**Supplemental Note 1: Supporting evidence and predicted roles for poorly annotated *Z. mobilis* genes**

This note summarizes the supporting evidence and predicted physiological roles for 56 poorly annotated *Zymomonas mobilis* ZM4 genes (highlighted in **bold** in this document). The gene-gene associations were identified by cofitness (r, see main text for details on cofitness and Supplemental Table 4 for the full list of poorly annotated genes with significant cofitness to another gene). Our criteria for defining genes as poorly annotated are described in the main text.

**ZMO0055** encodes a predicted permease annotated as DUF81 and has homology to a sulfite permease from *Cupriavidus necator* H16 ([Weinitschke, Denger et al. 2007](#_ENREF_24)). Mutants in **ZMO0055** have reduced fitness phenotypes in in defined media with L-glutamic acid or L-glutamine as nitrogen sources, and in rich media with the stressors copper chloride or sodium hypochloride. **ZMO0055** is cofit with genes involved in sulfate assimilation: sulfate adenylyltransferase (ZMO0005, r = 0.75; ZMO0004, r = 0.61) and uroporphyrin-III C-methyltransferase (ZMO0006, r = 0.77). While it is difficult to rationalize why the **ZMO0055** permease has reduced fitness in these particular conditions, we speculate that **ZMO0055** transports or exports some sulfur containing compound and is required for the activity of the sulfate assimilation pathway under some conditions.

**ZMO0100** is highly cofit with the adjacent and divergently transcribed ZMO0101 (r = 0.95). ZMO0100 encodes a transcription factor that probably regulates ZMO0101, a predicted NAD-dependent epimerase/dehydratase. Both genes are required for fitness in the presence of plant hydrolysate ([Skerker, Leon et al. 2013](#_ENREF_22)), a complex chemical stress, suggesting that ZMO0101 may participate in the synthesis of the cell wall.

**ZMO0107** encodes a hypothetical protein that has weak homology to NDP-sugar transferases and is cofit with putative sphingosine kinase ZMO1391 (r= 0.83) ([Lee, Um et al. 2005](#_ENREF_14)). **ZMO0107** and ZMO1391 may be involved in making glycolipids that affect lipopolysaccharide permeability, which might explain the sensitivity of mutants in ZMO0107 to beta-lactam antibiotics and gentamicin.

**ZMO0112** encodes a hypothetical protein with significant cofitness (r = 0.80) to glutamine cyclotransferase (ZMO1877), an enzyme that post-translationally circularizes N-terminal glutamine residues. ZMO1877 has been purified and its glutamine cyclotransferase activity has been experimentally validated ([Carrillo, Parthier et al. 2010](#_ENREF_3)). To date, there is no known physiological function for N-terminal glutamine cyclization in bacteria. ZMO1877 and **ZMO0112** have reduced fitness in the presence of eugenol, sodium sulfate, polymyxin B, MreB perturbing compound A22, sodium cholate, bacitracin, thioridazine, and verapamil hydrochloride. In contrast, mutants in both genes have enhanced fitness in a number of beta-lactam antibiotics (cefazolin, cloxacillin, amoxicillin, cephalothin, cefsulodin, and ceftazidime hydrate). **ZMO0112** has a predicted signal peptide sequence ([Petersen, Brunak et al. 2011](#_ENREF_20)) that leaves a N-terminal glutamine, suggesting that the glutamine cyclotransferase encoded by ZMO1877 may act on **ZMO0112**, and that this post-translational modification is necessary for **ZMO0112** to function in the cell wall.

The Rrf2 family regulator **ZMO0116** is cofit with a peroxidase (**ZMO1573**, r = 0.76) and catalase (ZMO0918, r = 0.75). We propose that **ZMO0116** may regulate a response to oxidative stress.

**ZMO0132** and **ZMO0133** are part of an uncharacterized six gene cluster (**ZMO0132**:ZMO0137) and all six genes are predicted to encode proteins with a Sel1-like tetratricopeptide (TPR) domain. The TPR repeat is thought to mediate protein-protein interactions and is present in both eukaryotes and prokaryotes. In bacteria, proteins with Sel1-like domains are extracellularly located in both *E. coli* ([Pastorello, Rossi Paccani et al. 2013](#_ENREF_19)) and *Francisella tularensis* ([Chong, Child et al. 2013](#_ENREF_5)). **ZMO0132** and **ZMO0133** are cofit with some cell wall related genes including ZMO0847 (carbohydrate-selective porin OprB). Mutants in **ZMO0132** and **ZMO0133** are hypersensitive to a number of diverse acids. Taken together, our data suggests that **ZMO0132** and **ZMO0133** are outer membrane-associated proteins that are required for *Z. mobilis* tolerance to low pH conditions.

