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# Increased Competitive Fitness of *Bacillus subtilis* under Nonsporulating Conditions via Inactivation of Pleiotropic Regulators AlsR, SigD, and SigW

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**Previous studies implicated loss of motility and mutations of the *alsR* and *sigW* regulatory genes in enhanced fitness of the *Bacillus subtilis* evolved strain WN716 over that of its ancestral strain WN624. The fitness of strains carrying knockout mutations *alsR*::*spc*, *sigD*::*kan*, and/or *sigW*::*erm* was measured and compared to that of the congenic ancestral strain by competition experiments.**

In order to understand the mechanisms leading to increased fitness during bacterial evolution under a particular set of environmental conditions, a long-term study was undertaken in which *Bacillus subtilis* was allowed to evolve for 6,000 generations in a rich medium that repressed selection for sporulation (9, 10). During this experiment, new strains which displayed numerous altered phenotypes, including significantly different colony and cell morphologies, loss of postexponential phenotypes, such as sporulation, competence, acetoin production, and motility, multiple auxotrophies, and increased competitive fitness, emerged (11). One of these evolved strains, *B. subtilis* WN716, was examined in greater detail using a combination of phenotypic characterizations, transcription microarray analyses, and whole-genome resequencing (3, 11). From these analyses, it appeared that loss of function of two different pleiotropic transcriptional regulators was implicated in the greater fitness of strain WN716: (i) SigW, an alternative extracytoplasmic function (ECF) sigma factor controlling resistance to bacteriocins and cell envelope-damaging compounds (5), and (ii) AlsR, a LysR family positive transcriptional regulator of the *alsSD* operon, encoding enzymes of the acetoin pathway (16). However, we were unable to assign loss of motility to the mutation in the *sigD* gene encoding the alternative sigma factor SigD, which controls expression of motility, chemotaxis, and cell wall proteins (1, 3, 11). In order to test the hypothesis that loss of motility and AlsR and SigW functions had led to an increase in strain WN716's fitness during its evolution, this communication describes (i) the construction of congenic knockout mutants in which SigW, SigD, and/or AlsR was disrupted, either singly or in all possible combinations, (ii) phenotypic characterization of the resulting mutant strains, and (iii) competition experiments to test the fitness of the mutants compared to that of the ancestral strain from which the evolution experiments were initiated.

The *B. subtilis* strains used are described in Table 1. Strains carrying knockout mutations inactivating *alsR*, *sigD*, or *sigW* were generous gifts from Elisabeth Härtig and John Helmann. The plasmid pCm::Tc for switching of the *amyE*::*cat* antibiotic resistance cassette to *amyE*::*tet* (17) was obtained from the *Bacillus* Genetic Stock Center, Columbus, OH. The media used were Luria-Bertani (LB) (12) and the sporulation-repressing (R) medium previously used for laboratory evolution experiments (9). For normal cultivation or motility assays, the medium was supplemented with agar at final concentrations of 1.5% or 0.3%, respectively. Where appropriate, the following antibiotics were added to the medium (final concentration): chloramphenicol (Chl; 5 μg/ml), erythromycin (Erm; 5 μg/ml), fosfomycin (Fos; 50 μg/ml), kanamycin (Kan; 10 μg/ml), spectinomycin (Spc; 100 μg/ml), or tetracycline (Tet; 10 μg/ml). Techniques used for *B. subtilis* transformation (2), enumeration of viable cells (15), assaying of phenotypic properties, and performance of competitions (14) have been described previously.

The evolved strain WN716 exhibited a number of altered phenotypes likely controlled by the *alsR*, *sigD*, or *sigW* gene products, including a small-colony phenotype and lack of motility on R medium, cell filamentation, sensitivity to bacteriocins and fosfomycin, loss of acetoin production, and acidification of liquid medium by overproduction of acetate (Fig. 1). Therefore, the phenotypes displayed by ancestral strains WN624, WN628, and WN715 (11), as well as by evolved strain WN716 (11), were compared to those of congenic strains carrying knockout mutations of *alsR*::*spc* (strain WN1241), *sigD*::*kan* (strain WN1242), *sigW*::*kan* (strain WN1243), and *sigW*::*erm* (strain WN1244) (Fig. 1). Strain WN716 was observed to resemble the *alsR*::*spc* knockout strain in its production of acid (methyl red [MR] positive) but not acetoin (Voges-Proskauer [V-P] negative), its reduced motility on R motility agar, and its small-colony phenotype on R agar (Fig. 1). Strain WN716 resembled the *sigD*::*kan* knockout strain in its cell filamentation in R liquid medium and resembled the *sigW*::*kan* and *sigW*::*erm* knockout strains in its sensitivity to fosfomycin and the bacteriocin produced by *B. subtilis* subsp. *spizizenii* (Fig. 1). Note, however, that strain WN716 had not completely lost motility, as would be predicted for a *sigD* mutant strain, but was more similar to the *alsR*::*spc* knockout strain in that motility was reduced on R motility agar but was normal on LB motility agar (Fig. 1).

