Supplemental Material for Waters and Nicholson, 2015
"Experimental evolution of *Bacillus subtilis* to enhanced growth at low atmospheric pressure: genomic changes revealed by whole genome sequencing (WGS)"

Supplemental Materials and Methods:

WGS: raw data processing and identification of mutations. Raw left- and right-end reads were analyzed using the Galaxy platform (1-3) and were run through the following tools before mapping: FASTQ Groomer (4), FASTQC, and Rename Sequences (based on the FASTX-toolkit by Assaf Gordon)(4). For mapping the left- and right-hand reads, BWA (5) was used using the reference *Bacillus subtilis* strain 168 genome sequence downloaded from the NCBI genome website (NCBI accession number NC_000964.3). The resulting SAM files were converted to BAM format using SAMtools (6). Coverage was determined by using BEDTools ("create a histogram of genome coverage", maximum depth set to 1,000,000) (7) and plotting the data using the R program; this revealed a high coverage across each strain's genome of >100x with a mean of ~5000x. SAMstat (8) and flagstat (6) were run to determine mapping quality, mapped reads and properly paired reads that were mapped. SNPs and InDels were called using the Unified Genotyper Tool (9). For quality control, two separate mappings were conducted with the reads from each strain: a mapping without filtering reads and one with the reads filtered based on quality before mapping. Filter FASTQ (9) (minimum quality 28, maximum quality 50, maximum bases allowed outside of quality range was 5) was used in the latter case and resulted in 41.09% and 43.28% of the left and right hand reads being kept, respectively for WN1106, and 49.07% and 44.51% of the respective left and right hand reads of WN624. Both filtered and unfiltered mappings resulted in the same SNP and InDel high quality calls; however, the percentage of reads that were properly paired for the filtered data was very low (<1%), but still resulted in ~ 10x coverage of the genome. Before verification by Sanger sequencing, all high mapping quality (>1,000) single-nucleotide polymorphisms (SNPs) and insertion/deletions (InDels) in strain WN1106 were verified using BAMview, a visualization tool for viewing BAM files.

Supplementary Results and Discussion:

***bacD* analysis.** The SNP in the coding region of *bacD* causes a residue change of E97Q located in the N-terminal region of the BacD enzyme. BacD is an L-amino acid ligase, which *in vivo* is responsible for the ATP-dependent ligation of L-alanine and L-anticapsin and the production of the antibiotic bacilysin (10). *In vitro* this enzyme is promiscuous in its substrate recognition (10), and its 3-dimensional structure has been determined (11). A structural alignment between wild-type and mutant protein indicates that there are no disruptions to any helix region. However, as would be expected, the surface charge in this region is changed. Due to the mutation occurring in a stretch of residues between two loops (loops 2 &3) that are involved in orientation of substrates and catalysis within the binding pocket, it is possible this charge difference could cause slight structural alterations and affect the catalysis, but this notion requires further studies. However, it is unclear how a mutation in BacD would affect growth of *B. subtilis* at LP. This mutation swept through the 5 kPa evolution experiment populations at ~ 900

generations and could be a neutral hitchhiker mutation as it co-occurs with the mutations in *resD, walK* and *yvlD*. All four of these mutations occur during the second sweep of the *rnjBΔ9* deletion (**Fig. 3**). Further analysis of the role of wild-type and mutant BacD at LP are needed to determine if this mutation is neutral, beneficial or possibly having ill effects.

***fliI* analysis.** The gene *fliI* is located within the large (>26 kbp) *fla/che* operon of *B. subtilis* and codes for a cytoplasmic flagella-specific ATPase of the Type III secretion system responsible for flagella component export (12-14). It is thought that flagellar subunits are exported in an ATP-dependent unfolding process facilitated by FliI in a complex with the chaperone, FliJ, and the regulator of both, FliH (12, 14). *fliI* mutants are known to be deficient in flagella (12, 15-17), resulting in a non-motile phenotype. Therefore, a motility phenotype was investigated for the P35T FliI mutation of WN1106 compared to the ancestor, WN624. First, liquid overnight cultures of both strains were spotted and compared under a microscope; WN624 cells exhibited aerotaxis, i.e. cells swarmed to trapped air bubbles, whereas the WN1106 cells appeared non-motile when viewed. A subsequent standard 0.3% motility agar spot test was performed, confirming the motility of the ancestor and the non-motile phenotype of the LP-evolved WN1106 when grown for 6 hr. at 37°C (data not shown).