ZMO0282, ZMO0283, and **ZMO0285** form an operon and encode an RND-class efflux system. These genes are highly cofit (r > 0.90 for all pairwise comparisons) and are required for optimal fitness in many stress conditions suggesting that this efflux system pumps a broad range of structurally diverse toxins outside the cell. We also noticed that this operon is conserved next to and negatively cofit with a tetR-like regulator, ZMO0281 (r = -0.34 to -0.39), which probably represses its expression.

The hypothetical protein **ZMO0331** is cofit with ZMO1088, rare lipoprotein A (r = 0.75). **ZMO0331** contains a domain related to peptidase M15A (PF05951) and a signal sequence for translocation by the twin-arginine pathway, which is consistent with a role in the cellular envelope. Also, many of the closer homologs of **ZMO0331** are adjacent to peptidoglycan binding proteins.

The operon **ZMO0444**:**ZMO0447** comprises four poorly annotated proteins: GSDL-like lipases or acylhydrolases **ZMO0444** and **ZMO0445**, membrane-bound O-acyltransferase ZMO0446, and hypothetical protein **ZMO0447**. **ZMO0444**, ZMO0446, and **ZMO0447** are cofit (all r > 0.95) and mutants in these genes are sensitive to a wide range of stresses. We propose that these genes act together to affect membrane integrity.

**ZMO0478** encodes a response regulator that is cofit with the cotranscribed peptidoglycan-binding protein encoded by ZMO0479 (r = 0.74) and a histidine kinase encoded by ZMO0480 (r = 0.83). **ZMO0478**:ZMO0480 are required for optimal fitness during acid stress, UV irradiation, and in the presence of paraquat dichloride, sodium sulfate, sodium sulfite, phosphomycin, bacitracin, methotrexate, and FCCP. This three gene operon is homologous to similar systems in the alpha-proteobacteria *Sphingomonas wittichii* RW1 (Swit\_4742:Swit\_4740) and *Caulobacter crescentus* CB15 (CC3325:CC3327). In addition, in vitro phosphotransfer assays in *Caulobacter crescentus* demonstrated that the histidine kinase CC3327 specifically signals to CC3325 ([Skerker, Prasol et al. 2005](#_ENREF_23)), further establishing the biochemical and functional relationship between these genes. Given the diversity of conditions under which these genes have reduced fitness and the presence of a conserved peptidoglycan-binding protein between the histidine kinase and response regulator, we propose that these genes sense or respond to changes (such as those induced by stress or cell division) in the cell wall.

**ZMO0495** (DUF490) is highly cofit with and is conserved next to (downstream of) ZMO0496 (r = 0.97), which encodes an outer membrane protein. According to PFam, DUF490 has distant homology to asmA, which affects lipopolysaccharide structure and/or the assembly of outer membrane proteins. Also, a homolog of **ZMO0495** from *Xanthomonas axonopodis* pv. *citri* (XAC4203) is required for biofilm formation and mutants in this gene had altered LPS and reduced EPS ([Li and Wang 2011](#_ENREF_15)). Mutants in both **ZMO0495** and ZMO0496 are very sensitive to a variety of stresses, especially aromatic acids. We propose that **ZMO0495** and ZMO0496 both act to affect the outer membrane.

**ZMO0767** (no domains, but PSI-BLAST finds hits to VIMSS 589130, a porin or outer membrane protein) is cofit with **ZMO1319** (tetratricopeptide repeat, r = 0.81). Both genes are detrimental to fitness on a variety of antibiotics and important for resisting acid stresses. We suspect that both genes play a role in the outer membrane.

