Hypothetical Roles in Plant Host Interaction

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Abstract

Sinorhizobium meliloti strain 1021, a nitrogen-fixing, root-nodulating bacterial microsymbiont of alfalfa, has a 3.5 Mbp circular chromosome and two megaplasmids including 1.3 Mbp pSymA carrying nonessential ‘accessory’ genes for nitrogen fixation (nif), nodulation and host specificity (nod). A related bacterium, psyllid-vectored ‘Ca. Liberibacter asiaticus,’ is an obligate phytopathogen with a reduced genome that was previously analyzed for genes orthologous to genes on the S. meliloti circular chromosome. In general, proteins encoded by pSymA genes are more similar in sequence alignment to those encoded by S. meliloti chromosomal orthologs than to orthologous proteins encoded by genes carried on the ‘Ca. Liberibacter asiaticus’ genome. Only two ‘Ca. Liberibacter asiaticus’ proteins were identified as having orthologous proteins encoded on pSymA but not also encoded on the chromosome of S. meliloti. These two orthologous gene pairs encode a Na+/K+ antiporter (shared with intracellular pathogens of the family Bartonellaceae) and a Co++, Zn++ and Cd++ cation efflux protein that is shared with the phytopathogen Agrobacterium. Another shared protein, a redox-regulated K+ efflux pump may regulate cytoplasmic pH and homeostasis. The pSymA and ‘Ca. Liberibacter asiaticus’ orthologs of the latter protein are more highly similar in amino acid alignment compared with the alignment of the pSymA-encoded protein with its S. meliloti chromosomal homolog. About 182 pSymA encoded proteins have sequence similarity (≤E-10) with ‘Ca. Liberibacter asiaticus’ proteins. These proteins are involved with amino acid uptake, cell surface structure, chaperonins, electron transport, export of bioactive molecules, cellular homeostasis, regulation of gene expression, signal transduction and synthesis of amino acids and metabolic cofactors. The presence of multiple orthologs defies mutational analysis and is consistent with the hypothesis that these proteins may be of particular importance in host/microbe interaction and their duplication likely facilitates their ongoing evolution.

Introduction

Within the order Rhizobiales of the class alpha-Proteobacteria, Sinorhizobium meliloti is a member of a diverse bacterial family, Rhizobiaceae [1–3]. Other genera contained within the Rhizobiaceae and indeed the Rhizobiales type genus [3] include Agrobacterium and Rhizobium [4]. A growing number of species of the family Rhizobiaceae and of the order Rhizobiales are becoming recognized based on 16 S rRNA gene sequence data. Many species interact with plants and other eukaryotes to produce important agricultural, economic and/or environmental consequences. ‘Ca. Liberibacter asiaticus’ is recognized as the causal agent of huanglongbing (HLB), also known as citrus greening disease. This phytopathogen was introduced into the New World from Asia [5,6], and is transmitted by the citrus psyllid, Diaphorina citri [7]. The full genomic sequence of ‘Ca. Liberibacter asiaticus’ was determined by deep sequencing of total DNA obtained from a single citrus psyllid which contained more than 10^8 cells of ‘Ca. Liberibacter asiaticus.’ Phylogenetic analysis indicated that ‘Ca. Liberibacter asiaticus’ and S. meliloti are close bacterial relatives [2]. ‘Ca. Liberibacter asiaticus’ has a single small chromosome of 1.23 Mbp with 36.5% GC content compared to the chromosome of S. meliloti which is 3.65 Mbp and 62.7% GC [2,8]. ‘Ca. Liberibacter asiaticus’ is not available in culture but can colonize intracellularly and move systemically in the phloem vessels of citrus trees [9,10] following introduction by the citrus psyllid, D. citri. ‘Ca. Liberibacter asiaticus’ also systemically and intracellularly colonizes citrus psyllid salivary glands [11]. The genome of ‘Ca. Liberibacter asiaticus’ has many adaptations to facilitate an intracellular lifestyle in both plant and insect cells [2,12].

S. meliloti 1021, free living in the soil, also lives in specialized root nodules in symbiosis with alfalfa, Medicago sativa L., where S. meliloti reduces atmospheric nitrogen and thereby confers plant nitrogen sufficiency. S. meliloti 1021 has a composite genome comprised of the chromosome and two megaplasmids, pSymA and pSymB, of 1.3 Mbp and 1.7 Mbp [8]. Megaplasmids pSymA and pSymB encode 1293 and 1570 protein-coding genes, respectively [8,13]. Predicted gene products of all open-reading frames (ORFs) in the genome of ‘Ca. Liberibacter asiaticus’ were compared by protein BLAST against all of the predicted gene products encoded by chromosomal genes of S. meliloti 1021 [2]. However, predicted
gene products of all ORFs encoded on the plant symbiosis- controlling S. meliloti megaplasmid pSymA had not been previously subjected to protein BLAST compared with the predicted gene products encoded in the ‘Ca. Liberibacter asiaticus’ genome. As an “accessory” genome component [14], pSymA may have originated from an ancestral plasmid and may have maintained individual and some small blocks of genes originally from host chromosomes. It has long been known that the pSymA megaplasmid of S. meliloti, in contrast to the circular chromosome or the other S. meliloti megaplasmid pSymB, specializes in carrying genes essential for establishing and maintaining intimate intracellular plant interactions with alfalfa. The megaplasmid pSymA itself is self-transmissible and upon transfer confers nodulation (nod) nitrogen fixation (nif) and other symbiotic abilities in related bacteria with a similar genetic background or complement of chromosomal genes [15–17]. The chromosome of the tumor-inducing bacterial phytopathogen Agrobacterium tumefaciens encodes characteristics practically identical to those of Rhizobium microsymbions, and members of the species also maintain pTi as an accessory genomic component. When bacterial cells recruit or accept transfer of the Ti plasmid or pSymA, either tumor-inducing pathogenicity or nitrogen-fixing symbiotic ability, respectively, is conferred on the host bacterium. Agrobacterium and Rhizobium were combined into a single genus within the Rhizobiaceae largely for this reason and to reject taxonomy based largely on plant interactions determined by extrachromosomal elements, i.e., to avoid plasmid-based nomenclature [4].

