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ORIGINAL ARTICLE

Muramidases found in the foregut microbiome of the Tammar wallaby can direct cell aggregation and biofilm formation

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We describe here the role of muramidases present in clones of metagenomic DNA that result in cell aggregation and biofilm formation by *Escherichia coli*. The metagenomic clones were obtained from uncultured Lachnospiraceae-affiliated bacteria resident in the foregut microbiome of the Tammar wallaby. One of these fosmid clones (p49C2) was chosen for more detailed studies and a variety of genetic methods were used to delimit the region responsible for the phenotype to an open reading frame of 1425 bp. Comparative sequence analysis with other fosmid clones giving rise to the same phenotype revealed the presence of muramidase homologues with the same modular composition. Phylogenetic analysis of the fosmid sequence data assigned these fosmid inserts to recently identified, but uncultured, phylogroups of Lachnospiraceae believed to be numerically dominant in the foregut microbiome of the Tammar wallaby. The muramidase is a modular protein containing putative *N*-acetylmuramoyl-L-alanine amidase and an endo-β-*N*-acetylglucosaminidase catalytic module, with a similar organization and functional properties to some Staphylococcal autolysins that also confer adhesive properties and biofilm formation. We also show here that the cloned muramidases result in the production of extracellular DNA, which appears to be the key for biofilm formation and autoaggregation. Collectively, these findings suggest that biofilm formation and cell aggregation in gut microbiomes might occur via the concerted action of carbohydrate-active enzymes and the production of extracellular DNA to serve as a biofilm scaffold.
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Introduction

The microbial populations resident in the gastrointestinal (GI) tract of herbivores support the hydrolysis and fermentation of the major structural components of plant biomass, and their end products provide the host animal with a source of energy- and protein-yielding nutrients. Extensive research in rumen microbiology has shown that discrete microbial populations are distributed between the liquid and solid phases of the habitat, which include the biofilms adherent to plant digesta particles and host mucosal surfaces (Akin, 1976; Anderson, 2003). Adhesion mechanisms and biofilm formation on surfaces by commensal gut bacteria are crucial for many ecological functions, including plant biomass degradation, protection against environmental fluctuations and/or pathogens, and for establishing host–microbe interactions (Costerton *et al.*, 1995). Various mechanisms of cell adhesion to plant and epithelial surfaces have now been elucidated, which include surface-associated carbohydrate-binding modules, glycocalyx containing extracellular polymeric substances and organelle-type structures (for example, Pegden *et al.*, 1998; Morrison and Miron, 2000, Miron *et al.*, 2001; Mosoni and Gaillard-Martinie, 2001; Rakotoarivonina *et al.*, 2005; Weimer *et al.*, 2006). In other microbial environments, the extracellular polymeric substance matrix, which can constitute up to 90% of the biofilm biomass, is a complex mixture of exopolysaccharides, proteins and other macromolecules (Sutherland, 2001). More recently, it has been shown that extracellular DNA (eDNA) is a major component of the extracellular polymeric substance often produced by microorganisms considered to be opportunistic pathogens (Flemming and Wingender, 2001; Spoering and Gilmore, 2006; Qin *et al.*, 2007). The detection of significant levels

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of eDNA in soil (Niemeyer and Gessler, 2002) and marine environments (Dell'Anno *et al.*, 2002; Dell'Anno and Danovaro, 2005), combined with the recent identification of eDNA in the biofilms isolated from these environments (Suzuki *et al.*, 2009; Wu and Xi, 2009), suggests that the phenomenon of eDNA as a scaffold for biofilm formation may be more ubiquitous in nature than was previously realized. However, the genetics and molecular biology underpinning cell adhesion and biofilm formation in the GI tract, including the possibility of production of an eDNA-dominated extracellular polymeric substance matrix, have not been examined in detail.

Recent advances in (meta)genomic technologies and the desire to better utlilize lignocellulosic biomass as a feedstock for the production of second-generation biofuels has led to resurgent interest in herbivore gut microbiome structure–function relationships (Morrison *et al.*, 2009). In that context, the Australian macropods (kangaroos and wallabies) have attracted attention because of their evolution in geographic isolation of other extant eutherian herbivores, and their adaptations to herbivory include a tubiform foregut that retains a microbiome coordinating efficient plant biomass degradation. Despite the crucial role of the foregut microbiome with respect to plant polysaccharide degradation, and their influence on intestinal health and host animal well-being, there has been scant functional and comparative analysis of the macropod foregut microbiome. To that end, recent studies using metagenomics approaches have revealed the microbiome of Australia's 'model' marsupial, the Tammar wallaby (*Macropus eugenii*), which possesses bacterial lineages and glycoside hydrolase profiles that differ from other herbivores (Pope *et al.*, 2010). In this study, we report the observation that fosmid clones of metagenomic DNA extracted from the Tammar wallaby foregut promote cell aggregation and biofilm formation in *Escherichia coli*. Further genetic and functional investigations showed that the fosmids giving rise to this phenotype all bear a gene encoding a modular carbohydrate-active enzyme (muramidase), and that the muramidase directs cell aggregation and biofilm formation via eDNA. These findings provide new evidence as to how biofilms of commensal bacteria may develop within gut microbiomes.

Materials and methods

Bacterial strains, plasmids and media

The strains, plasmids and primers used in this study are listed in Table 1. Cells were grown at 37 °C on solid or liquid Luria Bertani (LB) medium supplemented when required with the appropriate antibiotics. Media were routinely solidified with 1% (w v^{-1}) agar, unless otherwise specified. The antibiotics used for bacterial selection included ampicillin (200 μg ml^{-1}), chloramphenicol (12.5 μg ml^{-1}) and kanamycin (50 μg ml^{-1}).