**ZMO0780**:**ZMO0779** encode a membrane fusion protein and an outer membrane efflux protein and are conserved next to a putative (inner membrane) efflux transporter (ZMO0778). The three genes form a conserved operon and are strongly cofit (all pairwise r >= 0.96). This suggests that they comprise a complete system for exporting some compounds from the cytoplasm to the outside of the cell. These genes are very important for resisting a variety of stresses, with the strongest sensitivity to 4-hydroxymandelic acid and related aromatic acids, sodium sulfate, sodium sulfite, 5-fluoroorotic acid, vancomycin, and aztreonam, and they are strongly detrimental to fitness in the presence of cloxacillin.

**ZMO0803** contains a tetratricopeptide-like helical domain, which is often involved in protein-protein interactions, and a sporulation-related domain (PF05036) that may bind peptidoglycan. **ZMO0803** is cofit with ZMO1186 (mltB, r = 0.87), a lytic transglycosylase that probably recycles peptidoglycan. So we propose that **ZMO0803** may help to regulate peptidoglycan recycling. **ZMO0803** is also cofit with the hypothetical protein **ZMO1892** (r = 0.87), which contains a putative lipoprotein attachment site (PS51257), which suggests that **ZMO1892** may be involved in this process as well.

**ZMO0910** is predicted to encode an ATPase of an ABC transporter and is part of a gene cluster (ZMO0908:ZMO0911) that has similarity to genes involved in the export of capsular polysaccharides, such as the CtrABCD sialic capsule transport system of *Neisseria meningitidis* ([Frosch, Edwards et al. 1991](#_ENREF_8)). **ZMO0910** is cofit with the polysaccharide export protein ZMO0911 (r = 0.96) and the ABC transporter ZMO1467 (r = 0.86). In most bacterial genomes, the orthologs of **ZMO0910** (COG TagH) and ZMO1467 (COG TagG) are encoded in the same operon. Overall, our fitness data strongly suggests that ZMO1467 and **ZMO0910** encode components of the same ABC transporter and that this transporter is involved in the transport of capsular polysaccharides. These genes are important for fitness in most rich media conditions, but not in minimal media, and were strongly detrimental to fitness during growth on amoxicillin.

**ZMO0934** (zliE) is cofit with the lysR-type regulator **ZMO1206** (r = 0.98). ZliE has no homology to any InterPro domain but is reported to affect the expression of extracellular sucrose-metabolizing enzymes ([Kondo, Toyoda et al. 1994](#_ENREF_13)). Both ZliE and **ZMO1206** are important for resisting some stresses (e.g., hydrogen peroxide) and detrimental in the presence of other stresses. ZliE is upstream of and possibly co-transcribed with ZliS (ZMO0932), which also affects the expression of the sucrose enzymes, but ZliE and ZliS are not cofit (r = -0.13). The stress phenotypes suggest that ZliE may affect the secretion of proteins more generally and **ZMO1206** may be involved in the regulation.

**ZMO0947** (glycosyl transferase family protein) is expected to be involved in cell wall synthesis. **ZMO0947** is cofit with another poorly annotated protein, the hypothetical protein **ZMO0502** (r = 0.81). **ZMO0502** does not contain any InterPro domains but some homologs of **ZMO0502** are fused to a gtrA-like domain (PF04138) that is probably involved in the synthesis or export of cell wall polysaccharides. Both **ZMO0947** and **ZMO0502** are expected to localize to the cytoplasmic membrane (psortb ([Yu, Wagner et al. 2010](#_ENREF_26))). Thus, we propose that both **ZMO0947** and **ZMO0502** are involved in the export or synthesis of a component of the cell wall.

The operon **ZMO0964**-ZMO0965-ZMO0966 encodes a putative efflux system. These genes are important for resisting a variety of stresses but are also detrimental to fitness on some antibiotics. **ZMO0964**:ZMO0965 are strongly cofit (r = 0.98) while ZMO0966 has a somewhat different fitness pattern from the others (r = 0.68, r = 0.67). This system is homologous to vceC-vceA-vceB of *Vibrio cholerae*, which form a multi-drug efflux pump ([Woolley, Vediyappan et al. 2005](#_ENREF_25)). In *Zymomonas mobilis*, this system is most important for resisting organic acids, but it also is important for growth in unamended rich media with no shaking and is detrimental to fitness on a range of stresses (especially beta lactam antibiotics, although mutants in ZMO0966 lack those phenotypes).

