.9 |
| CDI-148A-8 | AmIV | 95.5 | 0.9 | 2,557 | 87.3 | 46 | 3.04 | 56.0 |
| CDI-13A-11 | AmIV | 95.5 | 0.0 | 2,670 | 88.1 | 66 | 3.20 | 56.6 |
| Average* | | 93.4 | 0.2 | 2,419.7 | 88.1 | 68.23 | 2.87 | 56.9 |

*The genome of *A. muciniphila* MucT was not included in the averages.*
the enzymes associated with the anaerobic pathway of corrin ring biosynthesis. This
cluster also contains a gene whose product is annotated as a hypothetical protein,
which shows some similarity to a putative cobalt transporter. The content and
arrangement of these genes were similar to those of the only other named member of
the Akkermansia genus, Akkermansia glycaniphila PytT, which was previously isolated
from a python. Additionally, all 75 genomes contained most of the genes associ-
ated with the upstream (tetrapyrrole precursor biosynthesis, e.g., Amuc_0090,
Amuc_0091, Amuc_0417, Amuc_0896, and Amuc_1730) and downstream (nucleotide
loop assembly, e.g., Amuc_1678 to Amuc_1683) stages of vitamin B₁₂ biosynthesis.
Genes annotated as a TonB-dependent transporter (e.g., Amuc_1684) and an extracel-
lular solute-binding family 5 protein (e.g., Amuc_1685) that may be involved in vitamin
B₁₂ import were also identified adjacent to the nucleotide loop assembly genes in all
except 1 genome.

A. muciniphila MucT was previously classified as a cobinamide (Cbi) salvager because
it lacks the genes coding for the enzymes to synthesize the corrin ring of vitamin B₁₂.

FIG 1 Pangenome of 75 Akkermansia genomes generated using anvi’o. Each concentric circle represents a bacterial genome,
with purple circles belonging to the AmI phylogroup, blue to AmII, orange to AmIII, and green to AmIV. Blank areas in each circle
indicate the absence of a particular GC in that genome. A total of 6,557 GCs were observed across all genomes. Genomes are ordered
by ANI, as depicted by the pink heatmap in the upper right. Host organisms are indicated below the heatmap, in white (human) or
black (mouse) boxes. Similarly, genome sources are indicated in white, gray, and black (this work) boxes. The outermost ring
is colored according to the presence (red) or absence (gray) of functional COG annotations. The next ring indicates the number of
genomes in which that particular GC was observed. Singleton (blue) and core (green) genes are indicated outside the concentric
circles. Corrin ring biosynthesis genes are indicated in the AmII (blue) and AmIII (orange) genomes.
but it needs this cofactor for methionine synthesis, nucleotide synthesis, queuosine synthesis, and propionato metabolism (28). Indeed, genes associated with these cellular functions were conserved across all phylogroups (Data Set S1). Interestingly, the vitamin B12-independent methionine synthase II gene (metE) was present in 25 of 40 AmI genomes but not in any of the other genomes, including that of the type strain MucT. Together, these observations suggest that all Akkermansia strains examined here are able to acquire and likely to remodel corrinoids from the environment for use, but some are also able to synthesize this important cofactor de novo.

**Cultivation and validation of vitamin B$_{12}$ biosynthesis.** To determine whether specific Akkermansia species/strains are indeed able to synthesize vitamin B$_{12}$ de novo, we isolated several Akkermansia strains from healthy adults and compared their nearly full-length 16S rRNA gene sequences with those reported by Guo et al. (14) in ARB (29), to determine phylogroup affiliation. Across phylogroups AmI, AmII, and AmIII, 16S rRNA gene sequences were all >97% identical but nevertheless clustered into the known phylogroups (Fig. S3). Based on this approach, we identified 8 AmI isolates and 2 AmII isolates in our culture collection. Because our MAGs did not contain any full-length 16S rRNA gene sequences, we could not positively identify AmIV members among the isolates.

Next, using the AmII and AmIII genomes and the genome of A. glycaniphila Pyt$, we designed degenerate PCR primers targeting 4 genes, $cbl$, $cbiC$, $cbiD$, and $cbiFGH$, of the corrin ring biosynthesis GC, which encode a cobalt-factor II C-20-methyltransferase, a cobalt-precorrin-8 methylmutase, a cobalt-precorrin-5B C-1-methyltransferase, and a cobalt-precorrin-4 methyltransferase/precorrin-3B C-17-methyltransferase, respectively (Table 2). These genes were selected because they are predicted to give the best indication of cobamide production, as described by Shelton et al. (28). As expected, only isolates from the AmII phylogroup (CSUN-17 and CSUN-34) and A. glycaniphila Pyt$
showed positive amplification, whereas all AmI isolates (including A. muciniphila MucT) failed to show amplification (Table 3). Sequencing and BLAST searching of these PCR amplicons from CSUN-17 against A. glycaniphila strain ERS 1290231 and Desulfovibrio vulgaris strain Hildenborough confirmed the identity of these gene fragments (Table S1), clearly demonstrating the presence of select cbir genes in the AmII phylogroup.