Table 1 Bacterial strains, plasmids and primers used in this study

Strains	*Description*	*Source or reference*
EPI300-T1^{R}	TransforMax electrocompetent *Escherichia coli*	Epicentre
S1918	Deleted for genes encoding type 1 fimbriae, does not produce curli	Brown (1992)
EPI300 (p49C2)	EPI300-T1^{R} with plasmid p49C2	This study
EPI300 (pC1)	EPI300-T1^{R} with plasmid pC1	This study
S1918 (pC2.a)	S1918 with plasmid pC2.a	This study
S1918 (pC2.b)	S1918 with plasmid pC2.b	This study
S1918 (pC2.c)	S1918 with plasmid pC2.c	This study
S1918 (p9-45)	S1918 with plasmid p9-45	This study
S1918 (p9-5T)	S1918 with plasmid p9-5^{T}	This study
S1918 (pUC19)	S1918 with plasmid pUC19	This study
Plasmid	*Description*	*Source or reference*
p49C2	pCC1FOS with a metagenomic fragment containing muramidase	This study
pC1	pCC1FOS vector control: no insert	This study
pC2.a	pUC19 with a random fragment generated from p49C2	This study
pC2.b	pUC19 with a random fragment generated from p49C2	This study
pC2.c	pUC19 with a random fragment generated from p49C2	This study
p9-45	pUC19 with *muramidase* gene constructed with primers 4F+5R	This study
p9-5^{T}	pUC19 with truncated *muramidase* gene constructed with primers 5F^{T}+5R	This study
pUC19	Control vector; no insert	This study
Primers	*Restriction*	*Sequence (5′–3′)*
4F	*Sma*I	TCTCATCACGAGGTGCTTTTT
5R	*Sma*I	TCACACATGGTTACGCTTTG
5F^{T}	*Sma*I	GCCGTTACTATTGAATGTGC

Wallaby sampling
Foregut contents were collected from a captive colony of Tammar wallabies (*Macropus eugenii*), maintained at CSIRO Sustainable Ecosystems (Canberra, Australia). In total, three female Tammar wallabies aged between 1.5 and 4 years were euthanized with an overdose of pentobarbitone sodium (CSIRO Sustainable Ecosystems Animal Ethics Approval Number 06-20) in May 2007. Foregut contents were transferred onto sterile containers and immediately frozen at −20 °C.

Cell dissociation and DNA extraction
For DNA analysis, microbial cells were initially passed through a dissociation step before DNA isolation, designed to remove any microbial cells adherent to digested material and thus capture as much of the microbial diversity as possible. Briefly, 5–10 g of biomass sample was centrifuged at 12 000 *g* for 2 min to remove supernatant and the particulates were resuspended in a final volume of 15 ml dissociation buffer (0.1% Tween 80, 1% methanol and 1% tertiary butanol (v v^{-1}), pH 2). The particulate material was vortexed for 30 s and then centrifuged at low speed for 20 s to sediment plant material; the supernatant was transferred onto a new cell-collection tube. This process was repeated no less than twice and the microbial biomass was recovered by centrifugation at 12 000 *g* for 5 min. The resulting pellet was resuspended in 1 ml of cell wash buffer (10 mM Tris-HC1 (pH 8.0), 1 M NaCl) and was centrifuged for a final time at low speed for 20 s to sediment any contaminating plant particles. The resulting supernatant was centrifuged at 12 000 *g* for 5 min to recover cells.

Metagenomic DNA extraction was based on enzymatic disruption, followed by purification and isolation of nucleic acids using cetyl trimethylammonium bromide/NaCl and phenol:chloroform:isoamylalcohol extraction. Approximately 200 mg (wet weight) of microbial biomass was resuspended in 700 µl TE buffer and incubated at 75 °C for 10 min to inactivate nucleases. The cell suspensions were then incubated at 37 °C for 1.5 h in the presence of lysozyme (1 mg ml^{-1}) and mutanolysin (20 U), followed by a further 1.5 h with achromopeptidase (1 mg ml^{-1}) added. Both sodium dodecyl sulphate and proteinase K were then added to final concentrations of 1% (w v^{-1}) and 0.20 mg ml^{-1}, respectively, and the mixture was incubated at 45 °C for a further 1.5 h. After this step, NaCl was added to give a final concentration of 0.7 M before 0.2 volumes of NaCl-cetyl trimethylammonium bromide buffer (0.7 M NaCl, 10% (w v^{-1}) cetyl trimethylammonium bromide) was added, and the suspension incubated at 70 °C for 10 min. The resulting lysates were then extracted with equal volumes of chloroform and phenol:chloroform:isoamylalcohol; the DNA was precipitated with 2 volumes of 95% (v v^{-1}) ethanol and washed with 70% (v v^{-1}) ethanol. The DNA pellets were air-dried and resuspended in TE buffer (pH 8.0) to give a final concentration of ~0.5 µg $µl^{-1}$.

