SUPPLEMENTAL TEXT FILE S2: Supplemental Methods

RNA sampling and isolation. *C. acetobutylicum* RNA RNA was isolated as described (1) using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was isolated from *C. botulinum* strains using a similar protocol except for the following changes. Following lysozyme incubation and vortexing with glass beads, TRI-RT-LS solution (Molecular Research Center (MRC), Inc., Cincinnati, OH) was added instead of TRIzol (Invitrogen). Following centrifugation and the transfer of the supernatant to a new tube, 50 µl of BAN (Bromoanisole; MRC) was added to the sample, vortexed for 30 sec, and incubated at room temperature for 5 min. Tubes were spun again for 15 min at 12,000xg at 4°C, and the supernatant transferred to a new tube. *C. botulinum* RNA was isolated by Marite Bradshaw in the Johnson laboratory.

RNA pools. For the validation of a select number of predicted sRNAs, a number of different RNA pools were created. For the quantitative (Q)-RT-PCR analysis of *C. acetobutylicum* sRNAs, a pool was created for each timepoint (i.e., unstressed 6 hrs, 12 hrs, 18 hrs, & 30 hrs, and 30 min & 1 hr after each stress) from the four replicates for each type of condition (i.e., unstressed, butanol stress, and butyrate stress). To create the pool for each timepoint, 2 µg of total RNA from each replicate were combined, and the total volume was brought up to 60 µl with nuclease-free water. Each pool was then reverse transcribed into cDNA, as described below. Finally, 12 µl of cDNA from each timepoint pool was combined into an overall pool called Pool CAC1. Q-RT-PCR was then carried out on Pool CAC1.

For the Northern validation of *C. acetobutylicum* sRNAs, a different type of an RNA pool was created consisting of all timepoints and conditions from one biological replicate of all three types of cultures (i.e., unstressed, butanol stress, and butyrate stress). Equal mass of total RNA

from each timepoint and culture was combined for each replicate to create this type of pool, called Pool CAC2. Several such pools were made and used. For the Northern gel, 20 µg of total RNA from Pool CAC2 were loaded, as described below.

For the Q-RT-PCR and Northern validation of *C. botulinum* sRNAs, pools were created for each strain by combining 5 µg of total RNA from each timepoint (i.e., 4, 5, 6, 8, 10, 12, and 24 hrs). The pools are called Pool CLK, Pool CBO, and Pool CLC for *C. botulinum A3 Loch Maree, C. botulinum A ATCC 3502*, and *C. botulinum A Hall*, respectively. For Q-RT-PCR, 2 µg of total RNA was reverse transcribed from each pool, and for Northern analysis, 20 µg of total RNA was loaded onto Northern analysis gels.

cDNA generation and Q-RT-PCR analysis. RNA was reverse transcribed and Q-RT-PCR was performed as described (2). Primer sequences used are listed in Additional File 4. As an additional negative control, Q-RT-PCR was performed using primer sets for 15 sRNA predictions in *C. acetobutyicum* on RNA which was not reverse transcribed. For these 15 primer sets, C_t values ranged from 30-34, indicating minimal DNA contamination.

Rapid amplification of cDNA ends (RACE) reactions (5' RACE and 3' RACE). To determine the length of sCAC610, 5' RACE and 3' RACE were performed with 4µg of total RNA from *C. acetobutylicum*. 5' RACE was performed using the GeneRacer™ Kit (Invitrogen) according to manufacturer's instruction with the following modifications. Calf Intestinal Phosphatase (CIP) treatment at the start was omitted because prokaryotic RNA was used and a control without Tobacco Acid Pyrophosphatase (TAP) treatment was included (3). First-strand cDNA was synthesized with random hexamer primers using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Nested PCR was performed with 0.5µl of RT reaction as template with the following reaction conditions, 0.7µM of GeneRacer™ 5'

Primer (5′-CGACTGGAGCACGAGGACACTGA-3′), 0.7 µM of Gene Specific primer (5' ATCCTTACAGCCCTTAGATATACACAG 3'), 0.25 mM $MgCl_2$, 0.2 µM of dNTPs, 1x PCR buffer II, 2.5 U of AmpliTaq Gold® DNA Polymerase (Invitrogen) in 50 µl reaction volume. PCR product (~200bp) was subcloned into pCR®-TOPO® TA Vector using PCR®8/GW/TOPO® TA Cloning Kit (Invitrogen) and subjected to DNA sequencing. For 3' RACE total RNA was polyadenylated using Poly(A) Tailing Kit (Ambion) according to manufacturer's instructions. Polyadenylated RNA was then reverse transcribed into cDNA using SuperScript™ III RT Module of GeneRacer™ Kit according to manufacturer's instructions. Nested PCR was performed with 0.5 µl of RT reaction as template using 0.7 µM of GeneRacer™ 3′ Nested Primer (5′-CGCTACGTAACGGCATGACAGTG-3′) and 0.7µM of Gene Specific primer (5'-CTTGTGTTATGCTATAATAATTAGCTTA-3') with reaction conditions as mentioned above. PCR product (~80 nt) was then subcloned and sequenced as above.

References for this supplemental file.

1. **Jones, S. W., C. J. Paredes, B. Tracy, N. Cheng, R. Sillers, R. S. Senger, and E. T. Papoutsakis.** 2008. The transcriptional program underlying the physiology of clostridial sporulation. Genome Biol **9:**R114.
2. **Borden, J. R., S. W. Jones, D. Indurthi, Y. Chen, and E. T. Papoutsakis.** 2010. A genomic-library based discovery of a novel, possibly synthetic, acid-tolerance mechanism in Clostridium acetobutylicum involving non-coding RNAs and ribosomal RNA processing. Metab Eng **12:**268-81.
3. **Argaman, L., R. Hershberg, J. Vogel, G. Bejerano, E. G. Wagner, H. Margalit, and S. Altuvia.** 2001. Novel small RNA-encoding genes in the intergenic regions of Escherichia coli. Curr Biol **11:**941-50.