The operon ZMO2008-ZMO0982-**ZMO0981** encodes a putative ABC transporter and is cofit (all pairwise r above 0.95). This subfamily of ABC transporters (dpp type) are often annotated as dipeptide or oligopeptide transporters, but mutants in the operon are sick in defined media with no peptides, which seems inconsistent with that annotation. These mutants are also sensitive to a variety of stresses and detrimental to fitness on some antibiotics (especially beta lactam antibiotics). Furthermore, some homologous operons include essential proteins (gatA ([Christen, Abeliuk et al. 2011](#_ENREF_6))) or putative cell-wall remodeling genes or beta-lactamases. We propose that this operon may be involved in forming the cell envelope.

**ZMO1015** (DUF330) is the last gene in the conserved operon **ZMO1018**:**ZMO1015** and is cofit with **ZMO1016** (r = 0.78), **ZMO1017** (r = 0.82), and **ZMO1018** (r = 0.73). **ZMO1018**:**ZMO1016** are similar to ABC-type transporters (ttg2BAC; **ZMO1016** is also referred to as mammalian cell entry related or mce). Homologous operons are involved in toluene resistance or uptake of glutamate or export of homogentistate, but these contain ttg2D instead of DUF330. Based on comparative genomics, DUF330 was proposed to be a part of an ABC transporter system involved in remodeling the cell envelope ([Casali and Riley 2007](#_ENREF_4)). Mutants in the **ZMO1018**:**ZMO1015** operon are sensitive to diverse stresses and are detrimental to fitness on a few stresses (especially beta lactam antibiotics), which is consistent with it affecting the cell envelope.

**ZMO1317** is annotated as a hypothetical protein but it is has a nucleotide kinase domain (PF02223). It is cofit with a putative NTP hydrolase (ZMO1318, r = 0.92), which is actually a paralog (38% identity). Mutants in both genes are sensitive to diverse stresses but are not sick in most experiments. These genes are also homologous to the uncharacterized *E. coli* protein yghT; mutants in yghT gene are sensitive to the fluoroquinolone antibiotic norfloxacin ([Nichols, Sen et al. 2011](#_ENREF_17)). Gene context suggests a role in cell wall synthesis, which could be a mechanism by which these genes would affect the permeability of the cell to diverse substrates.

The putative response regulator and transcription factor **ZMO1322** is cofit with the histidine kinase ZMO1323 (r = 0.75). This system is homologous to the pH response chvIG of *Agrobacterium tumefaciens* ([Li, Jia et al. 2002](#_ENREF_16)), and mutants in **ZMO1322**:ZMO1323 are sensitive to a variety of organic acids but also to other stresses.

The lysR-type regulatory gene **ZMO1336** is cofit with and conserved next to the divergently transcribed gene **ZMO1337** (r = 0.78). **ZMO1337** is probably an iron binding protein related to eukaryotic pirin (PF02678). Mutants in these genes are specifically sensitive to hydroquinone. We speculate that **ZMO1336** activates the expression of **ZMO1337** and that **ZMO1337** might detoxify hydroquinone.

The putative efflux system ZMO1432:ZMO1429, is important for hydrolysate tolerance ([Skerker, Leon et al. 2013](#_ENREF_22)) and for anaerobic growth, and is strongly cofit (median r = 0.97). ZMO1432 might be an aromatic acid exporter (PF04632); **ZMO1431** belongs to DUF1656; **ZMO1430** is related to hlyD and might be involved bringing the outer membrane and inner membrane together; and ZMO1429 is an outer membrane efflux protein. **ZMO1431** has two transmembrane helices and a possible signal peptide (as predicted by TMHMM: http://www.cbs.dtu.dk/services/TMHMM/) and is probably also involved in transport. The substrate of this system remains unclear.

**ZMO1510** is annotated as modification methylase HemK family protein but is most likely a methyltransferase modifying release factor (TIGR03534). Consistent with that, it is most cofit with elongation factor P (ZMO0328, r=0.86), and it is downstream of release factor 1 (prfA, ZMO1509).