As S. meliloti is a close phylogenetic relative of ‘Ca. Liberibacter asiaticus,’ it is likely that the two bacteria deploy a similar repertoire of mechanisms for avoiding defenses elicited in host plant cells by their invasion, or, in the case of beneficial root nodule bacteria, recruitment or “welcomed entry” [18]. The goal of the present study was to compare predicted gene products encoded by the genome of ‘Ca. Liberibacter asiaticus’ with those encoded by genes carried on the megaplasmid pSymA of S. meliloti strain 1021. The rationale for identifying protein products of ‘Ca. Liberibacter asiaticus’ genes orthologous to those encoded by genes on pSymA is simply that such genes and their protein products may play direct or indirect role(s) in establishing and maintaining an intimate intracellular interaction with eukaryotes. We have identified a number of such interesting protein products.

**Results**

**Maintenance of Homeostasis**

In general, proteins encoded by pSymA genes are more similar in sequence alignment to those encoded by S. meliloti chromosomal orthologs than to orthologous proteins encoded by genes carried on the ‘Ca. Liberibacter asiaticus’ genome. There are two very notable exceptions where orthologs are shared only by pSymA and ‘Ca. Liberibacter asiaticus’ and not with the chromosome of S. meliloti. YP_003064907, the predicted protein product of a ‘Ca. Liberibacter asiaticus’ gene, is orthologous to NP_4345608.1 (E = 5e–71) encoded by pSymA yet lacks an orthologous protein encoded by the chromosome of S. meliloti (Table 1). SMART analysis predicts that YP_003064907, has a cation efflux domain (E = 1.3e–31) from residues 15 to 313 of the 313 amino acid protein. This predicted protein also has BLAST matches with cobalt, zinc and cadmium efflux proteins (COG3965) [19]. pSymA-encoded NP_4345297.2 is orthologous to YP_003064775 (E = 3e–30) encoded on the chromosome of ‘Ca. Liberibacter asiaticus’, but neither has an ortholog encoded by the S. meliloti chromosome (Table 1). SMART analysis of ‘Ca. Liberibacter asiaticus’ YP_003064775, annotated as a nhaA gene, predicts a sodium/ proton antiporter domain (E = 2.1e-133) at residues 4 to 379 of the 381 amino acid protein [20,21]. These two protein pairs are the only two protein pairs uniquely shared by pSymA and ‘Ca. Liberibacter asiaticus’. A third orthologous protein pair comprised of NP_436118.1 (pSymA) and YP_003063337 (‘Ca. Liberibacter asiaticus’) is much more similar in amino acid sequence (E = 1e–134) than is NP_436118.1 and its ortholog encoded by the S. meliloti chromosome, NP_394912 (E = 1e–22). Bioinformatic analyses revealed that YP_003065337 is a potassium efflux protein. Control of intracellular pH is ascribed in its domain architecture. SMART analysis of YP_003065337 confidently predicts two domains as follows: an amino-terminal sodium/proton exchanger domain (E = 2.7e–72) from residues 4 to 394 and a non- overlapping TrkA domain (E = 3e–25) for redox regulation, from amino acids 451–566 of the 609 amino acid protein. ‘Ca. Liberibacter asiaticus’ YP_003065245, a potassium uptake protein, has E = 0 alignments with both pSymA (NP_436237.1) and S. meliloti chromosomal orthologs (Table 1). These proteins likely contribute to the maintenance of intracellular homeostasis through the regulation of pH and the control of mono- and divalent cation concentrations.

**Signal Transduction**

There are several orthologous proteins encoded by genes shared between pSymA and the ‘Ca. Liberibacter asiaticus’ and S. meliloti chromosomes that are predicted to function in sensing and signal transduction. For example, YP_003064826, a PAS/PAC sensor signal transduction kinase has a pSymA ortholog and a S. meliloti chromosomal ortholog. Several two-component response regulators or sensor histidine kinases are encoded both by genes on pSymA and the genome ‘Ca. Liberibacter asiaticus’, and include YP_003065306, which has a characteristic N-terminal CheY-like sensor and Trans_reg_C which are DNA-binding transcriptional activator/effectors domains. YP_003064633, a two component sensor histidine kinase/response regulator hybrid protein, is a FixL-like oxygen-controlled histidine kinase homologous to several pSymA-encoded proteins (E = 3e–49 for the most similar ortholog) (Table 1).

**ABC-cassette Type Amino Acid Transporters**

ATP Binding Cassette type amino acid transporters comprise the largest class of genes shared by ‘Ca. Liberibacter asiaticus’, pSymA and the S. meliloti chromosome (Table 2). For most ABC-type transporter proteins encoded by ‘Ca. Liberibacter asiaticus’, genes there are multiple homologs on pSymA, dispersed over the megaplasmid (Table S1). YP_003064586 is an example of an ABC-type transporter protein from ‘Ca. Liberibacter asiaticus’. In addition to NP_435288.1 there are 10 more pSymA-encoded proteins homologous to YP_003064586 (E = 4e–67 up to 1e–14), and all are annotated as general amino acid transporters with an ATP binding site and other characteristics of an ABC type amino acid transporter. The corresponding genes are dispersed across the pSymA (Fig. 1A; Table 2).

YP_003064753 is a proline/glycine betaine ABC transporter protein with eight pSymA-encoded homologs (Table S1). YP_003064754, another proline/glycine betaine permease/ABC transporter permease protein, has two pSymA-encoded homologs. ‘Ca. Liberibacter asiaticus’ YP_00306517 and YP_003064532, both ATP-binding ABC-type transporter proteins, each have four homologs encoded by genes on pSymA. YP_003065525, a putative amino acid-binding periplasmic protein, is homologous to two pSymA-encoded predicted proteins. YP_003065526, an ABC-type amino acid transporter permease, has 7 homologs encoded by pSymA genes. These are examples of multiple, redundant shared
homologs of ABC-type amino acid transport systems encoded by psymA. ‘Ca. Liberibacter asiaticus’ YP_003064636 is an exception since this putative transmembrane ABC-type transporter permease has only one psymA-encoded homolog, NP_435480.1 (Table 2). The multiplicity of ABC-type transporter homologs on psymA presumably provides a repertoire of ABC transporters for S. meliloti, and is consistent with a high magnitude of importance for ABC transporter systems in S. meliloti. In contrast, ‘Ca. Liberibacter asiaticus’ lacks the general overall redundancy of ABC-type transporters present in S. meliloti.