Fosmid library construction and activity screening
High-molecular-weight DNA was randomly sheared by repeated pipetting (~100 ×) and end-repaired to give blunt-ended 5′-phosphorylated DNA, following the manufacturer's instructions for the fosmid cloning kit (Epicentre, Madison, WI, USA). The end-repaired DNA was then size fractionated using a sucrose density gradient (20–50% (w v^{-1})) and centrifugation at 25 000 r.p.m. in SW-40 Ti rotor (Beckman, Brea, CA, USA) at 25 °C for 24 h. Aliquots of the gradient fractions were examined by agarose gel electrophoresis and those fractions containing fragments approximately 36–40 kb in size were recovered and blunt-end cloned within the fosmid vector pCC1Fos and introduced into *E. coli* EPI300-TI^R cells as per the manufacturer's instructions (Epicentre).

During the plating and propagation of the *E. coli* strain EPI300-TI^R fosmid library, a small percentage (~0.5%) of the colonies were found to adhere very tightly to the LB^{+cm} agar plates. When the clone library was replated on large agar plates in a 384-well-like format for functional screens and incubated at 37 °C for 36–48 h, the same colonies remained firmly adherent to the agar plate and resistant to removal by physical scraping. The clones displaying this phenotype were also cultured in LB^{+cm} broth medium and found to aggregate in a 'pellet' at the bottom of the culture tube, which was very resistant to dispersion by vortexing. On the basis of these observations, fosmid DNA was extracted from each of these strains and used to re-transform *E. coli* strain EPI300-TI^R to confirm the phenotype. On the basis of the re-transformation experiments and confirmation of the phenotype, fosmid p49C2 was selected for more detailed analysis.

Fosmid p49C2 mutant library construction and DNA insert sequencing
Fosmid p49C2 was subjected to random transposon mutagenesis with the EZ-Tn5 <*ori*V/KAN-2> Insertion Kit as per the manufacturer's instructions (Epicentre). Approximately 1 µg of DNA was used to transform strain EPI300 by electroporation, and transposon mutant clones were readily identified because the host strain lost the adhesion phenotype upon plating onto $LB^{+cm,kan}$ agar and incubation at 37 °C for 36–48 h. The transposon insertions giving rise to a loss of the adhesion phenotype were mapped to a region of ~10 kb within p49C2 (Figure 2).

Fosmid p49C2 DNA was then fragmented to 4–9 kb fragments by sonication, end-repaired with T4 DNA polymerase and ligated into pUC19 (Fermentas, Burlington, Ontario, Canada), and the library was used to transform *E. coli* strain S1918 to

Amp^R by electroporation. Plasmid pC2.a, which contained a 7-kb fragment of metagenomic DNA and conferred adhesion to *E. coli* strain S1918, was recovered and used for the initial aggregation and biofilm assays described below. Once the DNA sequence data for p49C2 were obtained, two additional plasmids (pC2.b and pC2.c; see Figure 2) were constructed using routine subcloning methods, and also confirmed to confer adhesion to *E. coli* S1918.

The transposon insertion mapping, DNA sequence and subclone data produced with p49C2 were all used to further delimit the region conferring the adhesion and aggregation phenotype. The Phusion High Fidelity polymerase (Finnzymes, Espoo, Finland) and primers listed in Table 1 were used to amplify a 2-kb region within p49C2 and the fragment was cloned to construct plasmid p9-45 (Figure 2). Truncated clones were also constructed covering the same genetic region (for example, p9-5^T; Table 1); these clones did not produce the adhesion and aggregation phenotype. After confirmation that *E. coli* S1918 p9-45 possessed the adhesion/aggregation phenotype, *E. coli* S1918 bearing pC2.a, p9-45 or p9-5T were used to produce more quantitative measures of biofilm formation and autoaggregation, and to elucidate the possible role of eDNA in biofilm formation, respectively (described below).

DNA sequencing and assembly of p49C2 and other adhesive fosmid clones

The metagenomic DNA insert within p49C2 was sequenced bidirectionally using DNA prepared from p49C2-transposon-bearing clones (including the eight insertional mutant clones) and using specific primer binding sites (Epicentre), BigDye terminators v3.1 and ABI PRISM 3730 sequencer. A total of 177 sequence reads were manually edited and assembled using Contig Assembly Program (Huang and Madan, 1999). The resulting assembly produced 13 contigs, with only three reads remaining as singlets, and the largest contig being 15.9 kb long. This draft assembly was used in combination with additional sequence data produced for p49C2 by 454 pyrosequencing (as described below).

In addition to p49C2, another four fosmids that conferred the strong adhesion and aggregation phenotype to *E. coli* EPI300-TI^R cells were subjected to 454 pyrosequencing. The individual fosmids were induced to increase their copy number following Epicentre protocols, and the fosmid DNA purified using Qiagen MiniPrep columns. Equimol amounts of the fosmids were pooled together ($\sim$20 μg total DNA) and both a 3-kb paired-end library and a 454 standard shotgun library were constructed. Both libraries were sequenced (by the US Department of Energy's Joint Genome Institute, Walnut Creek, CA, USA) with the 454 Life Sciences Genome Sequencer GS FLX and assembled using Newbler. From these five fosmids, a total of 165 351 bp was recovered and five individual scaffolds could be constructed from 13 contigs linked via paired ends.