The *fliI* SNP does not occur in the highly conserved Walker A and B motifs involved in nucleotide binding of ATPases (18, 19), but resides in the N-terminal domain region at the inter-subunit interface (20). FliI is known to oligomerize to a homohexameric ring (21) that is structurally homologous to the α/β-subunit of F_1-ATPases and the ATPase EscN from *E. coli* (14, 22). The N-terminal first 100 a.a. of FliI forms a six-stranded β-barrel that interacts with FliH (23) and may be responsible for oligomerization (20). Okabe et al. (20) investigated with in-frame deletion analysis the first 100 a.a. of *Salmonella* FliI; deletions of amino acids 20-40 caused non-motility and evidence suggested that amino acids in this region were involved in ATPase suppression and docking interaction between the export gate and the FliH-FLiI complex (20). By analogy, the mutation of *B. subtilis* P35T in this region might be affecting oligomerization and/or docking efficiency with the exportation port.

It has been found previously, that long-duration continuous culturing of *B. subtilis* may lead to mutations affecting motility due to lack of chemical gradients in a shaken flask (24); loss of motility has been reasoned to be beneficial to bacterial life-in-a-flask due to energy costs (25), as flagellar rotation and assembly are expensive in terms of materials, energy, and protonmotive force (26). As this SNP arose and dominated early in all detectable cells lines from resequencing the 5 kPa E.E. stocks (**Fig. 3**), it is thought that the loss of motility, due to the P35T mutation, gave a higher fitness advantage during the 5 kPa evolution experiment, however, not due to overcoming the inhibitive nature of LP *per se*, but by eliminating an unproductive drain on energy expenditure during cultivation, thus allowing cells to devote more resources to an increased growth rate.

***parC* analysis.** The SNP in the *parC* coding region changes an aspartate to histidine at position 205 (D205H). The *parC* gene codes for ParC, the topoisomerase IV subunit A, an essential gene (27) involved in decatenation and also, evidence suggests, replication fork movement (28, 29); the mutation occurs in the tower domain of the protein, whose primary function is believed to be structural, but which also contributes to DNA binding. Sequencing analysis of when this mutation occurred during the 5 kPa

evolution experiment reveals that a minority of the population possessed this mutation during the first sweep of the *rnjBΔ9* deletion (**Fig. 3**) and also possessed the *ytoI* SNP (see below). It can be reasoned that this population most likely included the ancestral cell line leading to strain WN1106.

***ytoI* analysis.** The mutation in *ytoI* results in a valine to phenylalanine change at residue 77 (V77F). This mutation occurred early during evolution at 5 kPa, co-occurring in the population with the *fliI* and *rnjBΔ9* deletion mutations (**Fig. 3**). YtoI is an uncharacterized protein with similarities to other uncharacterized proteins. It is unknown how this mutation affects the ability of *B. subtilis* to grow at LP, and as it co-occurred with *fliI*, *parC*, and the *rnjBΔ9* deletion, may be a neutral hitchhiking mutation; further investigations into the *ytoI* mutation in a clean background (i.e. with no other mutations) at 5 kPa are currently underway to investigate its putative role in LP growth of *B. subtilis*.

***resD* analysis.** The SNP detected in *resD* occurred late in the LP-evolution experiment, at Generation 850 (**Fig. 3**). ResDE is the anaerobic 2-component system in *B. subtilis* (30), with ResD functioning as the response regulator. ResD belongs to the OmpR-like response regulator family (33). We have previously reported that strain WN1106 has a decreased relative fitness compared to the ancestor under hypoxia but standard atmospheric pressure, indicating that any LP advantage WN1106 gained during the 5 kPa evolution experiment was most likely not a result of anaerobic adaptive changes (34). Two OmpR-like response regulators which have had their structures determined were used to analyze the mutation in ResD: PhoB from *E. coli* (35) and MtrA from *Mycobacterium tuberculosis* (36). Structural alignments revealed that the mutation of a proline to glutamine at position 110 in ResD corresponds to a stretch of amino acids involved in molecule-molecule interactions involved in homodimerization (35). However, ResD, unlike other OmpR-like response regulators, does not form homodimers either in its phosphorylated or unphosphorylated form (37). Although ResD does not form homodimers, some ResD-dependent promoters contain multiple tandem binding sites for ResD. One example is the promoter for *ctaA*, in which three ResD binding sites are required for full expression (37).

***rnjB* analysis.** Early during the LP-evolution experiment (Generation 250), a 9-bp in-frame deletion occurred in the coding region of *rnjB*, resulting in loss residues 183-AKI-185 (**Fig. 3, Table 2**). The *rnjB* gene encodes RNase J2, which together with RNase J1 (also known as RnjA) forms a complex (RnjA/RnjB complex) that is one of the major mRNA global processing systems in *Bacillus subtilis* (reviewed in ref. 38). The known primary function of RnjB is that of an endoribonuclease (39, 40) with RnjA, the essential riboenzyme in the complex, having the main exoribonuclease function as well as an endoribonuclease function (38, 40, 41). Both RnjA and RnjB are zinc metallo-hydrolases of the metallo-β-lactamase family and belong to the subfamily of β-CASP proteins, which act on RNA and DNA substrates (reviewed in ref. 42). The crystal structure of RnjA has been resolved, revealing an enzymatic pocket capable of accommodating monophosphorylated 5' ends, but not 5' triphosphate ends (43). RnjB shares ~ 48% sequence homology to RnjA; however, the overall surface charge difference between the two enzymes is significant: RnjA and RnjB have a pI values of 6.5 and 9, respectively (43). This charge difference is thought to be a contributing factor to the difference in enzymatic activity between the two (i.e. endo- versus exo- nuclease); it has also been shown that despite the non-essentiality of RnjB, the protein does exert an endonuclease

specificity role on RnjA; when RnjA is complexed with itself and not RnjB the cleavage site of certain RNA substrates differs, the reverse is also true of RnjB endoribonuclease recognition sites *in vitro* (40).