Metabolic cofactors are also likely to be imported by ‘Ca. Liberibacter asiaticus’ and S. meliloti from the plant host. For example, essential vitamins are taken up by ABC-type transporters encoded by psymA and the ‘Ca. Liberibacter asiaticus’ chromosome 2.

### Table 1. Proteins shared between the Sinorhizobium meliloti plasmid psymA, and the ‘Ca. Liberibacter asiaticus’ and Sinorhizobium meliloti chromosomes and with roles in the maintenance of homeostasis or signal transduction.

| pSymA     | L. asiaticus   | E   | AA | %    | S. meliloti | E   | Annotation                                      |
|-----------|----------------|-----|----|------|-------------|-----|------------------------------------------------|
| psymA vs L. asiaticus | psymA vs S. meliloti |
| Maintenance of Homeostasis |
| NP_435608.2 | YP_003064907  | 5E-71 | 306 | 65 | ******** | NA | cation diffusion facilitator transporter |
| NP_436297.2 | YP_003064775  | 3E-30 | 333 | 44 | ******** | NA | Na+/H+ antiporter |
| NP_436118.1 | YP_003065337  | 1E-134 | 605 | 62 | NP_384912  | 1E-22 | Potassium efflux protein |
| NP_436237.1 | YP_003065245  | 0E+00 | 624 | 67 | NP_384938  | 0E+00 | Potassium uptake protein |
| Signal Transduction |
| NP_436498.1 | YP_003064826  | 3E-16 | 239 | 49 | NP_384498  | 6E-22 | signal transduction histidine kinase |
| NP_436497.1 | YP_003065306  | 1E-22 | 220 | 47 | NP_385120  | 6E-35 | 2-component response regulator |
| NP_435549.2 | YP_003065316  | 1E-12 | 225 | 46 | NP_386067  | 2E-22 | 2-component sensor histidine kinase |
| NP_435864.1 | YP_003064863  | 3E-49 | 394 | 53 | NP_385908  | 1E-53 | 2-component sensor histidine kinase |
| NP_435916.1 | YP_003064863  | 2E-16 | 268 | 43 | NP_385564  | 4E-19 | 2-component sensor histidine kinase |

Genbank numbers, E-values, number of amino acids (AA) in the homologous region of the protein and the percentage of similar amino acids (%) in pair wise comparisons are provided. The annotations are for the proteins in ‘Ca. Liberibacter asiaticus’. doi:10.1371/journal.pone.0038725.t001

Figure 1. Distribution of genes on the S. meliloti pSymA megaplasmid that encode proteins orthologous to YP_003064586, annotated as an ABC-Type amino acid transporter in ‘Ca. Liberibacter asiaticus’ (A), that encode proteins orthologous to YP_003064948, annotated as a 3-ketoacyl-(acyl carrier protein) reductase in ‘Ca. Liberibacter asiaticus’ (B) and that encode proteins orthologous to YP_003064695, annotated as a LysR type regulator of transcription in ‘Ca. Liberibacter asiaticus’ (C).

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some. YP_003064972, an ATP-binding component of an ABC transport complex for thiamine, has two homologs on both pSymA and the S. meliloti chromosome.

### Synthesis of Metabolic Cofactors and Amino Acids

In addition to transporter proteins, there are a large number of genes shared between 'Ca. Librribacter asiaticus', S. meliloti and pSymA that encode proteins involved in the synthesis of metabolic cofactors (Table 2). 'Ca. Librribacter asiaticus' YP_003064901 encodes adenosylmethionine-8-amino-7-oxononanoate transaminase (E = 5e-35). The enzyme 8-amino-7-oxononanoate synthase (AONS), which is pyridoxal 5’-phosphate-dependent, catalyzes 8-amino-7-oxononanoate synthase, needed for biosynthesis of biotin. 'Ca. Librribacter asiaticus' YP_003064762 is a serine hydroxymethyltransferase (SHMT) that catalyzes production of L-serine and tetrahydrofolate with an exceptionally low E-value when compared with its pSymA ortholog NP_436412.1 (E = 3e-33).

### Synthesis of Amino Acids and Metabolic Cofactors

| pSymA | L. asiaticus | E | AA | % | S. meliloti | E | Annotation |
|-------|-------------|---|----|---|------------|---|------------|
| NP_435288.1 | YP_003064586 | 6E-87 | 257 | 76 | NP_385581 | 1E-100 | ABC transport |
| NP_436046.1 | YP_003064753 | 9E-41 | 230 | 59 | NP_386072 | 1E-50 | proline/glycinebetaine ABC transporter |
| NP_436047.1 | YP_003064754 | 3E-12 | 138 | 57 | NP_385141 | 3E-19 | proline/glycinebetaine ABC transporter |
| NP_435405.1 | YP_003065117 | 2E-24 | 243 | 51 | NP_387074 | 7E-65 | ABC transporter, nucleotide binding |
| NP_435492.2 | YP_003064552 | 2E-24 | 238 | 53 | NP_387225 | 1E-92 | put ATP binding ABC transporter |
| NP_436433.1 | YP_003065525 | 3E-12 | 243 | 42 | NP_387380 | 8E-21 | put amino acid-binding periplasmic ABC |
| NP_435285.1 | YP_003065526 | 7E-24 | 202 | 59 | NP_387382 | 2E-27 | amino acid ABC transporter permease |
| NP_435480.1 | YP_003064636 | 1E-12 | 275 | 41 | NP_385492 | 3E-17 | ABC transporter permease |
| NP_435301.1 | YP_003064972 | 5E-22 | 204 | 53 | NP_388055 | 1E-66 | thiamine transporter ATP-binding subunit |

### Proteins Encoded by Liberibacter and pSymA

In addition to the proteins involved in the import or potential export of amino acids and metabolic cofactors, 'Ca. Librribacter asiaticus' shares genes with pSymA and the S. meliloti chromosomes.