It is known that many fermentative bacteria, including A. muciniphila MucT, use vitamin B12 to activate methylmalonyl-coenzyme A (CoA) synthase to convert succinate to propionate (30, 31). Therefore, to demonstrate vitamin B12 biosynthesis in vitro, we

| Gene or strain | Primer name | Primer (5′ to 3′)a | Expected amplicon size (bp) | Source | Reference |
|----------------|-------------|-------------------|-----------------------------|--------|-----------|
| cbirL          | Precorrin-2 forward | TYTTCAGCATGTSCCGYGAC | 358 | This work |
| cbirL          | Precorrin-2 reverse | GCCGGCTRCGGTAGGTYTT | 358 | This work |
| cbirC          | cbirC forward | ATCCACACCACGGCRGAC | 500 | This work |
| cbirC          | cbirC reverse | GCGGTCGACGGGTTRGT | 500 | This work |
| cbirFGH        | cbirG forward | GTSAGCAGCGGTYYTG | 340 | This work |
| cbirFGH        | cbirG reverse | ATGAGGSCCGTCGCCGCCGA | 340 | This work |
| cbidD          | cbidD forward | GACCCGACTGCACSCA | 379 | This work |
| cbidD          | cbidD reverse | TAGGCTCTCTTGCTG | 379 | This work |
| 16S rRNA       | 8F          | AGAGTTTGATCCTGGCTCAG | Variable | 79 |
| 16S rRNA       | 515F        | GTGCCAGCMGCCGCGGTAA | Variable | 79 |
| 16S rRNA       | 806R        | GGACTACHVGGGTWTCTAAT | Variable | 80 |
| 16S rRNA       | 1492R       | TACGGTTACCTTGTACGA | Variable | 81 |

aY = C or T, S = C or G, B = G, T, or C, and R = G or A.

TABLE 3 Presence of select genes associated with corrin ring biosynthesis in CSUN Akkermansia isolates, as determined by PCR

| Isolate | Phylogroup | Presence or absencea | cbirL | cbirC | cbidD | cbirFGH |
|---------|------------|----------------------|-------|-------|-------|---------|
| CSUN-7  | AmI        | –                    | –     | –     | –     | –       |
| CSUN-12 | AmI        | –                    | –     | –     | –     | –       |
| CSUN-17 | AmII       | +                    | +     | +     | +     | +       |
| CSUN-23 | AmI        | –                    | –     | –     | –     | –       |
| CSUN-27 | AmI        | –                    | –     | –     | –     | –       |
| CSUN-28 | AmI        | –                    | –     | –     | –     | –       |
| CSUN-33 | AmI        | +                    | +     | +     | +     | +       |
| CSUN-34 | AmI        | +                    | +     | +     | +     | +       |
| CSUN-31 | AmI        | –                    | –     | –     | –     | –       |
| CSUN-36 | AmI        | –                    | –     | –     | –     | –       |
| A. muciniphila ATCC BAA-835 | AmI | – | – | – | – |
| A. glycaniphila ERS 1290231 | NAb | + | + | + | + |

a,b: PCR product of the predicted amplicon size.

bNA, not applicable.
quantified the production of succinate and propionate (and acetate) in the presence and absence of vitamin B$_{12}$ in mucin medium (Fig. 3). Our predictions were that the AmI phylogroup (represented by $A$. muciniphila MucT) would produce acetate and succinate in the absence of vitamin B$_{12}$ and acetate and propionate when B$_{12}$ was present. For AmII, we predicted that acetate and propionate would be produced regardless of whether the culture medium was supplemented with vitamin B$_{12}$. Results showed that the AmI isolate produced propionate in a vitamin B$_{12}$-concentration-dependent manner (Fig. 3B and C). Also, as expected, the CSUN-17 isolate (AmII) produced significant amounts of acetate and propionate in the absence and presence of exogenous vitamin B$_{12}$, but production was more rapid with supplementation (Fig. 3D to F). These results strongly suggest vitamin B$_{12}$ biosynthesis by strain CSUN-17, which represents the AmII phylogroup.