Phylogenetic analysis of metagenomic DNA and gene predictions

The assembled fosmid sequences were first phylogenetically binned (classified) using Phylopythia (McHardy *et al*., 2007). Generic models for the ranks of domain, phylum and class were combined with sample-specific models as described in Pope *et al*. (2010). Putative genes and open reading frames were called with a combination of MetaGene (Noguchi *et al*., 2006) and BLASTx. All called genes were annotated via the IMG/M-ER annotation pipeline and loaded as independent data sets into IMG/M-ER (Markowitz *et al*., 2008) (http://img.jgi.doe.gov/cgi-bin/m/main.cgi), a data-management and analysis platform for genomic and metagenomic data based on IMG (Markowitz *et al*., 2006). In-depth manual analysis of the peptidoglycan-binding genes was performed using HMMER hmmsearch with pfam_Is hidden Markov models (full-length models) to identify complete matches to their respective families. All hits with *E*-values less than 10^{-4} were counted. For phylogenetic analysis of the identified muramidase-encoding genes, multiple protein sequence alignments were produced using ClustalW and then examined with the protein maximum-likelihood program by applying the Jones–Taylor–Thornton probability model of change between amino acids.

Suspension autoaggregation assay

Liquid LB cultures of *E. coli* S1918 harbouring plasmids pUC19 (control), pC2.a or p9-45 were prepared by overnight incubation at 37 °C in the presence of 0.1 mM isopropyl β-D-1-thiogalactopyranoside. Cultures were adjusted to an optical density of 1.0 at 600 nm (OD_{600}), mixed well at the start of the assay (0 min) and left to stand at room temperature. Bacterial settling was monitored over time by measuring the OD_{600} of 100 μl samples ($n = 3$) collected from the upper part of the culture ($\sim$0.5 cm below the air–liquid interface). Data are shown as mean % of initial absorbance ± s.e.m. The degree of autoaggregation is inversely proportional to turbidity.

Biofilm assay

Biofilm formation on polyvinyl chloride surfaces was measured using 96-well microtitre BD falcon plates as described previously (Valle *et al*., 2008). Briefly, *E. coli* S1918 cells harbouring plasmids pUC19 (control), pC2.a or p9-45 were cultured in LB^{+amp} broth (supplemented when required with 0.1 mM isopropyl β-D-1-thiogalactopyranoside and/or 2 mg ml^{-1} DNase I) for 24 h at 37 °C, washed to remove unbound cells and stained with 0.1% crystal violet. Quantification of adhesive cells was

performed by the addition of acetone–ethanol (20:80 (vol vol^{-1})) and measurement of the dissolved crystal violet at an absorbance of 595 nm. Data are shown as mean absorbance at 595 nm (A_{595}) ± s.e.m. of minimum six replicate wells. All experiments were performed in triplicate.

Accession numbers

The whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under accession number ADGC00000000. The *muramidase* gene sequence recovered from p49C2 is deposited under the accession number GU479396.

Results and Discussion

Identification and behaviour of E. coli *strains bearing fosmids and subclones encoding the adhesion and aggregation phenotype*

Approximately 0.5% of the metagenomic clones resulted in the *E. coli* host strain adhering tenaciously to the agar plate surface (see Supplementary Movie 1) and the formation of a tightly adherent 'pellet' within LB broth cultures (Figure 1).

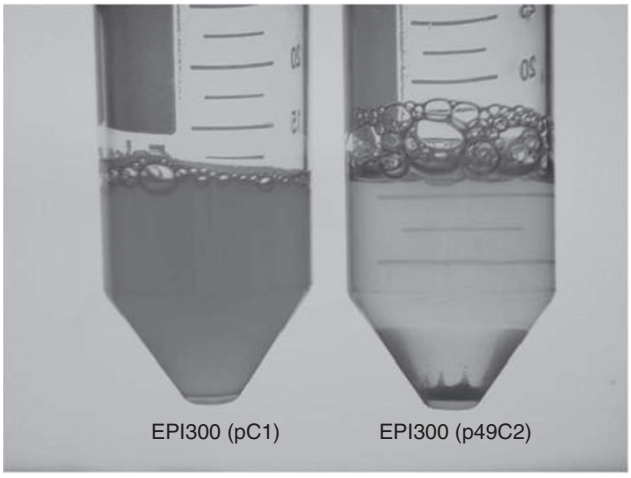

Figure 1 Cell–cell aggregation characteristics and settling from static liquid suspensions of *E. coli* EPI300 (p49C2) (right column) and EPI300 (pC1) (vector control).

To further delimit the gene(s) responsible for the observed phenotypes, random transposon insertion mutagenesis, subcloning and DNA sequencing were performed using metagenomic clone p49C2, and a compilation of these results is illustrated in Figure 2. Although EZ-Tn5 transposon insertion sites were mapped throughout the entire length of the metagenomic DNA insert in p49C2, those insertions disrupting the adhesion phenotype were confined to a relatively small region located within the middle third of the insert. Subclones of p49C2 DNA were constructed in pUC19 and the three subclones retaining the adhesion phenotype (pC2.a–c) were all mapped to span the same region identified by EZ-Tn5 insertion mutagenesis. Furthermore, the overlapping regions subcloned in pC2.a–c indicated that an ~2-kb region shared by these subclones was necessary and sufficient to confer the adhesion phenotype to *E. coli*. This hypothesis was confirmed by the construction of plasmid p9-45, which contains a 2-kb PCR amplicon spanning the region shared by plasmids pC2.a–c.

On the basis of these results, more quantitative analyses were performed only with pC2.a (Figure 3) and p9-45 (Figure 5). In liquid suspension assays, *E. coli* S1918 pC2.a cells showed more rapid settling dynamics (Figure 3a) and biofilm formation on polyvinyl chloride (Figure 3b) than *E. coli* S1918 cells containing pUC19 alone. The S1918 pC2.a aggregates were also extremely robust and could not be dispersed by vortexing. It is also notable that the *E. coli* strain used in this study (S1918) is deleted for genes encoding type 1 fimbriae, does not produce curli and has never been shown to express antigen 43 (Brown, 1992; Schembri and Klemm, 1998). Thus, the adhesion and autoaggregation phenotype conferred by the metagenomic DNA involves a process distinct and separate from these well-characterized systems.