**Table 2.** Proteins shared between the Sinorhizobium meliloti plasmid pSymA, and the ‘Ca. Librribacter asiaticus’ and Sinorhizobium meliloti chromosomes with roles in ABC-Type transport and the synthesis of amino acids and metabolic cofactors.

| Genbank numbers, E-values, number of amino acids (AA) in the homologous region of the protein and the percentage of similar amino acids (%) in pair wise comparisions are provided. The annotations are for the proteins in 'Ca. Librribacter asiaticus'.
for several proteins used to export small bioactive molecules (Table 3). These include YP_003064686 an ABC-type transporter with excellent Blast alignments with chromosomal virulence gene (Table 3). These include YP_003064686 an ABC-type transporter for several proteins used to export small bioactive molecules with a yhbG domain, is similar to homologs, likely involved in exporting lipid virulence factors. Consistent with important roles for these proteins in the cell wall and outer membrane or Nod factor components.

### Regulation of Transcription and Translation

| pSymA vs L. asiaticus | E   | AA | %  | S. melloti vs S. meliloti | E   | Annotation |
|----------------------|-----|-----|----|--------------------------|-----|------------|
| Export of Bioactive Molecules |      |     |    |                          |     |            |
| NP_435263.1          | YP_003064686 | 9E-62 | 505 | 51 | NP_385265 | 2E-87 | put ABC transporter ATP-binding |
| NP_435718.1          | YP_003065157 | 4E-30 | 239 | 51 | NP_385995 | 1E-35 | ABC transporter nucleotide binding |
| NP_435856.2          | YP_003065113 | 5E-33 | 241 | 51 | NP_385127 | 2E-45 | Serine protease DO like protease |
| Chaperonins          |      |     |    |                          |     |            |
| NP_435305.2          | YP_003065051 | 2E-15 | 175 | 47 | NP_385003 | 7E-27 | chaperone protein DnaJ |
| NP_435342.1          | YP_003065326 | 8E-13 | 70  | 67 | NP_386217 | 1E-18 | Cold shock protein |
| NP_435641.1          | YP_003065262 | 0E+00 | 526 | 77 | NP_384898 | 0E+00 | Chaperonin GroEL |
| NP_435311.1          | YP_003065261 | 4E-22 | 95  | 71 | NP_384899 | 8E-39 | co-chaperonin GroES |

### Table 3.

Proteins shared between the *Sinorhizobium meliloti* plasmid pSymA, and the ‘Ca. Liberibacter asiaticus’ and *Sinorhizobium meliloti* chromosomes with roles in the export of bioactive molecules, as chaperonins and in the regulation of transcription and translation.

Chaperonins

Several chaperonin proteins are encoded by orthologous genes shared by ‘Ca. Liberibacter asiaticus’, pSymA and *S. melloti* (Table 3). YP_003065051 is a DnaJ molecular chaperone protein with one orthologous protein encoded by pSymA, NP435305.2. Cold shock protein YP_003065326 has three similar proteins encoded by pSymA. YP_003065262 (GroEL) is orthologous with both pSymA NP_435641.1 (E = 0) and with pSymA NP_435310.1 (E = 1e-106). Co-chaperonin GroES YP_003065261 is orthologous with pSymA-encoded NP_535311.1 (E = 4e-22) and NP_435462.1 (E = 9e-21).

### Regulation of Transcription and Translation

‘Ca. Liberibacter asiaticus’, pSymA and *S. melloti* share several proteins that regulate transcription (Table 3). YP_003064695 is a member of the LysR family of transcription regulator proteins. Its importance for plant interaction is indicated by at least 14 LysR-like proteins encoded by and distributed across pSymA (E = 1e-21 to E = 1e-10) (Fig. 1C; Table S1). Other genes encoding transcriptional regulators are not as extensively duplicated on pSymA, consistent with LysR family transcription regulators being of unique importance in plant interaction in *S. melloti* [23]. The ‘Ca. Liberibacter asiaticus’ genome, by contrast, encodes only YP_003064695, annotated as transcriptional regulator with a LysR region. YP_003065539, a putative sigma-54-dependent transcription regulator protein is similar to NP_435689 in pSymA annotated as NifA. NifA is a DNA-binding ATPase and CheY-like two-component sensor-based response regulator that is known to
activate expression of genes involved in nitrogen metabolism. YP_003065196, encodes a transcription-activating MucR-like protein [24], with two orthologs on pSymA. MucR proteins regulate production of extracellular polysaccharides which could contribute to biofilm formation in phloem cells of citrus inhabited by ‘Ca. Liberibacter asiaticus’. YP_003063563 and pSymA NP_436495 (E = 7e-49) are encoded by orthologous genes for a UvrD2 DNA helicase II, or superfamily I DNA and RNA helicase. ‘Ca. Liberibacter asiaticus’ YP_003065000 and pSymA orthologs NP_436130.1 and NP_436426.1 encodes a XerD recombinase/ integrase, an enzyme known to allow formation of correctly charged asparagine or glutamine tRNAs in organisms which lack either or both a asparaginyl-tRNA synthetase or a glutaminyl-tRNA synthetase.

Cell Surface

‘Ca. Liberibacter asiaticus’ and pSymA share several genes encoding proteins that contribute to a functional architecture of the cell surface (Table 4). A UDP-glucose-4-epimerase, YP_003064881, a putative prokaryotic sensory diguanylate cyclase/phosphodiesterase protein, has two orthologs on pSymA, NP_435318.2 (E = 1e-42) and NP_436089.2 (E = 7e-42). YP_003065366 encodes an OmpA/MotB-like protein similar with S. meliloti NP_435714.1 (E = 8e-14) and the S. meliloti chromosome NP_384380 (E = 2e-16). Pilus assembly gene clusters on pSymA have orthologs on the ‘Ca. Liberibacter asiaticus’ chromosome, Proteins YP_003065134 (NP_436096, E = 5e-50), YP_003065135 (NP_436097, E = 3e-49), YP_003065137 (NP_436098, E = 1e-178; NP_435955, E = 2e-35), YP_003065140 (NP_435332.2, E = 9e-45) and YP_003065141 (NP_436102.1, E = 3e-29) are all components of a pilus assembly for ‘Ca. Liberibacter asiaticus’. YP_003065138, a pilus-associated response regulator receptor protein, likely regulates production of pili based on signals received from the environment.

A transglycosylase, YP_003064721 in ‘Ca. Liberibacter bacterium’ asiaticus’ and pSymA-encoded NP_436359.1 (E = 3e-40), could produce cell wall degradation products, YP_003064721 signal peptide protein orthologous to pSymA NP_436359.1 and is a putative membrane-bound lytic murine transglycosylase (E = 3e-40).