Due to the complexity of cobalamin, involving different possible lower-ligand structures, analytical verification of an unknown type of cobalamin can be challenging. Therefore, we used two bioassays to verify de novo biosynthesis of vitamin B$_{12}$ by strain CSUN-17. The first bioassay utilized Lactobacillus leichmannii ATCC 7830, which cannot grow without vitamin B$_{12}$ supplementation (32). For the second bioassay, mutant strains of Escherichia coli ($E$. coli $\Delta$metE and $\Delta$metE $\Delta$metH strains) that require vitamin B$_{12}$ for methionine biosynthesis were utilized (33–36). Results of both bioassays confirmed the biosynthesis of vitamin B$_{12}$ by strain CSUN-17 and not by $A$. muciniphila MucT, as only CSUN-17 lysates could support the growth of the vitamin B$_{12}$ auxotrophs (Fig. 4).

**DISCUSSION**

$A$. muciniphila is a common gut bacterium that is highly regarded as a beneficial member of the human gut microbiome, with important probiotic potential (10, 37). Various studies have described positive associations between the abundance of Akkermansia organisms and intestinal health (3, 4). For example, $A$. muciniphila affects glucose metabolism and intestinal immunity, and its abundance in the gastrointestinal...
tract is inversely correlated with diseases, including Crohn’s disease, ulcerative colitis, and acute appendicitis (38–41). Although a number of 16S rRNA gene variants have been observed (12) and dozens of isolates have been obtained (14), human-associated Akkermansia strains have largely been considered a single species and the functional potential beyond mucin degradation has gone largely unexplored. Here, we demonstrate that there are significant genomic and physiological differences among the human-associated Akkermansia strains. Through comparative genomic analysis, we identified four phylogroups of human-associated Akkermansia strains, expanding the known genomic diversity of this lineage. Although all 16S rRNA gene sequences examined here and elsewhere (38) are >97% identical, use of an ANI of 95% across genomes as a species-level delineation (42, 43) would suggest that each phylogroup represents a different species of Akkermansia. When we examined gene content, several phylogroup-specific genes that are predicted to code for functional differences

![Graph A](image1)

**FIG 4** Biosynthesis of vitamin B₁₂ by strain A. muciniphila strain CSUN-17, as confirmed by bioassays using *L. leichmannii* ATCC 7830 (A) and mutant strains of *E. coli* (B). Growth of *L. leichmannii* strain ATCC 7830, an *E. coli* ΔmetE strain, and an *E. coli* ΔmetE ΔmetH strain was measured in growth medium with and without vitamin B₁₂ supplementation and with cell lysates from both *A. muciniphila* MucT and *A. muciniphila* strain CSUN-17. Growth was measured as OD₆₀₀. Values were averaged from three biological replicates, and error bars represent the standard deviations.

![Graph B](image2)
among phylogroups were identified, further supporting species delineation. Most notably, we identified a complete set of genes involved in \textit{de novo} biosynthesis of cobalamin, or vitamin B$_{12}$, in two of the four phylogroups. We were able to validate these predictions \textit{in vitro} using novel strains obtained from healthy adults. These findings demonstrate an ecologically important function (44) not previously associated with human-associated \textit{Akkermansia} strains, fundamentally altering our understanding of the diversity and physiology of this lineage. More broadly, these results continue to demonstrate the importance of merging next-generation sequencing approaches with traditional cultivation approaches to elucidate the basic biology of microorganisms of significance.

A recent comparative genomic analysis examining 11,000 bacterial genomes for cobamide production revealed that approximately 37\% of bacteria are predicted to synthesize cobamides, although 86\% require them for at least one cellular function (28, 45). Additionally, Degnan et al. found that most vitamin B$_{12}$-dependent human gut bacteria lack the ability to synthesize vitamin B$_{12}$ (45). The type strain \textit{A. muciniphila} Muc$^T$ was included in the analysis by Shelton and colleagues (28) and was described as a Cbi salvager able to use exogenous sources of vitamin B$_{12}$. Indeed, based on previous \textit{in vitro} coculture experiments, \textit{A. muciniphila} Muc$^T$ can use at least three types of cobamides, i.e., cyanocobalamin supplied in the culture medium, pseudovitamin B$_{12}$ produced by \textit{Eubacterium hallii} L2-7 (30), and an unknown form produced by \textit{Anaerostipes caccae} (46). Presumably, \textit{Akkermansia} strains are able to import these various forms of cobalamin and use them directly or remodel the lower ligand to suit their needs. With our findings, some \textit{Akkermansia} strains can now be considered producers of corrinoids, altering our understanding of how they interact with other members of the human gut microbiome and potentially their human host. However, questions remain regarding the type of cobalamin produced by AmII members and, more generally, the specificity and efficiency of cobamide import and remodeling by all \textit{Akkermansia} strains.