The adhesion phenotype is conferred by a gene encoding a muramidase with two catalytic modules and is flanked by phage-related genes

Preliminary annotation of the genes encoded by p49C2 is illustrated in Figures 2 and 4a. The region determined by transposon insertion and subcloning to confer the adhesion and aggregation phenotype

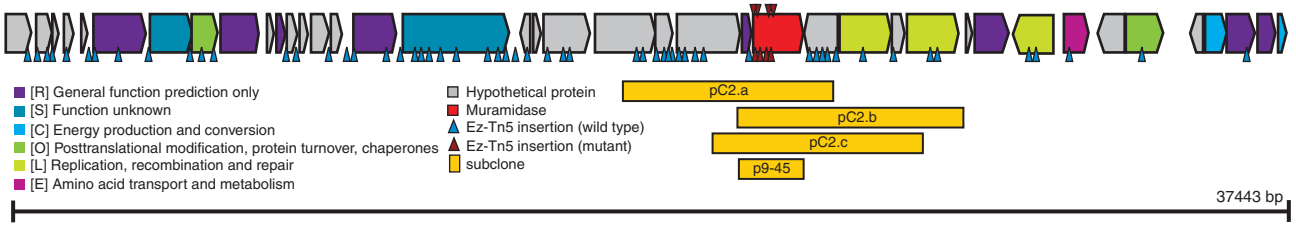

Figure 2 Gene map of adhesive fosmid p49C2 derived from transposon mutagenesis and DNA sequence analysis. Identified open reading frames (ORFs) are shown in arrows, which are colour coded according to function category assigned by top COG (Clusters of Orthologous Groups of proteins) hits. Vertical red arrows denote transposon insertions in p49C2 that result in the loss of the adhesion/autoaggregation phenotype. Vertical blue arrows indicate transposon insertions where the wild-type phenotype remains. Yellow rectangles outline the location of adhesive subclones constructed from p49C2, which were used in aggregation and biofilm assays.

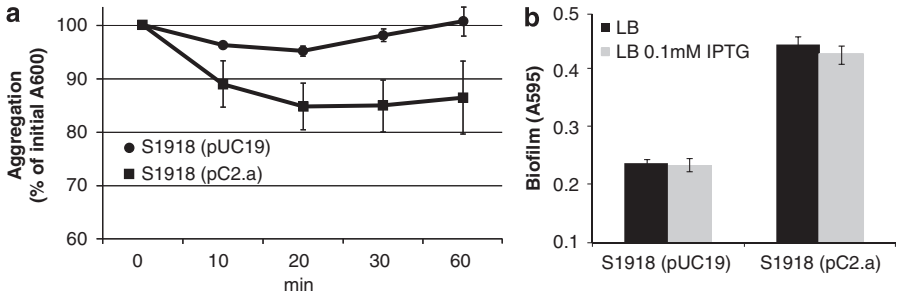

Figure 3 (**a**) Autoaggregation assay shows the settling profiles from liquid suspensions of *E. coli* strains S1918 (pC2.a) and S1918 (pUC19) (vector control). (**b**) Biofilm formation by *E. coli* strains S1918 (pC2.a) and S1918 (pUC19) (vector control). All strains were cultured in LB^{+amp} either in the presence or in the absence of isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 mM) to induce gene expression. Biofilm formation was examined in polyvinyl chloride (PVC) microtitre plates. The data represent the average absorbance at 595 nm (± s.e.m.).