YP_003065163, a penicillin-binding peptidoglycan synthase, is orthologous to NP_436481.1 (E = 0). A transmembrane protein that also binds penicillin, YP_003065516 is orthologous to pSymA NP_436482.1 (E = 1e-93). ‘Ca. Liberibacter asiaticus’ YP_003063536 (Table 1), a glucoseamine-fructose-5-phosphate aminotransferase, needed for N-acetyl D-glucosamine synthesis, aligns with pSymA-encoded NP_435728.1 (E = 0).

Electron Transfer

An important conserved microsynteny orthologous gene (MOG) cluster, occurs in the ‘Ca. Liberibacter asiaticus’ genome and in the genomes of four other members of the Rhizobiadetes that were analyzed previously [26]. This conserved MOG encodes thirteen subunits that comprise a ‘mitochondrial-like’ Type I respiratory complex (Table 5). Genes encoding this respiratory complex are also found on pSymA, and orthologous proteins specified by ‘Ca. Liberibacter asiaticus’ chromosomal genes include the following: Chain A, NuoA2 NADH: ubiquinone oxidoreductase YP_003065265 and NP_436080.1 (E = 9e-14); Chain B, or NuoB2 YP_003065266 and NP_436079.1 (E = 6e-49); Chain C, or NuoC2 YP_003065267 and NP_436078.1 (E = 6e-35); Chain D, or NuoD2 YP_003065268 and NP_436077.1 (E = 1e-10); Chain E or NuoE2, YP_003065269 and NP_436076.1 (E = 4e-24) and Chain F or NuoF2 YP_003065271 and NP_436075.1 (E = 1e-10). Chains G, H and I/J or NuoG2, NuoH2, a multi-subunit ubiquinone oxidase have the following orthologs, YP_003065272 and NP_436074.1 (E = 2e-62); YP_003065273 and NP_436071.1 (E = 1e-51), YP_003065274 and NP_436072.1 (E = 1e-22), YP_003065275 and NP_436087.2 (E = 8e-11). YP_003065278 is orthologous with pSym proteins NP_436085.1 and NP_436083.4 annotated as, respectively, a multidomain K/L oxidoreductase, a NADH ubiquinone oxidase (COG1009), or a multi-domain NAD+/K+ antiporter, largely synonymous, involved in energy generation, cation efflux or intracellular pH control. ‘Ca. Liberibacter asiaticus’ YP_003065279 and pSymA NP_436082.1 (E = 2e-92) are chain M of ubiquinone oxidoreductase. ‘Ca. Liberibacter asiaticus’ YP_003065280 and pSymA NP_436081.1 are chain N (E = 4e-57). Nearly all orthologous genes shared between pSymA and S. meliloti chromosome have lower E-values aligned with each other than do orthologs shared between pSymA and the ‘Ca. Liberibacter asiaticus’ chromosome (Tables 1–4). However the amino acid sequence similarities between protein components of the respiratory complex I encoded by pSymA, and the ‘Ca. Liberibacter asiaticus’ genome and the S. meliloti chromosome are highly comparable with each other (Table 5). The organization of the genes in genomic regions encoding the NADH dehydrogenase complex is the same for S. meliloti and ‘Ca. Liberibacter asiaticus’ (Fig. 2). NuoG and NuoL are encoded by genes in the expected cluster on the S. meliloti chromosome. But the c-values obtained by pBLAST of pSymA NuoG and NuoL vs S. meliloti are better for genes found elsewhere on the S. meliloti chromosome. The best match for pSymA NuoG (NP_43074) on the S. meliloti chromosome is a NAD-dependent formate dehydrogenase alpha subunit (NP_387115; E = 2e-99). The NADH dehydrogenase subunit G (NP_385378; E = 8e-77) is the second best result. This protein is transcribed and translated from a gene with the expected position in the NADH dehydrogenase MOG and aligns well with the ‘Ca. Liberibacter asiaticus’ NuoG (e = 0). A similar finding was obtained when pSymA NP_436085 encoding the NuoL subunit was used in a blast search of the chromosome of S. meliloti. The best matches were to proteins annotated as monovalent cation/H+ antiporter subunits located at different positions on the chromosome. NADH dehydrogenase subunit L (NP_385383; E = 7e-25) encoded by the chromosome was significantly less well-aligned. As was seen for NuoG, this protein is transcribed from a gene with the expected position in the NADH dehydrogenase MOG and aligns well with the ‘Ca. Liberibacter asiaticus’ NuoL (e = 0). Thus these proteins likely function in the role of NuoL. The NADH dehydrogenase gene cluster on pSymA has a different arrangement of genes (Fig. 2). Genes encoding NuoA-NuoG in the center of the NuoG cluster are flanked on one side by genes encoding NuoJ–NuoN and by genes, transcribed from the opposite strand, that encode NuoH and NuoI on the other side.

Other proteins are encoded by genes shared by ‘Ca. Liberibacter asiaticus’, pSymA and the chromosome of S. meliloti, and have roles in energy generation. YP_003065437, is a probable electron transfer flavoprotein-quinone oxidoreductase or FixC. YP_003064703 is orthologous with both pSymA-borne NP_435265.2 and NP_435950.1, which are zinc-dependent NADPH quinone oxidoreductases, COG0604, and are important in energy production. YP_003064781 and YP_003064772,
encoded by adjacent genes, are orthologs of NP_436008.1 and NP_436007.1 (E = 3e-73 and 2e-77 respectively). Both genes have two adjacent ortholog pairs on both pSymA and the _S. meliloti_ chromosome. YP_003065174 and NP_435375.1 (E = 3e-76) are annotated as energy yielding FAD-dependent dehydrogenases. YP_003065041 is coproporphyrinogen III oxidase and is orthologous to a pSymA-borne locus NP_435937.1. The protein has two domains; N-terminal Radical-SAM super family and a C-terminal HemN_C superfamily, which obtains electrons from a flavodoxin-like system regenerated by a nicotinamide cofactor-dependent flavodoxin [27]. ‘Ca. Liberibacter asiaticus’ YP_003064696 is a NADPH thioredoxin reductase orthologous to NP_436285.1.