Cobalamin produced by bacteria and archaea in the large intestine is not readily available to the human host, for two main reasons (44). First, the receptors responsible for cobalamin absorption are found in the small intestine, which is not as densely colonized by bacteria as the large intestine in times of health. Second, although bacteria produce many different types of cobalamin, their contribution to the available pool of cobalamin is small because many of the forms produced by bacteria are not recognized by human receptors. Thus, bacteria are thought of more as competitors for dietary cobalamin than as suppliers. However, if a bacterium colonized the small intestine and produced an appropriate form of cobalamin, then the cofactor would possibly be available to the human host. With regard to \textit{Akkermansia} strains, we do not yet know the form of cobalamin produced by AmII members but \textit{Akkermansia}-like organisms have been observed throughout the human gastrointestinal tract, including in the small intestine (reviewed in reference 38). Interestingly, phylogenetic analyses consistently group AmII and AmIII isolates (14) with clones and other sequences previously observed in the small intestine (38). Because our genomic sequence data and isolates were obtained from fecal samples, we could not determine whether the different phylogroups colonize different segments of the gastrointestinal tract, although it is intriguing to speculate.

Although we do not yet know whether humans can directly benefit from vitamin B$_{12}$ produced by \textit{Akkermansia}, there are indirect benefits resulting from the altered metabolites produced when vitamin B$_{12}$ is available. Specifically, the type and quantity of short-chain fatty acids (SCFAs) produced during fermentation influence host health (47–49). For example, propionate is known to help regulate appetite by stimulating the release of peptide YY (PYY) and glucagon-like peptide-1 (GLP-1) by human colonic cells (50). Less is known about the potential benefits of succinate in the human gut, but succinate does improve glucose homeostasis in the mouse cecum via intestinal gluconeogenesis (51). In contrast, succinate has been shown to trigger a type 2 immune inflammatory response, initiated by epithelial tuft cells, in the human small intestine.
Thus, possessing the ability to synthesize vitamin B$_{12}$ de novo would suggest that the AmII and AmIII phylogroups have the potential to consistently produce more propionate than succinate during mucin fermentation and, as a result, influence gut epithelial cell behavior. If the AmII and/or AmIII phylogroups do colonize the small intestine, then the ability to consistently produce propionate over succinate could have significant health implications.

In addition to propionate metabolism, Akkermansia strains are predicted to use vitamin B$_{12}$ as a cofactor for methionine biosynthesis using methionine synthase type I (MetH). All genomes possessed the metH gene; however, select AmI genomes (25/40 genomes) also contained the vitamin B$_{12}$-independent methionine synthase II (metE) gene, suggesting that these select AmI strains can generate methionine in the absence of vitamin B$_{12}$. Given that the AmI phylogroup does not synthesize vitamin B$_{12}$, this would allow production of this essential amino acid when exogenous corrinoids are unavailable. How readily available corrinoids are to Akkermansia strains, either from other bacterial producers or from the host diet, is not known, but possessing both variants may be an adaptive strategy for AmI strains.

A. muciniphila is being explored as a commercial probiotic and/or therapeutic agent (41). Recent studies reported large-scale cultivation of *A. muciniphila* on a defined medium that is safe for human consumption (23) and evaluated the stability and viability of the bacterium in dark chocolate (53). However, our results indicate that there are still gaps in our understanding of the diversity and physiology of human-associated *Verrucomicrobia* strains that need to be explored.

Here, we carried out a pangenomic analysis of 75 *Akkermansia* genomes and identified at least four species-level phylogroups (AmI to AmIV), with differing functional potentials. However, a polyphasic taxonomic characterization that includes robust phenotypic and genomic analyses is needed to verify species designations. Quantification of SCFAs produced by select strains in the presence and absence of vitamin B$_{12}$ supplementation strongly suggested cobalamin biosynthesis by AmI strains. Two bioassays using bacterial strains dependent on vitamin B$_{12}$ for growth confirmed de novo biosynthesis of this important cofactor by select *Akkermansia* strains. This work alters our understanding of how *Akkermansia* interacts with its human host and other members of the human gut microbiome in its unique environment. Future work will focus on other genomic similarities and differences identified in our analysis but will also continue to explore vitamin B$_{12}$ production and acquisition using our culture collection. We are also continuing to isolate novel strains from healthy adults, attempting to obtain representatives of each phylogroup that has been observed or others that have yet to be observed.