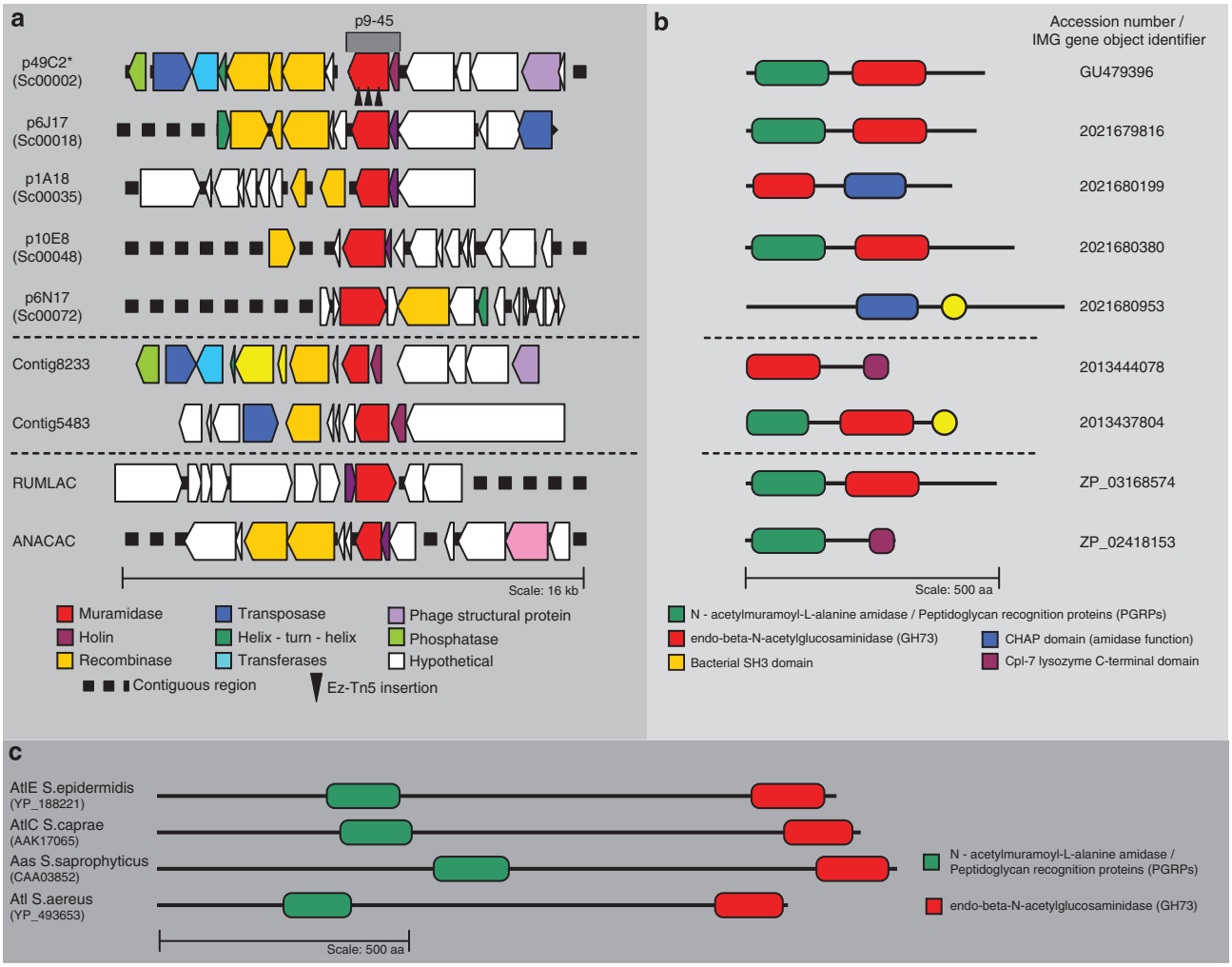

Figure 4 (**a**) Comparison of open reading frame (ORF) maps reconstructed from the five sequenced fosmids shown to cause autoaggregation in *E. coli*, as well as two contigs assembled from the Sanger-generated metagenome data set (Pope *et al*., 2010), and hypothetical proteins from the genomes of *Anaerostipes caccae* DSM 14662 (ANACAC) and *Ruminococcus lactaris* ATCC 29176 (RUMLAC). The putative *muramidase* genes are shown in red. (**b**) Comparisons of the putative muramidases described in (**a**), showing the modular catalytic domains that hydrolyze different moieties of peptidoglycan. (**c**) Pfam models of several autolysins (peptidoglycan-hydrolyzing enzymes) characterized from different *Staphylococcus* spp. previously identified to exhibit adhesive properties. Note the interconnected *N*-acetylmuramoyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase modules, similar to those observed for the putative muramidases responsible for cell aggregation and biofilm formation in host *E. coli* cells.

was shown to contain a single gene encoding a putative muramidase (Figure 2). The muramidases belong to the lysozyme family (EC 3.2.1.17) and hydrolyze the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine within bacterial peptidoglycan. More detailed comparison of the putative muramidase encoded by p49C2 with global hidden Markov models from the Pfam database revealed the presence of an *N*-acetylmuramoyl-L-alanine amidase and an endo-β-*N*-acetylglucosaminidase catalytic module (Figure 4b). The other fosmid clones conferring the adhesion and aggregation phenotype in *E. coli* were also shown to possess homologues of the *muramidase* gene present in p49C2 (Figures 4a and b). Furthermore, the genes flanking the *muramidase* gene in all these fosmid clones encode phage-like functions (Figure 4a). For instance, the gene immediately upstream of the muramidase in all the fosmid clones encodes a putative holin protein, which is predicted to be associated with toxin secretion. Other phage-related genes, including recombinases and transposases, were also identified in these fosmid clones in close proximity to the muramidase-encoding gene. Despite these similarities in the regions flanking the *muramidase* gene, the remainder of the fosmid clones were not found to share a high degree of similarity with respect to gene organization and (or) predicted function (data not shown). For these reasons, it appears that the *muramidase* gene might be associated with a cassette of genes that are phage related, and subject to generalized transduction and recombination events.

An examination of the data set produced by Sanger sequencing of metagenomic DNA from the Tammar wallaby microbiome (Pope *et al.*, 2010) identified 20 additional *muramidase* genes, including several encoded on contigs with a high degree of sequence similarity and gene organization when compared with the sequenced fosmids (Figure 4a). Furthermore, a class of bifunctional autolysins found in a number of Staphylococci and implicated in cell adhesion and biofilm formation have been shown to possess both *N*-acetylmuramoyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase catalytic modules (Hell *et al.*, 1998; Allignet *et al.*, 2001; Heilmann *et al.*, 2007; Qin *et al.*, 2007) (Figure 4c). Our searches of the GenBank databases also showed that a 'hypothetical protein' present in the genomes of the Firmicutes-affiliated human gut bacteria *Anaerostipes caccae* DSM 14662 and *Ruminococcus lactaris* ATCC 29176 possess the same modular architecture (Figure 4b). Although neither of these bacteria have been examined with respect to cell aggregation and (or) biofilm formation, it is interesting to note that both bacteria have been described to form flocculent or 'ropy' sediments in broth culture that are suggestive of cell aggregation (Moore *et al.*, 1976; Schwiertz *et al.*, 2002). Taken together, these results suggest that the *muramidase* genes identified in the metagenomic resources produced from the Tammar wallaby foregut microbiome are present in other Gram-positive bacteria,