**ZMO1529**:**ZMO1525** (**ZMO1529**=COG-acrA, ZMO1528=COG-acrB, ZMO1527=COG-acrB, and **ZMO1525**=COG-tolC) are a putative efflux system. They are strongly cofit (median r = 0.93). Mutants in this operon are very sensitive to a variety of antibiotics, including bacitracin, the macrolides erythromycin, clarithromycin, and spiramycin, rifampicin, nigericin, actinomycin D, the beta-lactams ceftazidime, amoxicillin, cloxacillin, and cefsulodin, vancomycin, and MreB perturbing compound

A22. These genes may encode an efflux pump with broad specificity, or it could export a component of lipopolysaccharide or the cell wall.

We propose that **ZMO1530**, together with ZMO1922:ZMO1923 and ZMO1299, is involved in the synthesis of a polysaccharide capsule. **ZMO1530** is annotated as kpsF/gutQ family protein and has a sugar isomerase (SIS) domain followed by two CBS domains. It is related to kpsF, which is involved in capsular polysialic acid synthesis in some strains of *E. coli* (TIGR00393). ZMO1530 is cofit with capsular biosynthesis proteins ZMO1922 (r=0.92), ZMO1299 (r=0.88), and ZMO1923 (r=0.84). ZMO1299 (DUF227) is similar to an uncharacterized capsular biosynthesis protein bcbG of *Pasteurella multocida* and may be a kinase. ZMO1923 is annotated as a sulfatase but is related to rkpI, which is involved in synthesis of a K-like capsular polysaccharide in *Rhizobium meliloti* ([Kiss, Reuhs et al. 1997](#_ENREF_12)) and might actually be a phosphoglycerol transferase. We are not aware of any biochemical study of extracellular polysaccharides of *Zymomonas mobilis* but it can form a polysaccharide capsule ([Kirk 1994](#_ENREF_11)).

**ZMO1591** and **ZMO1590** are strongly cofit (r=0.95). **ZMO1591** is annotated as ABC transporter permease component and **ZMO1591** is DUF140 (multiple transmembrane helices); both genes are important for resisting plant hydrolysate and some aromatic compounds. These genes are related to a system for toluene resistance in *Pseudomonas putida* ([Kim, Lee et al. 1998](#_ENREF_10)). In *Z. mobilis*, we propose that **ZMO1591** and **ZMO1590** form an efflux pump.

As of the 2011 release of MicrobesOnline ([Dehal, Joachimiak et al. 2010](#_ENREF_7)), the hypothetical gene **ZMO1630** lacked any homologs or matches to domain databases. Since then, the genome sequence of *Z. mobilis* subsp. pomaceae ATCC 29192 has become available, which does contain a homologous protein (as detected by blastp against NR). The tiling microarray data shows that **ZMO1630** lies within an operon, ZMO1631:**ZMO1628**. In the fitness data, **ZMO1630** is cofit with **ZMO1628** (r=0.81) and to some extent with the ZMO1629 and ZMO1631 (r = 0.60 and r=0.38, respectively, which are the next closest hits). ZMO1631 is annotated as a tonB-like siderophore receptor protein. There is an orthologous operon in *S. oneidensis* MR-1 (based on similarity of **ZMO1628** to SO\_0449) without the tonB-like protein. ZMO1630 and SO\_0448 lack detectable homology but are about the same length, are in the same location in the operon, and have very similar membrane topologies, with signal peptides and 3 transmembrane helices (as predicted by TMHMM: http://www.cbs.dtu.dk/services/TMHMM/), so they may be highly diverged orthologs. In *Z. mobilis* ZM4, mutants in **ZMO1628**:**ZMO1630** are sensitive to catechol or protocatechualdehyde, which are similar compounds (both have benzene rings with two adjacent hydroxyl groups) and are siderophores. Our prediction is that the ZM4 system naturally promotes the uptake of a ferric siderophore, while in our conditions, **ZMO1628**:**ZMO1630** acts as an efflux pump for catechol and protocatechualdehyde.

Hypothetical protein **ZMO1718** (has homologs but no domains) is cofit with the tonB helper protein ZMO0162 (r=0.77). **ZMO1718** is also adjacent to (divergent from) other tonB-related proteins (**ZMO1717**, ZMO1716, ZMO1715, ZMO2025). We propose that ZMO0162 and surrounding proteins including **ZMO1718** are involved in the export of an outer membrane protein.

