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SHORT COMMUNICATION

Proteome insights into the symbiotic relationship between a captive colony of *Nasutitermes corniger* and its hindgut microbiome

Kristin E Burnum[1,4], Stephen J Callister[1,4], Carrie D Nicora[1], Samuel O Purvine[1], Philip Hugenholtz[2], Falk Warnecke[2], Rudolf H Scheffrahn[3], Richard D Smith[1] and Mary S Lipton[1]

[1]*Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, USA;* [2]*Microbial Ecology Program, DOE Joint Genome Institute, Walnut Creek, CA, USA and* [3]*Lauderdale Research and Education Center, University of Florida, Davie, FL, USA*

We analyzed the metaproteome of the bacterial community resident in the hindgut paunch of the wood-feeding 'higher' termite (*Nasutitermes*) and identified 886 proteins, 197 of which have known enzymatic function. Using these enzymes, we reconstructed complete metabolic pathways revealing carbohydrate transport and metabolism, nitrogen fixation and assimilation, energy production, amino-acid synthesis and significant pyruvate ferredoxin/flavodoxin oxidoreductase protein redundancy. Our results suggest that the activity associated with these enzymes may have more of a role in the symbiotic relationship between the hindgut microbial community and its termite host than activities related to cellulose degradation.

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Within the family Termitidae (higher termites), the P3 segment (hindgut paunch) microbiome is capable of fixing nitrogen and potentially degrading lignocellulose (Breznak and Brune, 1994). In contrast to the families of 'lower termites' (for example, Kalotermitidae, Rhinotermitidae), where flagellated protists and the termite are sources of cellulases (Cleveland, 1923; Watanabe *et al.*, 2002; Tokuda *et al.*, 2004), the contribution and sources of hydrolytic enzymes in the higher termite are not well defined. For example, a recent metagenomic study of the *Nasutitermes* P3 microbiome (Warnecke *et al.*, 2007) identified domains representative of several families of carbohydrate active enzymes (CAZy families) (Cantarel *et al.*, 2009) and then used liquid chromatography-tandem mass spectrometry-based proteomics to look for evidence of CAZy expression.

We report results from a liquid chromatography-tandem mass spectrometry-based global proteome characterization (Supplementary Materials and Methods) of the *Nasutitermes* (laboratory maintained colony collected in Dania Beach, FL) P3 microbiome, in which we searched for evidence supporting CAZy family expression. Metaproteomic and metagenomic data sets were obtained from different termite nests. We observed 886 proteins (Supplementary Information) identified using $\geqslant 2$ unique peptide sequences as a requirement for confident protein identification. The confidently identified proteins represent 1.2% of the predicted proteome (based on the metagenomic sequence); however, these proteins make up 235 protein families (Pfams) or 11.5% of the 2050 Pfams predicted by the metagenome. The limited coverage may reflect the small volume of P3 hindgut fluid available for analysis (100 μl equates to ~100 P3 hindguts), but suggests that the identified proteins are the most abundant and/or easily detected using liquid chromatography-tandem mass spectrometry. Among the 886 proteins, 70 are unannotated and 58 are characterized as hypothetical, predicted or putative in the annotation. The most represented proteins are flagellin-related hook-associated proteins and methyl-accepting chemotaxis proteins that contain 50 and 43 copies (redundancy), respectively. Chemotaxis is hypothesized to have an important role in compartmentalizing bacteria to specific regions of the termite gut according to the functional roles of the bacteria (Warnecke *et al.*, 2007).

Of the 886 proteins identified in this study, 197 had Enzyme Commission (EC) numbers, which allowed us to construct enzymatic pathways

Correspondence: MS Lipton, Biological Separations and Mass Spectrometry, Pacific Northwest National Laboratory, P.O. Box 999, K8-98, Richland, WA 99352, USA.
E-mail: mary.lipton@pnl.gov
[4]These authors contributed equally to this work.
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and gain insight into the molecular mechanisms defining the symbiotic relationship between the P3 hindgut microbiome and its termite host. Figure 1 shows expressed genes for transport, glucan binding, sugar fermentation, hydrogen metabolism, amino-acid synthesis, and nitrogen fixation and assimilation pathways. The transportation and fermentation pathways are of particular interest with regard to possible energy substrates available for the microbiome. For example, xylose (wood sugar) is transported into the cytoplasm by ATPases (EC3.6.3.17), and glucose, monosaccharide and disaccharide substrates are transported into the bacterial cells through the phosphoenolpyruvate-dependent sugar phosphotransferase system (EC2.7.1.69). Phosphotransferase catalyzes phosphorylation of these incoming sugar substrates simultaneously with their translocation across the cell membrane, after which phosphorylated glucose (glucose-6P) can be metabolized and phosphorylated disaccharide can be broken down by β-glucan hydrolases (EC2.4.1.-). The collective actions of many bacterial enzymes transport and metabolize reducing sugars to alcohol, energy (ATP) and acetate (Supplementary Table S1). Bacterial acetate is a dominant end product for energy and biosynthesis in the termite; however, molecular hydrogen (H_2) is an alternative energy source and is abundantly produced during both lignocellulose fermentation and N_2 fixation. H_2 is metabolized by iron-only hydrogenase enzymes (EC1.6.5.3) (Vignais and Colbeau, 2004).