As stated above, RNase Js are members of the β-CASP subfamily of proteins and there exists conservation in protein structure when compared. The deletion event in *rnjBΔ9* occurred in the highly conserved α5 helix in the β-CASP domain (43); therefore the deletion was studied *in silico* for structural perturbations to this region. Secondary and tertiary structure predictions were performed and indicate that the α5 helix region no longer forms and in its place an unstructured region exists. This region is predicted to act as a structural hinge between the metallo-β-lactamase and β-CASP domains (43).

***walK* analysis.** The SNP in *walK* occurred towards the end of the LP-evolution experiment at ~ 850-900 generations (**Fig. 3**). WalKR is an essential 2-component system controlling cell wall metabolism, with WalK serving as a transmembrane sensor kinase (31). WalK function is believed to be modulated by two integral membrane proteins, YycI and YycH, each of which possesses a transmembrane helix that has been reported to have interactions with the two transmembrane helices of WalK; mutations in these contacting residues have been shown to affect *yocH* transcript levels (32). The SNP in WalK (T195M) occurs in the second of these helices (transmembrane helix 2, TM2) and may affect the intermolecular contacts that regulate WalK function.

Supplemental Table S1. WGS statistics for strains WN624 and WN1106.

	Flagstat Data on Mapped Paired-End Reads	
	WN624	**WN1106**
Total Reads	104979183 + 0 in total (QC-passed reads + QC-failed reads)	106043431 + 0 in total (QC-passed reads + QC-failed reads)
Duplicate Reads	0 + 0 duplicates	0 + 0 duplicates
Mapped Reads	102249189 + 0 mapped (97.40%:-nan%)	101084682 + 0 mapped (95.32%:-nan%)
Paired Reads	104979183 + 0 paired in sequencing	106043431 + 0 paired in sequencing
Left Reads	52473457 + 0 read1	53013438 + 0 read1
Right Reads	52505726 + 0 read2	53029993 + 0 read2
Properly Paired Reads	99079843 + 0 properly paired (94.38%:-nan%)	99688928 + 0 properly paired (94.01%:-nan%)
Paired Reads Mapped	101628499 + 0 with itself and mate mapped	100387188 + 0 with itself and mate mapped
Single Mapped Reads	620690 + 0 singletons (0.59%:-nan%)	697494 + 0 singletons (0.66%:-nan%)

Supplemental Table S2. SNPs at which ancestor strain WN624 differs from the published *B. subtilis* strain 168 sequence (44).

Locus	Position (168)[a]	Mutation[b]	AA Change[c]	QB928 SNP matches:[d]
veg (upstream)	52646	C>T		168
scoC	1073117	C>A	V201L	168
oppD	1224525	T>G	G357V	WN624
sigI	1412484	T>G	L198R	168
mswC (riboswitch)	1424638	T>G		WN624
sepF	1610896	T>C		WN624
rluD	1618068	C>T	S287P	WN624
trmD	1675849	C>T		WN624
recA (upstream)	1764559	C>T		168
gltA	2011091	G>A		168
yozT	2041099	A>G		WN624
yoqA	2201409	A>G		WN624
uvrX	2271425	T>C		WN624
uvrX	2271505	C>T		WN624
uvrX	2271523	A>C		WN624
ypiB	2366016	T>C	H87R	168
rluB	2421606	T>C		WN624
yqeZ	2619105	C>T		WN624
ssrSB (upstream)	2814468	C>T		WN624
ilvC	2893906	G>A		168
citZ (upstream)	2982417	T>A		WN624
sftA	3051461	G>A	P375S	WN624
comP	3253956	T>C	E628G	168
yutE	3319154	C>T	A37T	WN624
gerAA	3391676	A>G	T299A	WN624
epsC	3527377	G>A	A276V	WN624
pgdS	3696869	T>C		WN624
sacA	3902306	C>A	F448L	WN624
yxjM	3993539	G>T		WN624
yxbD	4095811	C>T	I9V	WN624

[a]Nucleotide position in the *B. subtilis* 168 genome (AL009126.3)
[b]Nucleotide change occurring in the WN624 genome SNP
[c]Amino acid change, if any, resulting from the nucleotide change
[d]Corresponding nucleotide in the genome of *B. subtilis* QB928 (CP003783.1)

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