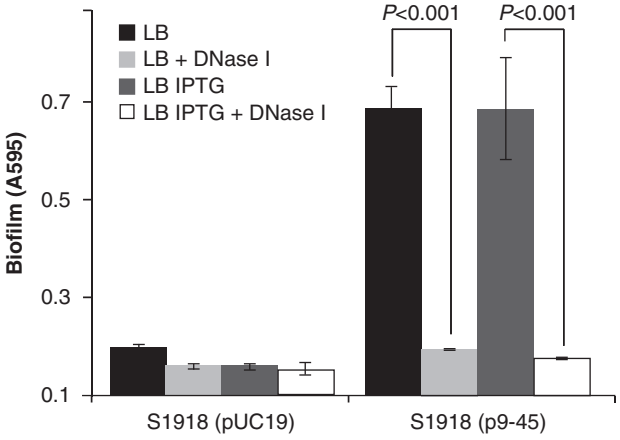

Figure 5 Effect of nuclease activity on biofilm formation by *E. coli* strain S1918 harbouring plasmid p9-45. Biofilm formation was examined in polyvinyl chloride (PVC) 96-well microtitre plates. All strains were cultured in LB^{+amp} in the presence or absence of isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 mM) to induce gene expression and in the presence or absence of DNase I (2 mg ml^{-1}). The data represent the average absorbance at 595 nm ± s.e.m.

all of which have been shown or described to form cell aggregates and (or) biofilms.

The muramidase *gene from p49C2 promotes cell aggregation and biofilm formation in* E. coli *through the production of eDNA*

Recent studies of the AtlE autolysin of *Staphylococcus epidermidis* have suggested that the protein supports biofilm formation by the lysis of a subpopulation of the bacterium that releases eDNA, which then functions as a scaffold for cell aggregation and biofilm formation (Qin *et al.*, 2007). To investigate whether the *muramidase* gene from p49C2 promoted biofilm formation by the release of eDNA, we examined the ability of *E. coli* S1918 p9-45 to form biofilms in the presence or absence of DNase I (Figure 5). The recombinant bacterium readily produced a biofilm when cultured using LB^{+amp} broth. The presence of DNase I did not affect the growth of the recombinant bacterium (data not shown), but its ability to form a biofilm was abolished in the presence of 2 mg ml^{-1} DNase I. Similarly, DNAse I treatment of the *E. coli* control (vector alone) did not promote (or reduce) biofilm formation in any manner (Figure 5). On the basis of these findings, we conclude that the recombinant muramidase does produce eDNA, which then serves as the primary scaffold for cell aggregation and biofilm formation. Furthermore, the recombinant *muramidase* gene appears to be necessary, and sufficient, in conferring the adhesion and autoaggregation phenotype in *E. coli.*

The muramidase *genes originate from the firmicutes and deep branching, uncultured lineages of the Lachnospiraceae*

Phylogenetic analysis of the individual *muramidase* genes retrieved from the Tammar wallaby metagenomic

resources originate from bacteria affiliated with the Firmicutes (Figure 6). As expected, these sequences closely grouped with *muramidase* genes from commensal gut bacteria isolated from other gut environments. Further phylogenetic assignments, using the nucleotide composition-based classifier Phylopythia (McHardy *et al*., 2007), confirmed that four of the five assembled fosmids, as well as four other *muramidase* genes recovered from the Sanger-generated metagenome data set, originate from novel lineages of uncultured bacteria affiliated with the Lachnospiraceae. These novel lineages of Lachnospiraceae were previously shown to be numerically predominant in the Tammar wallaby foregut microbiome by *rrs* and gene centric metagenomic analyses (Pope *et al*., 2010). The Lachnospiraceae family also includes *Clostridium* groups IV and XIVa, which are found (often in high abundance) throughout the GI tract of various herbivores as well as intestinal and oral surfaces in humans (Bryant, 1986a, b; Downes