Importantly, the sugar fermentation and nitrogen fixation pathways are coupled by the activity of pyruvate ferredoxin/flavodoxin oxidoreductase (EC1.2.7.-) through oxidative decarboxylation of pyruvate to acetyl-CoA under anaerobic conditions (Figure 1). The electrons generated from this reaction are transferred to ferredoxin (or some other electron carrier) and are used by nitrogenase in the reduction of N_2. Pyruvate ferredoxin/flavodoxin oxidoreductase was observed with the highest degree of redundancy among the 197 enzymes, that is, 24 observed copies (Supplementary Table S2). Within this group, 4 copies (out of a possible 6) are

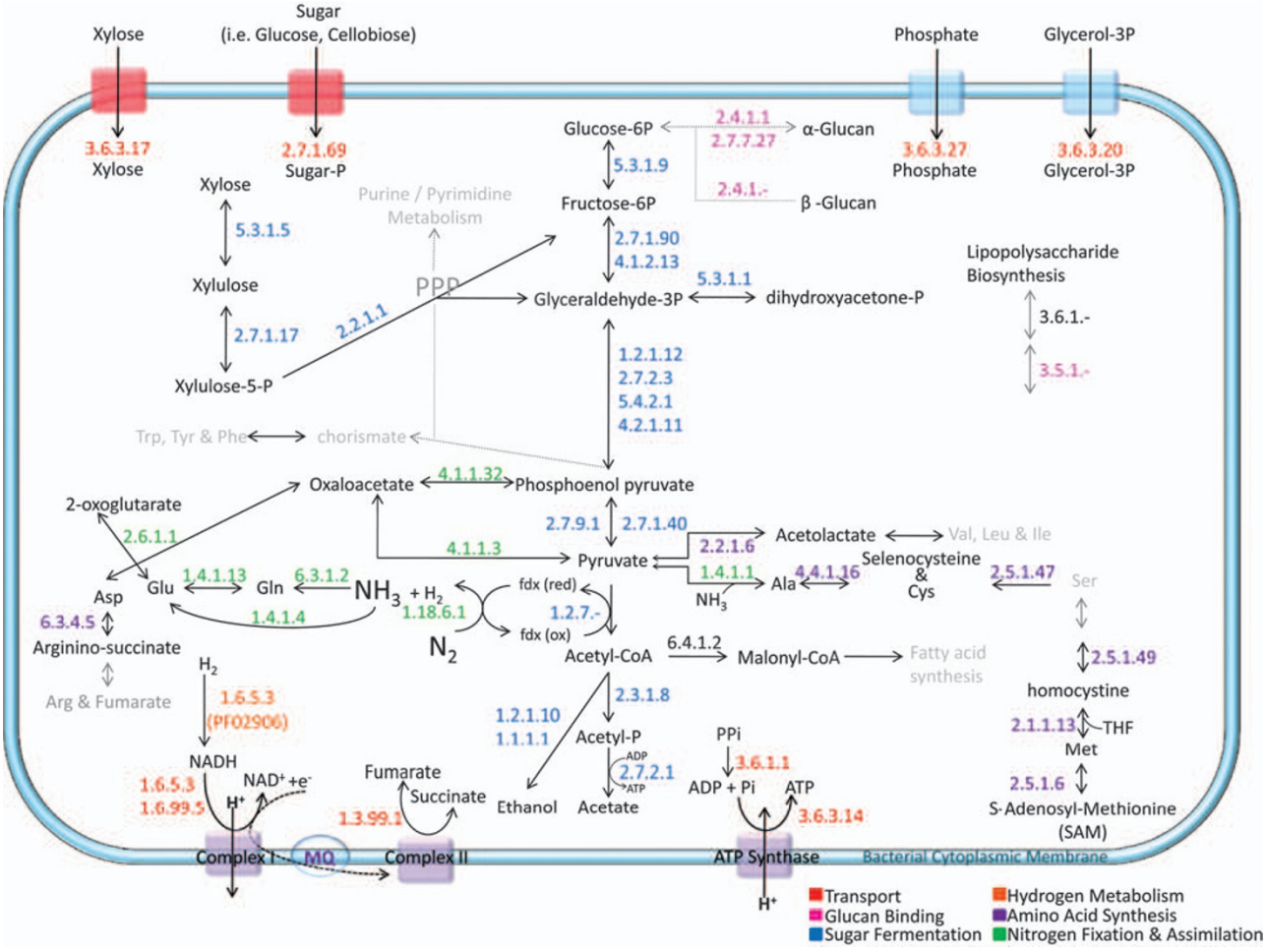

Figure 1 Molecular snapshot of the metabolic pathways within the termite gut microbial community. The enzymatic pathways of multiple enzymes are illustrated. Black arrows represent complete pathways; every enzyme was identified. Gray arrows and gray text represent incomplete pathways. The legend is color coded to the EC numbers. Ala, alanine; Arg, arginine; Asp, aspartate; Cys, cysteine; Gln, glutamine; Glu, glutamate; Ile, isoleucine; Leu, leucine; Met, methionine; Phe, phenylalanine; PPi, pyrophosphate; PPP, pentose phosphate pathway; Ser, serine; THF, tetrahydrofolate; Trp, tryptophan; Tyr, tyrosine; Val, valine.