**MATERIALS AND METHODS**

**Metagenomic studies.** (i) Recruitment and sampling. Samples used for metagenomic sequencing were obtained from healthy children 2 to 9 years of age, as described elsewhere (54). The participants were informed and provided consent under protocol 1314-223, approved by the institutional review board (IRB) at California State University, Northridge (CSUN). Verbal assent was obtained from each child, and written consent was obtained from one parent/guardian. Data Set S3 in the supplemental material provides unidentifiable demographic information for each child included in this study.

(ii) DNA extraction, library preparation, and sequencing. Parents collected fecal samples in the privacy of their homes, using sterile, double-tipped swabs, by swabbing toilet paper (or diapers) after use. Samples were frozen at −20°C within 24 h after collection and transported on blue ice to the laboratory (<30 min in transit), where they were stored at −80°C. This protocol is minimally invasive and has been successfully used in many similar, community-based research projects (55–57).

DNA was extracted from approximately ~0.1 g of collected samples using the Mo Bio PowerSoil DNA isolation kit (Mo Bio, Carlsbad, CA), following a modified extraction protocol (58). Extracts were then quantified using a Qubit 2.0 fluorometer with high-sensitivity reagents, and 100 ng of DNA from each sample was sheared into 300-bp fragments using a Covaris M220 ultrasonicator (59). The NEBNext Ultra DNA library preparation kit for the Illumina platform (60) was used to prepare dual-indexed metagenomic libraries from the sheared samples. Libraries were confirmed using a Bio-Rad Experion automated electrophoresis system, with Kapa quantitative PCR next-generation sequencing library quantification. Two sequencing runs with the multiplexed libraries were conducted on an Illumina HiSeq 2000 system (2 by 100 bp) at the University of California, Irvine, Genomics High-Throughput Facility.
(iii) Metagenomic sequence processing. Raw fastq files from each sample were trimmed using Trimmomatic (61) (with the following parameters: illr:inclip, TruSeq3-PE.fa:2:30:10; leading, 3; trailing, 3; slidingwindow, 4:15; minlen, 36). Trimmed sequences were then screened against the human genome (GRCh38) using DeconSeq (62), in order to remove any potential human DNA sequences. Nonhuman sequences were further cleaned using PRINSEQ (63) (with the following parameters: min_qual_mean, 20; ns_max_n, 3). Remaining sequences without a matepair were removed, and paired sequences were assembled using the default parameters for metagenomes in SPAdes (15). Resulting contigs of $\geq 2$ kbp were binned using MetaBAT (16), with default parameters, and the taxonomy and completeness of bins were verified against the Verrucomicrobia phylum using the taxonomy workflow of CheckM (64). We determined that bins confidently identified as “k_Bacteria (UID2982)” were Akkermansia, and we evaluated the quality of those bins further using MiGA (65). Assembled contigs ($>2$ kbp) from each child with a high-quality Akkermansia bin were submitted to IMG-M, where they were annotated using their workflow (25). Both IMG and Geneious 7.1.3 were used to manually inspect annotations of interest. Vitamin B$_12$-associated genes were detected by searching for annotations from Enzyme Commission (EC) numbers, IMG terms, the pfam database, and the COG database (25, 66, 67). The annotations included those used by Shelton et al. (28) and Degnan et al. (45).

(iv) Pangenome analysis. To explore the Akkermansia pangenome, we combined our 35 MAGs with 40 other publicly available genomes in anvilo (17, 18). Assembled fasta files were first converted to db files using the anvi-script-FASTA-to-contigs-db command, which uses Prodigal (68) to call open reading frames. Each db file was then annotated against the COG database (69) using the anvi-run-ncbi-cogs command with the use-ncbi-blst flag. After generating the genome storage file with the anvi-genomes-storage command, the anvi-pan-genome command was used with the same parameters (num-threads, 12; minbit, 0.5; mcl-inflation, 10; use-ncbi-blst) as outlined by Delmont and Eren (17, 70, 71). The pangenome was visualized using the anvi-display-pan command.

To calculate ANI in anviol, the anvi-compute-ani command, which utilizes PyANI (19), was used. To identify functions (i.e., COG annotations) that were differentially distributed among the phylogroups, we used the anvi-get-enriched-functions-per-panel-group command, with phylogroups (Aml to AmIV) as the category. To perform phylome analyses, we used the anvi-get-sequences-for-hmm-hits function, which performs an HMM search of the single-copy genes described by Campbell et al. (20) for each genome, aligns them using MUSCLE (72), and then concatenates them into a single fasta file. The fasta file was then input into FastTree (73) for phylogenetic reconstruction using default parameters, and trees were visualized using FigTree version 1.4.3 (https://github.com/rambout/figtree).