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**Table 4.** Proteins shared between the _Sinorhizobium meliloti_ plasmid pSymA, and the ‘Ca. Liberibacter asiaticus’ and _Sinorhizobium meliloti_ chromosomes with roles in the modification of cell surface structures.

| pSymA       | L. asiaticus | E     | A A | %                 | S. meliloti | E     | Annotation                           |
|-------------|--------------|-------|-----|-------------------|-------------|-------|--------------------------------------|
| pSymA vs L. asiaticus | pSymA vs S. meliloti |
| **Cell Surface Structure** | | | | | | |
| NP_435626.1 | YP_003064741 | 4E-11 | 188 | 46 | NP_384213 | 7E-18 | UDP-glucose-4-epimerase |
| NP_436089.2 | YP_003064881 | 7E-42 | 451 | 49 | NP_385065 | 2E-95 | sensory box/GGDEF family |
| NP_435318.2 | YP_003064881 | 1E-42 | 426 | 49 | NP_385065 | 3E-76 | sensory box/GGDEF family |
| NP_435574.1 | YP_003065366 | 8E-14 | 96  | 59 | NP_384380 | 2E-16 | OmpA/MotB/outer membrane |
| NP_436096.2 | YP_003065134 | 5E-50 | 254 | 57 | NP_384252 | 1E-62 | Pilus component |
| NP_436097.1 | YP_003065135 | 3E-49 | 179 | 72 | NP_384251 | 7E-68 | Pilus component |
| NP_436098.1 | YP_003065137 | 1E-178 | 408 | 83 | NP_384250 | 0E-00 | component of type IV pilus |
| NP_435332.2 | YP_003065140 | 9E-45 | 455 | 49 | NP_384247 | 2E-43 | put pilus assembly |
| NP_436102.1 | YP_003065141 | 3E-29 | 200 | 55 | NP_384246 | 2E-32 | put pilus assembly |
| NP_436101.1 | YP_003065138 | 1E-66 | 397 | 55 | NP_384249 | 7E-76 | Response regulator receiver (pilus) |
| NP_436359.1 | YP_003064721 | 3E-40 | 204 | 57 | NP_387423 | 1E-64 | lytic murein transglycosylase |
| NP_436481.1 | YP_003065165 | 0E+00 | 711 | 70 | NP_385436 | 0E+00 | Peptidoglycan synthetase |
| NP_436482.1 | YP_003065151 | 1E-93 | 416 | 60 | NP_386288 | 1E-100 | penicillin-binding transmembrane |
| NP_435728.1 | YP_003065356 | 0E+00 | 608 | 76 | NP_435728 | 0E+00 | glucosamine-6-Phos transporter |

Genbank numbers, E-values, number of amino acids (AA) in the homologous region of the protein and the percentage of similar amino acids (%) in pair wise comparisons are provided. The annotations are for the proteins in ‘Ca. Liberibacter asiaticus’.

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**Discussion**

The premise for identifying protein products of ‘Ca. Liberibacter asiaticus’ orthologous to those encoded by genes on pSymA was an hypothesis that such genes and their protein products in all likelihood play direct or indirect role(s) in establishing and maintaining an intimate intracellular interaction with eukaryotes. ‘Ca. Liberibacter asiaticus’ has an obligatory requirement for a

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**Figure 2.** Diagram showing the organization of the genes encoding the components of the NADH dehydrogenase complex on pSymA, ‘Ca. Liberibacter asiaticus’ and _S. meliloti_.

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eukaryotic host, and there are multiple orthologs of these genes in *Sinorhizobium meliloti*. These factors preclude mutagenesis studies at the present time to test this hypothesis. In particular, we discovered that there are several orthologous genes in the *'Liberibacter asiaticus' genome with an e-value that is 110 orders of magnitude lower than the alignment of the orthologous proteins encoded by *pSymA* and the *S. meliloti* chromosome. Thus, the maintenance of intracellular pH and ion homeostasis are likely critical functions of proteins encoded by *pSymA*, but not the *S. meliloti* chromosome. Other *'Liberibacter asiaticus' genes with orthologs on *pSymA* likely encode proteins with either direct or indirect nutritional or energy roles in establishing and maintaining an intimate intracellular interaction with eukaryotic hosts.

We identified 86 *'Liberibacter asiaticus' genes with 182 probable orthologs on *pSymA* and the *S. meliloti* chromosome. Most of the orthologous proteins encoded by *pSymA* and the *S. meliloti* chromosome produced alignments with substantially more significant E-values than orthologous proteins encoded by *'Liberibacter asiaticus' and *pSymA*. Individual genes encoded by the reduced genome of *'Liberibacter asiaticus' may have many orthologs or paralogs carried by the *pSymA* megaplasmid besides additional similar genes on the chromosome of *S. meliloti*. An example is YP_0003064948, a 3-ketoacyl-(acyl-carrier-protein) one-gated potassium efflux protein that exchanges potassium for sodium and protons and thereby modulates cytoplasmic pH [29,30].

### Table 5. Proteins shared between the *Sinorhizobium meliloti* plasmid *pSymA*, and the *'Ca. Liberibacter asiaticus' and *Sinorhizobium meliloti* chromosomes with roles in electron transfer.