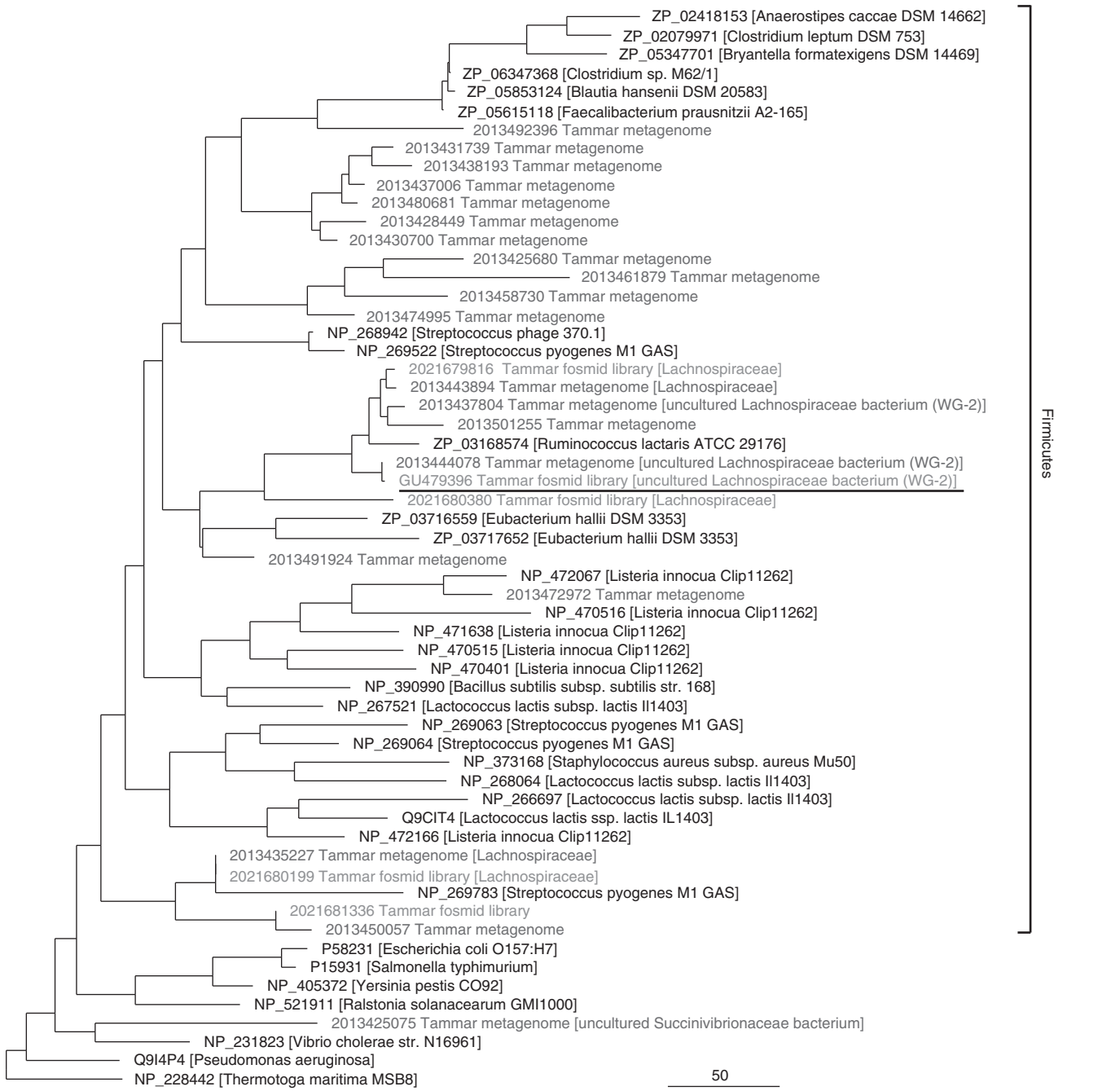

Figure 6 Phylogenetic analysis of the muramidase diversity encoded by the Tammar wallaby foregut microbiome. Muramidase sequences from the Tammar foregut metagenome are coloured in red, muramidase sequences from the sequenced fosmid clones are coloured in blue and various other sources in black. Phylopythia assignments for muramidase sequences from the Tammar foregut are given in parentheses. Metagenomic sequences and additional public sequences are identified by their JGI gene object identifier or GenBank GI number, respectively.

et al., 2002; Hold *et al*., 2002; Eckburg *et al*., 2005; Zhongtang *et al*., 2005; Frank *et al*., 2007). Furthermore, culture-independent analysis identified Lachnospiraceae-affiliated bacteria to be present in the biofilms adherent to plant biomass recovered from ruminants (Zhongtang *et al*., 2005) and the oral cavity in humans (Chhour *et al*., 2005).

In conclusion, biofilm formation by commensal bacteria in the GI tracts of animals and humans has long been recognized, but remained largely unexplored at a molecular and genetic level, despite the critical role these biofilms have in plant biomass degradation and host–microbe cross-talk. The studies presented here provide some of the first genetic and functional evidence as to how these biofilms might form and develop in the herbivore GI tract,by the lysis of bacterial peptidoglycan and release of eDNA. The role of eDNA in biofilm formation is widely recognized by pathogenic microbiologists, having been shown with species of *Pseudomonas* (Whitchurch *et al*., 2002; Nemoto *et al*., 2003; Allesen-Holm *et al*., 2006), *Streptococcus* (Petersen *et al*., 2004, 2005), *Staphylococcus* (Qin *et al*., 2007) and *Listeria* (Harmsen *et al*., 2010), several of which are directly related to the activities of muramidase-like genes (Qin *et al*., 2007; Lappann *et al*., 2010). Moreover, recent reports have linked eDNA to biofilm formation in bacteria isolated from both soil and marine environments (Suzuki *et al*., 2009; Wu and Xi, 2009), which suggests that this mechanism of biofilm formation is perhaps more common than previously thought. The detection of muramidases with similar modular and functional properties from a diverse range of Firmicutes considered to be gut commensal bacteria—including uncultured, novel lineages affiliated with the Lachnospiraceae—further supports this hypothesis.

Finally, the presence of numerous phage-related genes in proximity to the *muramidase* gene is indicative of generalized transduction and recombination events within the gut environment. However, phylogenetic analysis of the Tammar foregut *muramidase* genes showed affiliation to a diverse range of Lachnospiraceae gut bacteria that is suggestive of ancestral vertically transmitted genes—therefore making it difficult to distinguish if/when horizontal gene transfer events occurred. Although questions still remain with regard to the evolutionary and ecological role of eDNA and biofilm formation in gut environments, this study, undoubtedly, provides a new foundation for the examination of this phenomenon in gut microbial communities.

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