Cultivation studies. (i) Recruitment and sampling. Fecal samples used for cultivation of Akkermansia isolates were obtained from healthy adults using swabs, as described previously (56), under CSUN IRB protocol 1516-146. Written consent was obtained from each subject. Collected samples were refrigerated (4°C) and transferred to culture medium (see below) within 24 h after collection.

(ii) Enrichment, isolation, genomic DNA extraction, and 16S rRNA gene sequencing. Anaerobic mucin medium was modified slightly from that described by Derrien et al. (13) and contained 0.4 g/liter KH$_2$PO$_4$, 0.53 g/liter Na$_2$HPO$_4$, 0.3 g/liter NH$_4$Cl, 0.3 g/liter NaCl, 0.1 g/liter MgCl$_2$·6H$_2$O, 0.4 g/liter NaHCO$_3$, 1 mg/liter resazurin, and 10 ml/liter trace mineral solution, as described by Ferguson and Mah (74). The pH of the medium was adjusted to 6.5. The medium was prepared with boiled Milli-Q water under constant gassing with a gas mixture of N$_2$/CO$_2$ (80:20 [vol/vol]) (with the following parameters: min_qual_mean, 20; ns_max_n, 3). The culture medium was later modified to include 1 mM L-threonine and 10 g/liter tryptone (Oxoid), as described previously (31). Broth medium was prepared in serum tubes or bottles, which were sealed with butyl rubber stoppers and aluminum crimp caps prior to being autoclaved at 121°C and 15 lb/in$^2$ for 15 min. Prior to inoculation, the medium was autoclaved and held at 4°C. PCR mixtures were purified using the QIAquick PCR purification kit (Qiagen). Initial sequencing of the 16S rRNA gene was performed using either the 8F or 1492R primer on an ABI Prism
3730 DNA sequencer (Laragen Sequencing and Genotyping, Culver City, CA). If cultures were pure and yielded positive results for A. muciniphila in a BLAST search, then the nearly full-length 16S rRNA gene was sequenced with additional primers (515F, 806R, and 8F or 1492R) (Table 2). Sequences associated with each isolate were then assembled in Geneious 7.1.3 and imported into ARB (29), as discussed below. General demographic information about donors is provided in Table S2 in the supplemental material.

(iii) 16S rRNA gene phylogeny. To determine the phylogroup affiliations of our isolates, 16S rRNA gene sequences of the isolates described by Guo et al. (14) were first extracted from their genomic sequence data and imported into ARB (29). Once in ARB, gene sequences were aligned with the 16S rRNA gene sequence of A. muciniphila Muc⁴, with secondary structure constraints, and manually inspected, and sequences that were <1,000 bp were discarded. Similarly, 16S rRNA gene sequences of our novel isolates were imported and aligned in ARB. A custom alignment mask excluding nucleotide positions found in less than one-half of all isolates was generated, and masked alignments were imported into MEGA7 (75), where phylogenetic reconstruction was generated using the maximum-likelihood approach. Because we knew the affiliation of the isolates described by Guo et al. (14), we were able to place our isolates in this framework based on placement in the 16S rRNA gene tree.

(iv) Corrin biosynthesis PCR screen of isolates and gene sequencing. To amplify conserved regions of corrin-biosynthesis-associated genes, degenerate primers were designed (Table 2). Select corrin-biosynthesis-associated homologous sequences were aligned using BioEdit sequence alignment editor version 7.0.5 (http://www.mbio.ncsu.edu/BioEdit/page2.html) (locus tags of sequences used in the alignments are shown in Table S3). All gene sequences were obtained from JGI IMG/ER. Conserved regions were found using the accessory application ClustalW multiple alignment tool in BioEdit (76). For amplification of the corrin biosynthesis genes cbl, cbiC, cbiD, and cbiFGH, 1 µl of genomic DNA was added to 12.5 µl of GoTaq Green Master Mix (Promega) and 1 µl of 10 µM each primer, using a final PCR mixture volume of 25 µl. PCR reaction conditions were optimized, and a PCR screen of isolates was carried out in duplicate, using a PCR program of initial denaturation at 95°C for 2 min, 25 to 35 cycles of 95°C for 45 s, 52°C to 62°C for 30 s to 1 min (annealing), and 72°C for 45 s, final extension at 72°C for 5 min, and holding at 4°C. PCR amplicons were separated and visualized using a 1% agarose gel. PCR products were purified using the QiAquick PCR purification kit (Qiagen). For amplification of cbiFGH, the amplicon was excised from the gel and gel purified using the PureLink quick gel extraction and PCR purification combo kit (Invitrogen). The amplicons were sequenced as described above. BioEdit version 7.0.5 was used to analyze the sequences. Sequences of PCR amplicons from CSUN-17 were checked by BLASTx using the IMG and the NCBI database, to examine similarity to vitamin B12-associated genes from the genomes of A. glycaniphila strain ERS 1290231 and Desulfovibrio vulgaris strain Hildenborough (Table S1).