| pSymA vs L. asiaticus | E   | A   | %    | S. meliloti | E   | Annotation                  |
|-----------------------|-----|-----|------|-------------|-----|-----------------------------|
| NP_43608.1    | YP_0003065265 | 9E-14 | 109 | 53 | NP_385370 | 3E-14 | NADH dehydrogenase subunit A |
| NP_436079.1   | YP_0003065266 | 6E-48 | 141 | 79 | NP_385371 | 2E-47 | NADH dehydrogenase subunit B |
| NP_436078.1   | YP_0003065267 | 6E-33 | 142 | 60 | NP_385372 | 1E-29 | NADH dehydrogenase subunit C |
| NP_436077.1   | YP_0003065268 | 1E-110 | 393 | 64 | NP_385373 | 1E-106 | NADH dehydrogenase subunit D |
| NP_436076.1   | YP_0003065269 | 4E-24 | 169 | 53 | NP_385375 | 3E-22 | NADH-quinone oxidoreductase subunit E |
| NP_436075.1   | YP_0003065271 | 1E-104 | 420 | 64 | NP_385376 | 1E-105 | NADH dehydrogenase I subunit F |
| NP_436074.1   | YP_0003065272 | 2E-62 | 410 | 54 | NP_385378* | 2E-65 | NADH dehydrogenase subunit G |
| NP_436071.1   | YP_0003065273 | 1E-51 | 311 | 55 | NP_385379 | 2E-54 | NADH dehydrogenase subunit H |
| NP_436072.1   | YP_0003065274 | 1E-22 | 136 | 54 | NP_385380 | 4E-23 | NADH dehydrogenase subunit I |
| NP_436087.2   | YP_0003065275 | 8E-11 | 54  | 78 | NP_385381 | 6E-10 | NADHDehydrogenasesubunit J |
| NP_436085.1   | YP_0003065278 | 2E-23 | 461 | 42 | NP_385383* | 1E-22 | NADH dehydrogenase subunit L |
| NP_436083.4   | YP_0003065272 | 3E-20 | 180 | 52 | NP_385388* | 1E-19 | NADH dehydrogenase subunit L |
| NP_436082.1   | YP_0003065279 | 2E-92 | 485 | 54 | NP_385384 | 1E-100 | NADH dehydrogenase subunit M |
| NP_436081.1   | YP_0003065280 | 4E-57 | 427 | 50 | NP_385385 | 2E-75 | NADH dehydrogenase subunit N |
| NP_436008.1   | YP_0003064781 | 3E-73 | 249 | 71 | NP_436008 | 1E-128 | electron transfer flavoprotein beta |
| NP_435693.1   | YP_0003064781 | 4E-14 | 186 | 52 | NP_435693 | 8E-18 | electrontransfer flavoprotein beta |
| NP_436007.1   | YP_0003064782 | 2E-77 | 316 | 69 | NP_436007 | 1E-150 | Electron transfer flavoprotein alpha |
| NP_435692.1   | YP_0003064782 | 2E-20 | 245 | 48 | NP_386750 | 3E-21 | Electron transfer flavoprotein alpha |
| NP_435691.1   | YP_000306437 | 1E-18 | 326 | 43 | NP_385139 | 6E-09 | flavoprotein ubiquinone oxidoreductase |
| NP_435265.2   | YP_0003064703 | 2E-26 | 334 | 43 | NP_385059 | 1E-32 | quinone oxidoreductase |
| NP_435950.1   | YP_0003064703 | 4E-12 | 269 | 40 | NP_386798 | 5E-42 | quinonexoxidoreductase |
| NP_435375.1   | YP_0003065174 | 3E-76 | 425 | 56 | NP_384973 | 7E-99 | prob FAD-dependent oxidoreductase |
| NP_435937.1   | YP_0003065041 | 9E-20 | 187 | 51 | NP_384479 | 3E-19 | cothiorphinogen III oxidase |
| NP_436285.1   | YP_0003064696 | 1E-22 | 134 | 61 | NP_385673 | 1E-31 | thioredoxin reductase (NADPH) |

Genbank numbers, E-values, number of amino acids (AA) in the homologous region of the protein and the percentage of similar amino acids (%) in pair wise comparisons are provided. The annotations are for the proteins in *'Liberibacter asiaticus'*. *indicates proteins that were the second or third best match by Blast.

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reductase with 20 similar proteins encoded by pSymA. Thus, numerous physiological and niche adaptations conferred upon *S. meliloti* by pSymA may not be available to *Ca. Liberibacter asiaticus*. However, in a reduced genome such as that of *Ca. Liberibacter asiaticus*, genes that are retained may function with relaxed substrate specificity [31,32], compensating for the lack of multiple homologs in the *Ca. Liberibacter asiaticus* genome. The multiplicity of pSymA orthologs complicates any mutagenesis strategy to definitively establish their roles in *S. meliloti*.

Protein products not constitutively expressed may be considered as candidates for being involved with niche specialization or pathogenesis; *S. meliloti* has conditionally expressed genes that are especially important for nitrogen fixation and nodulation symbiosis. YP_003064695 is a putative regulator of transcription by *Ca. Liberibacter asiaticus*, with 14 related proteins in pSymA, many of which are annotated as LysR transcriptional regulatory proteins. Despite considerable conservation both structurally and functionally, LysR-type transcriptional regulators regulate a diverse set of genes, including those involved in virulence, metabolism, quorum sensing and motility [33,34]. In *S. meliloti*, LysR proteins function in exquisite control of a sequence of events leading to root colonization, initiation of nodulation and subsequent differentiation of nodules containing *S. meliloti* [23]. Fatty acid products of the 3-ketoacyl-(acyl-carrier-protein) reductase orthologs are also used to complete the structure of nodulation factors. This may be why the extensive collection of LysR regulatory elements and 3-ketoacyl-(acyl-carrier-protein) reductases encoded on pSymA are not found on the *Ca. Liberibacter asiaticus* chromosome. Thus, *Ca. Liberibacter asiaticus* may have much less capability for finely nuanced control of the expression of genes, consistent with its obligatory intracellular lifestyle. Parasitism of citrus by *Ca. Liberibacter asiaticus* follows direct injection of the pathogen into the host and results in systemic invasion of phloem tissues throughout the host [9,10], and differentiated nodules are not produced.

Genes with orthologs on both pSymA and the chromosome of *S. meliloti* are largely specialized for symbiosis and nitrogen metabolism in *S. meliloti* and include 48 ABC-type transporter genes. The genome of *Ca. Liberibacter asiaticus* encodes only 11 ABC-type transporter proteins that are orthologous to pSymAencoded proteins, compared with a total of about 40 ABC-type transporters encoded by the chromosome [2]. These transporters likely serve to import sugars, amino acids and precursors and purines and pyrimidines into *Ca. Liberibacter asiaticus* from the host. The genome of *Ca. Liberibacter asiaticus* is known to be deficient in genes used in the biosynthetic pathways of these compounds [2,12]. ABC-type transporters may also be used to export small molecules by *Ca. Liberibacter asiaticus*. YP_003064989 is an ABC-type transporter protein encoded by *Ca. Liberibacter asiaticus* that likely functions to export glucans for osmoprotection. A class of proteins that confer multiple drug resistance, are called ‘RND’ for Resistance, Nodulation and Division [35]. RND-type proteins confer drug resistance based on rapid efflux of drugs from the bacterial cell. YP_003065157, is an ATP-binding ABC-type transporter protein with a yhbG domain, and is orthologous to pSymA NodI, a protein that is required for nodulation of alfalfa by *S. meliloti*. NodI-like proteins secrete lipids and lipopolysaccharides such as cell wall, outer membrane or Nod factor components needed to stimulate the differentiation of nodules by the host. Very interestingly pSymA encodes six homologs of YP_003065157. Orthologs of ABC-type transporters encoded by *Ca. Liberibacter asiaticus* were identified with the “Transporter Classification Database” (http://www.tcdb.org/), YP_003065330, annotated as an ABC-type transporter with nucleotide binding and ATPase domains was homologous to Q2G2M4 [4.00E-88], annotated as a putative multi-drug export ATP-binding/permease protein, *Staphylococcus aureus* SAOUHSC_02005. YP_003065330 aligns best with nodI YP_435718.1 [2.00E-021] encoded by pSymA. Thus these proteins at the least share similar structural motifs.