**ZMO1723** encodes a hypothetical protein with homology to laccase and is cofit (r = 0.85) with the adjacent ZMO1722, a putative glutathione detoxification protein. Mutants in both **ZMO1723** and ZMO1722 have reduced fitness in plant hydrolysate and methylglyoxal ([Skerker, Leon et al. 2013](#_ENREF_22)), suggesting a common function for these proteins in tolerating oxidative stress.

**ZMO1733** encodes a transcriptional regulator (lysR family) that is cofit with genes in glutathione synthesis: ZMO1556 (gsh, r = 0.81) and ZMO1913 (gshB, r = 0.69). In addition, **ZMO1733** is cofit with the adjacent and divergently transcribed ZMO1732 (ahpC, annotated as alkyl hydroperoxide reductase, r = 0.66). Mutants in **ZMO1733**, ZMO1556, ZMO1913, and ZMO1732 have reduced fitness in almost all aerobic experiments but have no fitness defects in a subset of the anaerobic experiments. Lastly, the ortholog of **ZMO1733** in *Caulobacter crescentus*, CC3697, was recently shown to be OxyR ([Italiani, da Silva Neto et al. 2011](#_ENREF_9)). Taken together, our data strongly suggests that **ZMO1733** is OxyR and regulates ZMO1732 and other genes involved in the response to oxidative stress.

**ZMO1734** (annotated as UDP-glycosyltransferase family) is cofit with nucleotide sugar dehydrogenase ZMO0819 (r = 0.85). Additionally, comparative genomics places ZMO1734 in close proximity with other cell wall synthesis genes. Taken together, we propose that ZMO1734 and ZMO0819 might act together to make some component of the cell wall.

The response regulator **ZMO1738** is cofit with the adjacent histidine kinase ZMO1739 (r = 0.83). Mutants in ZMO1738 and ZMO1739 have reduced fitness in almost all conditions suggesting that this two-component system may be essential for viability in *Z. mobilis.* Furthermore, the next cofitness hits to ZMO1738 are mviN (ZMO1639, r = 0.80) and secD (ZMO1897, r = 0.78), which are essential genes in both *E. coli* ([Baba, Ara et al. 2006](#_ENREF_1)) and *C. crescentus* ([Christen, Abeliuk et al. 2011](#_ENREF_6)).

**ZMO1790** has ATP transporter ATPase and permease domains, but no conserved proximity to other functionally related genes. Some homologs are involved in lipid A export or are eukaryotic P-glycoproteins (efflux pumps). **ZMO1790** is strongly cofit with hemE (ZMO1998, r = 0.88) and other heme synthesis proteins. However, the role of **ZMO1790** is not obvious as there is a predicted heme exporter in *Z. mobilis*, ZMO1258 (cytochrome c maturation protein CcmC), that has rather different phenotypes.

As described in the main text, **ZMO1808** is rnfH.

The hypothetical gene **ZMO1875** is cofit with bolA (ZMO1874, r = 0.83) and its putative role in Fe-S cluster synthesis and/or repair was previously discussed ([Skerker, Leon et al. 2013](#_ENREF_22)).

**ZMO1916** is annotated by SEED as bioH ([Overbeek, Begley et al. 2005](#_ENREF_18)), but it is very diverged. The annotation may derive from the proposal that bioK, which is orthologous to **ZMO1916**, replaces bioH in cyanobacteria ([Rodionov, Mironov et al. 2002](#_ENREF_21)), or it may derive from proximity. To the best of our knowledge, there is no published experimental evidence as to the function of bioK. The role of **ZMO1916** during biotin synthesis is supported by cofitness to biotin synthase ZMO0094 (r = 0.949) and dethiobiotin synthase (ZMO1915, r = 0.87). However, it is difficult to rule out polar effects, as **ZMO1916** is upstream of ZMO1915.

**ZMO1997** is involved in heme/porphyrin/cobalamin synthesis (its top cofit hits are all in heme synthesis). It is downstream of hemE (ZMO1998) and is in PF03653 / UPF0093 / COG1981. SEED annotates **ZMO1997** as Protoporphyrinogen IX oxidase, novel form, HemJ (EC 1.3.-.-), based on a recently characterized homolog in *Acinetobacter baylyi* (BLAST reports 32% identity) ([Boynton, Gerdes et al. 2011](#_ENREF_2)).

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