(v) Quantification of SCFAs via HPLC. To quantify production of SCFAs with and without vitamin supplementation, A. muciniphila Muc⁴ (Aml) and CSUN-17 (AmIl) were grown in anaerobic mucin medium supplemented with 1 mM L-threonine, 10 g/liter tryptone (Oxoid), and 1% purified mucin, and at 4°C. PCR amplicons were separated and visualized using a 1% agarose gel. PCR products were purified using the QiAquick PCR purification kit (Qiagen). For amplification of cbiFGH, the amplicon was excised from the gel and gel purified using the PureLink quick gel extraction and PCR purification combo kit (Invitrogen). The amplicons were sequenced as described above. BioEdit version 7.0.5 was used to analyze the sequences. Sequences of PCR amplicons from CSUN-17 were checked by BLASTx using the IMG and the NCBI database, to examine similarity to vitamin B12-associated genes from the genomes of A. glycaniphila strain ERS 1290231 and Desulfovibrio vulgaris strain Hildenborough (Table S1).

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(iv) Quantification of SCFAs via HPLC. To quantify production of SCFAs with and without vitamin supplementation, A. muciniphila Muc⁴ (Aml) and CSUN-17 (AmIl) were grown in anaerobic mucin medium supplemented with 1 mM L-threonine, 10 g/liter tryptone (Oxoid), 1% purified mucin, and vitamin supplementation, depending on treatment conditions. For vitamin supplementation, we first performed the experiment using the ATCC MD-VS supplement at the recommended concentration (10 ml/liter). Because the concentration of vitamin B12 in the formulation is 100-fold less than those reported by Belzer et al. (30), we subsequently performed a second experiment with pure vitamin B12 (Sigma-Aldrich), using a final concentration of 100 ng/ml. For all experiments, overnight cultures were transferred three times in the appropriate medium, with the final transfer being used to inoculate 25 ml of medium at 5% in quadruplicate for each isolate and treatment. The optical density at 600 nm (OD₆₀₀) (determined with an Eppendorf BioPhotometer Plus) was recorded at inoculation and at 12, 16, and 20 h. An additional 1.25 ml of culture was removed at each time point and centrifuged at 15,000 × g for 10 min, and the cell-free supernatant was filtered through a 13-mm, 0.2-µm, Spartan high-performance liquid chromatography (HPLC) syringe filter. Samples were stored at −20°C until HPLC analysis.

HPLC was performed using a Waters Breeze 2 system (Waters Corp., Milford, MA) equipped with a refraactive index detector (model 2414). An Aminex HPX-87H column (Bio-Rad Laboratories) was used to measure the production of SCFAs. Sulfuric acid (5 mM) was used as the mobile phase, at a flow rate of 0.6 ml/min. Peak areas and retention times were compared against known standards. Samples were also compared against a medium-only control, to determine background levels of acetate, propionate, and succinate present in the starting medium before growth. Approximately 3 mM propionate was detected in the culture medium and subtracted from all respective measurements.

(vi) Vitamin B₁₂ bioassays. To confirm vitamin B₁₂ production by Akkermansia phylogroup AmIl, we used two bioassays involving bacterial strains that depend on vitamin B₁₂ for growth. For the first bioassay, the classic approach of Hoff-Jørgensen (32), using Lactobacillus leichmannii ATCC 7830 (formerly Lactobacillus delbrueckii subsp. lactic ATCC 7830), was employed. Briefly, L. leichmannii was cultured overnight in MRS broth (BD Difco) and incubated at 37°C under an atmosphere with 5% CO₂. To prepare L. leichmannii for the assay, 0.5 ml of the overnight culture was removed and centrifuged at 15,000 × g for 3 min, followed by three washes with sterile Milli-Q water. Washed cells were then incubated at 4°C for 45 min before being inoculated at 0.1% (vol/vol) into 10 ml of sterile vitamin B₁₂ assay medium (BD Difco) with standard concentrations (0 to 0.25 mg/ml) of cyanocobalamin (Sigma-Aldrich) or 0.1 µl of cell extracts of A. muciniphila Muc⁴ (Aml) or CSUN-17 (AmIl) (see below). Standard tubes and assay tubes were incubated for 18 to 24 h at 37°C under an atmosphere of 5% CO₂, and growth was measured as the OD₆₀₀. All experiments were conducted in triplicate and repeated at least twice.