Genes encoding type IV pilus may have evolved from structures used for DNA transfer but it is now known that virulence proteins may be secreted by components of a type IV pili [36]. Genes encoding type IV pili are shared between *Ca. Liberibacter asiaticus*, *S. meliloti*, and pSymA. Effectors directly involved with the establishment of *Ca. Liberibacter asiaticus* as an intracellular pathogen living within the phloem cells of citrus, may be transferred from the bacterium to its host by type IV secretion involving pilus assembly proteins. If pili are produced by *Ca. Liberibacter asiaticus*, they may also have roles in twitching motility and biofilm formation as in *Xylella fastidiosa* [37].

Why is the entire *S. meliloti* chromosomal respiratory complex I gene set duplicated on pSymA? Plants respond to bacterial pathogens by producing a sustained oxidative burst leading to apoptosis. The respiratory complex protein NuoG can also be used by *Myxococcus tuberculosis* to pump protons out of the cell to neutralize such superoxide radicals [30]. This may explain the duplication of the genes encoding the respiratory complex on pSymA and provide an additional role for NuoG in the *Ca. Liberibacter asiaticus* citrus interaction. Homeostasis with respect to sodium is also of critical importance to both *S. meliloti* and *Ca. Liberibacter asiaticus* as discussed above, and in addition to pumping protons out of the cell to create the proton motive force needed for the synthesis of ATP, respiratory complex I of at least some Gram negative bacteria can also transport sodium ions out of the cells [39]. Besides an obvious potential gene dosage effect, redundant copies carried on an extrachromosomal element provide a palette for gene evolution. However in spite of the potential for diversification of these genes afforded to pSymA, substantial diversification is not apparent since the E-values for the orthologs encoded by pSymA, *Ca. Liberibacter asiaticus* and *S. meliloti* are very similar.

Most *Ca. Liberibacter asiaticus*’ protein products that align significantly with proteins encoded by pSymA also align significantly with predicted protein products encoded by the *S. meliloti* chromosome. Thus, although presented as single copies for the most part in *Ca. Liberibacter asiaticus*, these genes are present in multiple copies per replicon in the free-living microsymbiont. The presence of multiple orthologs in pSymA of *Ca. Liberibacter asiaticus*’ proteins such as the amino acid transporter YP_003064586, the fatty acid dehydrogenase YP_003064940 and the LysR transcriptional regulator YP_003064695, suggests that their products are of particular importance in host/microbe interactions, and are likely to be examples of a single protein acquiring multiple functions in an organism with a reduced genome [32]. *Ca. Liberibacter asiaticus* very notably also has two or three genes shared with pSymA but not the *S. meliloti* chromosome. These genes are predicted to produce proteins that generate a proton gradient with ATP-producing potential, maintain sodium and potassium homeostasis, prevent over accumulation of divalent metal cations and maintain intracellular pH. Thus the maintenance of intracellular homeostasis is potentially a vital requirement for successful colonization of citrus and psyllids by *Ca. Liberibacter asiaticus*. In an obligate pathogen, cellular vitality and pathogenesis are tied together.
Materials and Methods

The genome of ‘Ca. Liberibacter asiaticus’ was compared with both the circular chromosome and megaplasmids, especially pSymA, of S. meliloti. To identify orthologous proteins, predicted amino acid sequences of ‘Ca. Liberibacter asiaticus’ strain psy62, RefSeq NC_012985; Sinorhizobium meliloti strain 1021 RefSeq NC_003047, and pSymA RefSeq NC_003037 were downloaded from NCBI. Using default BLAST parameters, each predicted amino acid sequence from the ORFs identified on pSymA of the S. meliloti 1021 genome was BLASTed against the predicted amino acid sequences of the ORFs on the chromosomes of ‘Ca. Liberibacter asiaticus’ and Sinorhizobium meliloti strain 1021. Perl scripts and Excel spreadsheets were created to identify hits between genomes with low, negative e-values and to extract annotations from Genbank. In addition, amino acid similarity and length of the blast hit was extracted from each top hit from the BLAST output. First, a general analysis was done extracting predicted protein products having BLAST alignment values of e-10 or lower. Similar or homologous proteins were also identified manually where possible to be consistent with the annotations from the authors of each respective genome. That is, annotations of ORFs were extracted from NCBI annotations of the genome of ‘Ca. Liberibacter asiaticus’ and megaplasmid pSymA of Sinorhizobium meliloti strain 1021, respectively. Thus, the matching homolog might not have the best e-value or match with the top hit as many proteins share the same domain structure or amino acid similarity, but are functionally quite different. Similarly, for proteins of interest that were unique to each genome, proteins were sorted for positive e-values, signifying that an orthologous protein was not encoded in a respective genome. Orthologous genes from the ‘Ca. Liberibacter asiaticus’ chromosome, the S. meliloti chromosome and megaplasmid pSymA were mapped on linear representations of the respective genophores opened at their origins of replication.

Supporting Information

Table S1 Proteins shared between the Sinorhizobium meliloti plasmid pSymA, and the ‘Ca. Liberibacter asiaticus’ and Sinorhizobium meliloti chromosomes. The protein IDs and annotations used by NCBI and the e-values of pairwise comparisons are provided. (XLSX)

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Author Contributions

Conceived and designed the experiments: LDK JYS JSH. Performed the experiments: LDK JYS JSH. Analyzed the data: LDK JYS JSH. Contributed reagents/materials/analysis tools: JYS. Wrote the paper: LDK JYS JSH.

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