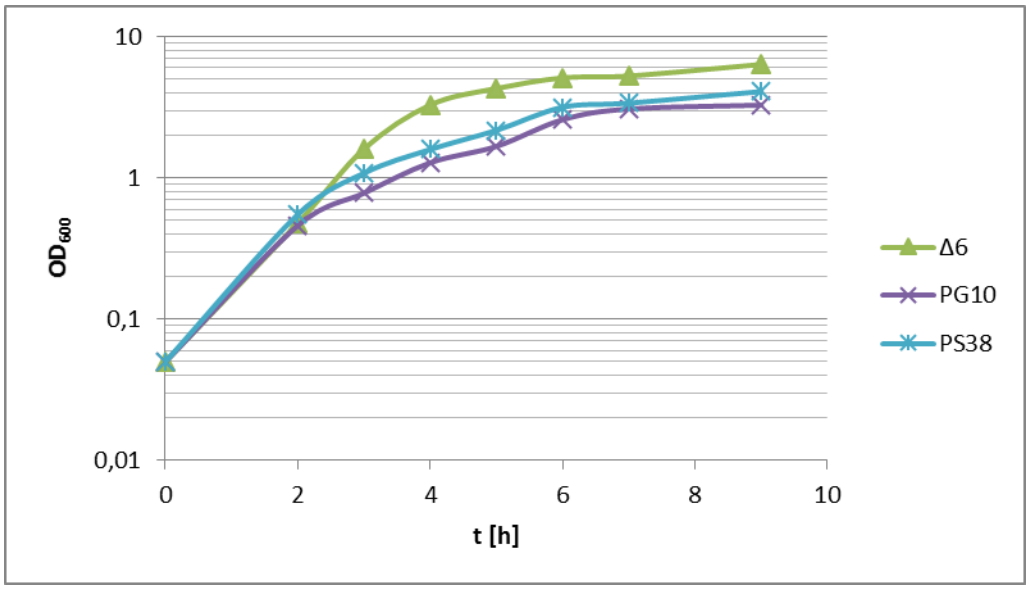
Supplemental Data S1: Phenotypic analysis of the genome-reduced strains

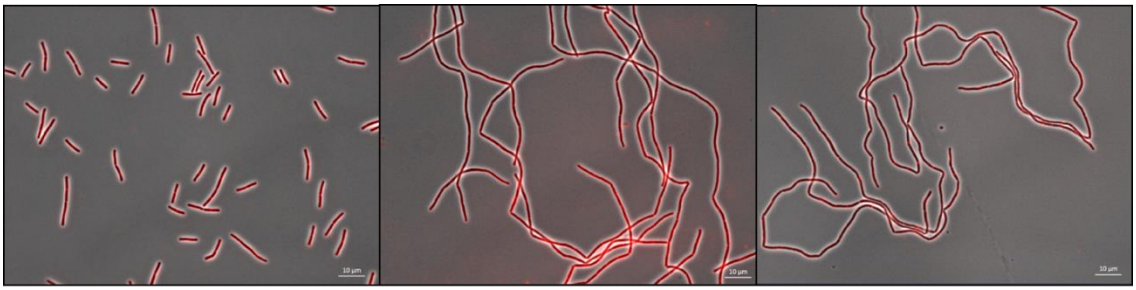
Growth of the genome-reduced strains in complex medium.

The two genome-reduced strains (PG10 and PS38) and the reference strain *B. subtilis* Δ6 were grown in Lysogeny broth supplemented with 0.5% glucose. The cultures were inoculated with exponentially growing cells and the experiment was performed at 37°C and 200 rpm agitation.



Light microscopy pictures of *B. subtilis* Δ6, PG10, and PS38.

The depicted cells are in the exponential growth phase (Lysogeny broth medium with 0.5% glucose) and their membranes were stained with Nile Red.



Δ6 PG10 PS38

Determination of the cell volume

Comparison of cell sizes for the reference strains Δ6 and the two genome-reduced strains. For details of the cytoplasmic volume determination see Supplemental Methods.

Strain	Length [µm] (median)	Width [µm] (median)	Volume [µm³] (median)	Cell division status of measured cells (single/dividing/chained cells in %)
Δ6	4.388	0.670	1.454	18/76.3/5.7
PG10	5.083	0.651	1.585	0/60.2/39.8
PS38	4.553	0.648	1.392	13/60.5/26.5

Genetic competence of the genome reduced strains

A major problem encountered with progressing genome reduction was a gradual decrease of genetic competence. After about 35 deletions only about 10 transformants per 1 µg plasmid DNA were obtained in comparison to more than 1,000 colonies with the starting strain. This problem was solved by integrating a *comK*/*comS* cassette under control of the mannitol-inducible *mtlP* promoter behind the *hisI* gene of the histidine operon. This did not only re-establish the original transformation rate; the new strain *B. subtilis* IIG-Bs27-24 showed a 20-fold higher transformation rate compared to the laboratory strain *B. subtilis* 168. Determination of genetic competence of the final deletion strains revealed no impact of the further deletions as compared to the strain into which the cassette was originally introduced (26,000 and 27,000 transformants per µg of DNA for PG10 and PS38, as compared to 1,200 and 29,000 transformants per µg of DNA for *B. subtilis* Δ6 and IIG-Bs27-47-24, respectively). This high level of genetic competence suggests that the deletion strains can be used for continued rounds of genome reduction and engineering.