Table 1 Glycoside hydrolases and carbohydrate-binding modules

CAZy family	*Pfam HMM name*	*Pfam accession*[b]	*Previously reported proteomics data*[a]	*Our analysis*	
			⩾1 peptides	*1 peptide*	*⩾2 peptides*
GH1	Glyo_hydro_1	Pfam00232	1	1	
GH3	Glyco_hydro_3	Pfam00933	1	3	
GH4	Glyco_hydro_4	Pfam02056	2	1	
GH5	Cellulase	Pfam00150	2	1	
GH8	Glyco_hydro_8	Pfam01270		1	
GH9	Glyco_hydro_9	Pfam00759		1	
GH10	Glyco_hydro_10	Pfam00331	1	1	
GH13	Alpha-amylase	Pfam00128	2	5	
GH23	SLT	Pfam01464		2	
GH42	Glyco_hydro_42	Pfam02449	1		
GH43	Glyco_hydro_43	Pfam04616		5	
GH57	Glyco_hydro_57	Pfam03065		2	
GH77	Glyco_hydro_77	Pfam02446		4	3
GH88	Glyco_hydro_88	Pfam07470	1	1	
GH94	Glyco_transf_36	Pfam06165	8	8	2
CBM4	CBM_4_9	Pfam02018	1		
CBM6	CBM_6	Pfam03422		1	
CBM11	CBM_11	Pfam03425		2	2
	Big_2	Pfam02368		4	
	Big_3	Pfam07523		1	
	CBM_X	Pfam06204	8	8	2
	Glyco_hydro_3_C	Pfam01915	3	2	1
	GT36_AF	Pfam06205	8	9	2
	CBM_48 (Isoamylase_N)	Pfam02922		1	

[a]Warnecke *et al.*, 2007.
[b]Many proteins contain more than one Pfam domain (Supplementary Tables 3 and 4 corresponding Pfams denoted in red).

annotated as EC1.2.7.- in the metagenome (Supplementary Figure S1, Supplementary Table 1). The role of this enzyme in acetate and nitrogen fixation and its high degree of redundancy suggest this enzyme's catalytic function is critical to the microbiome and the termite. The reported absence of pyruvate dehydrogenase activity (aerobic conversion of pyruvate to acetyl-CoA) in *Nasutitermes walkeri* tissues (Slaytor *et al.*, 1997) and other termites (O'Brien and Breznak, 1984) has led to a long-standing hypothesis that acetate production by the microbiome is critical for producing energy in the termite (reviewed by Breznak and Brune (1994)). More recently, activity of the pyruvate dehydrogenase complex was reported for mitochondria extracted from *N. walkeri* and *Coptotermes formosanus*; however, pyruvate dehydrogenase activity in whole tissue homogenates remained elusive (Itakura *et al.*, 2003).

After global characterization, we looked for supporting evidence of CAZy family expression. To improve the likelihood of observing these Pfams, we prepared samples from several fractionations of the hindgut fluid (Supplementary Materials and Methods and Supplementary Table S3) based on cellulase activity recently reported in similar fractions for *Nasutitermes* species (Tokuda and Watanabe, 2007). We searched our data for proteins having domains previously identified by Warnecke *et al.* (2007), using Pfam hidden Markov models (Krogh *et al.*, 1994) (Supplementary Materials and Methods) and associated with glycoside hydrolysis and carbohydrate binding (Table 1). We identified 48 proteins within 22 CAZy and Pfams, of which 13 were reported for *Nasutitermes* (Warnecke *et al.*, 2007). However, of these 48 proteins, only 8 within 6 families passed the ⩾2 unique peptide filtering criterion. The absence of confidently observed enzymes involved in cellulose degradation maybe because of these enzymes not being readily detected using liquid chromatography-tandem mass spectrometry (for example, secreted CAZy enzymes were insoluble and/or remained firmly bound to lignocellulose), the need for a more representative genome, or that native cellulose degradation may occur elsewhere in the termite. A recent report of an abundance of endo-β-1,4-glucanase in the midgut of *Nasutitermes* suggests that early stages of native cellulose degradation may depend on enzymes secreted by the termite host before complete degradation by the microbiome (Tokuda and Watanabe, 2007). In conclusion, the presented proteomic data shed further light on which genes are expressed to support the symbiotic relationship between the hindgut microbiome and its host.

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