To confirm vitamin B₁₂ production by Akkermansia strain CSUN-17, a vitamin B₁₂-dependent E. coli bioassay was performed using E. coli ΔmetE and ΔmetH mutant strains (33). E. coli MetE is a cobalamin-independent homocysteine transmethylase (34), and E. coli MetH is a cobalamin-dependent enzyme.

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methionine synthase (35, 36). The *E. coli ΔmetE* strain requires either methionine or vitamin B₁₂ supplementation for growth. The *E. coli ΔmetE ΔmetH* strain requires methionine supplementation for growth and was used as an additional control to demonstrate that methionine was not present in the *Akkermansia* extracts at levels that would support *E. coli ΔmetE* strain growth. The *E. coli ΔmetE* and *ΔmetE ΔmetH* strains were inoculated from LB agar plates into M9 minimal medium supplemented with methionine (1 mg/ml) and were grown for 24 h, to saturation. *E. coli* cultures were subsequently inoculated at 1% (vol/vol) into fresh M9 minimal medium with methionine (1 mg/ml) and were grown for 24 h. Cell pellets were then washed three times in M9 minimal medium without methionine supplementation before being inoculated to a starting OD₆₀₀ of 0.01 in M9 minimal medium without methionine and being incubated for 24 h at 37°C. *E. coli* growth was determined by measuring the OD₆₀₀. *E. coli* mutant strains were examined for growth under five different conditions, i.e., no vitamin B₁₂ supplementation, vitamin B₁₂ supplementation (0.125 ng/ml; Sigma-Aldrich), 7 µl *A. muciniphila* Muc² extract supplementation, 7 µl *A. muciniphila* strain CSUN-17 extract supplementation, and no bacteria (mucin medium control). *Akkermansia* extracts were prepared for *E. coli* bioassays as described below. Assays were carried out in 2-ml volumes in 15-ml Corning polypropylene tubes. Assays were carried out in biological triplicates, and the experiment was replicated. Negative controls without *E. coli* were included.

Cell extracts were prepared for both *A. muciniphila* Muc² (Aml) and CSUN-17 (Amll) by first growing each strain for 18 to 24 h at 37°C in 50 ml of 1% mucin medium, as described above. Extracts were then obtained from each culture by following the protocol described by Kumudha and Sarada (78), with slight modifications. Briefly, cells were pelleted by centrifugation at 10,000 × g for 10 min, and the supernatant was discarded. Cells were resuspended in 50 ml of Milli-Q water and autoclaved at 121°C for 10 min. Once cooled, cell extracts were centrifuged again (1,000 × g for 10 min) and adjusted to pH 6.0 with HCl, and each supernatant was filtered through a 25-mm, 0.2-µm polyethersulfone (PES) membrane Whatman syringe filter (GE Healthcare). Extracts were prepared fresh for each bioassay.

For both bioassays, all glassware was baked at 250°C for 2 h to remove organic residues, and standard solutions of cyanocobalamin (product no. V2876; Sigma-Aldrich) were prepared using sterile Milli-Q water. After preparation and filter sterilization through 25-mm, 0.2-µm, PES membrane filters, all standards were kept in the dark and stored at 4°C.

Data availability. Genomic sequence data from Guo et al. (14) are available at GenBank under BioProject no. PRJNA331216. Our quality-filtered metagenomic sequence data are available at GenBank under BioProject no. PRJNA525290. Additionally, assembled contigs of >2 kbp from children with an *Akkermansia* bin are available in IMG under GOLD study identification no. Gs0133482. It is important to note that contigs available in IMG include not only *Akkermansia* contigs but all contigs from each child. Data Set S4 has a list of all *Akkermansia* contigs in IMG that were included in our analysis. Nearly full-length 16S rRNA gene sequences of our isolates are available in GenBank under accession no. MKS77303 to MKS77312. Corrin gene sequences of isolate CSUN-17 are available in GenBank under accession no. MKS85566 to MKS85569.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.4 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.03 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.1 MB.

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We declare that we have no competing interests.

N.K. conducted wet laboratory work, analyzed and interpreted the data, and wrote the paper. K.G. performed bioinformatics analysis and analyzed and interpreted the data. N.R. conducted wet laboratory work and analyzed and interpreted the data. M.P. analyzed and interpreted the data. G.E.F. conceived of and designed the study, performed bioinformatics analysis, analyzed and interpreted the data, and wrote the paper. All authors read and approved the final manuscript.

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