To test the hypothesis that inactivation of *alsR*, *sigD*, and/or *sigW* could confer greater competitive fitness, the appropriate

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**TABLE 1** *B. subtilis* strains used in this study[a]

| Strain | Genotype and/or phenotype | Source, reference, and/or transformation process[b] |
|---|---|---|
| WN624 | *trpC2*, *amyE::spc*; Spc$^r$ ancestral strain | 9 |
| WN628 | *trpC2*, *amyE::cat*; Chl$^r$ ancestral strain | 9 |
| WN715 | *trpC2*, *amyE::cat*; Chl$^r$ strain isolated before WN716 population sweep | 11 |
| WN716 | *trpC2*, *amyE::spc*; Spc$^r$ strain evolved from WN624 to greater fitness | 11 |
| WN1148 | Strain AMBs2; *trpC2*, *pheA1*, *alsR::spc*; Spc$^r$ | E. Härtig (6) |
| WN1220 | *B. subtilis* subsp. *spizizenii* strain NRRL B-14821 | M. Roberts (13) |
| WN1237 | Strain HB4035; *sigD::kan* | J. Helmann (18) |
| WN1238 | Strain HB0042; *sigW::kan* | J. Helmann |
| WN1239 | Strain HB0020; *sigW::erm* | J. Helmann (4) |
| WN1241 | *trpC2*, *amyE::cat*, *alsR::spc*; Chl$^r$, Spc$^r$ | WN1148→WN628; Spc$^r$/Chl$^r$ |
| WN1242 | *trpC2*, *amyE::cat*, *sigD::kan*; Chl$^r$, Kan$^r$ | WN1237→WN628; Kan$^r$/Chl$^r$ |
| WN1243 | *trpC2*, *amyE::cat*, *sigW::kan*; Chl$^r$, Kan$^r$ | WN1238→WN628; Kan$^r$/Chl$^r$ |
| WN1244 | *trpC2*, *amyE::cat*, *sigW::erm*; Chl$^r$, Erm$^r$ | WN1239→WN628; Erm$^r$/Chl$^r$ |
| WN1262 | *trpC2*, *amyE::tet*, Tet$^r$ | pCm::Tc→WN628; Tet$^r$/Chl$^s$ |
| WN1265 | *trpC2*, *amyE::cat*, *alsR::spc*, *sigD::kan*; Chl$^r$, Kan$^r$, Spc$^r$ | WN1242→WN1241; Kan$^r$/Chl$^r$, Spc$^r$ |
| WN1266 | *trpC2*, *amyE::cat*, *alsR::spc*, *sigW::erm*; Chl$^r$, Spc$^r$, Erm$^r$ | WN1244→WN1241; Erm$^r$/Chl$^r$, Spc$^r$ |
| WN1267 | *trpC2*, *amyE::cat*, *sigD::kan*, *sigW::erm*; Chl$^r$, Kan$^r$, Erm$^r$ | WN1242→WN1244; Kan$^r$/Chl$^r$, Erm$^r$ |
| WN1268 | *trpC2*, *amyE::cat*, *alsR::spc*, *sigD::kan*, *sigW::erm*; Chl$^r$, Spc$^r$, Kan$^r$, Erm$^r$ | WN1242→WN1266; Kan$^r$/Chl$^r$, Spc$^r$, Erm$^r$ |

[a] Spc$^r$, spectinomycin resistant; Chl$^s$, chloramphenicol sensitive.
[b] →, transformation from donor to recipient strain. The first marker after the semicolon denotes the selected marker. The marker(s) after the forward slash denotes the screened marker(s).

strains were constructed and competed against the congenic ancestral strain WN1262 (with *amyE*::*tet* as its selective marker) in pairwise combinations in liquid R medium as described in detail previously (14). The results of these competition experiments showed that the *alsR*::*spc* single mutant was significantly more fit than the ancestor, whereas the *sigD*::*kan* and *sigW*::*erm* knockout strains were not significantly more fit at the *P* level of 0.05 (Fig. 2). All three strains carrying double mutations (*alsR*::*spc sigD*::*kan*,

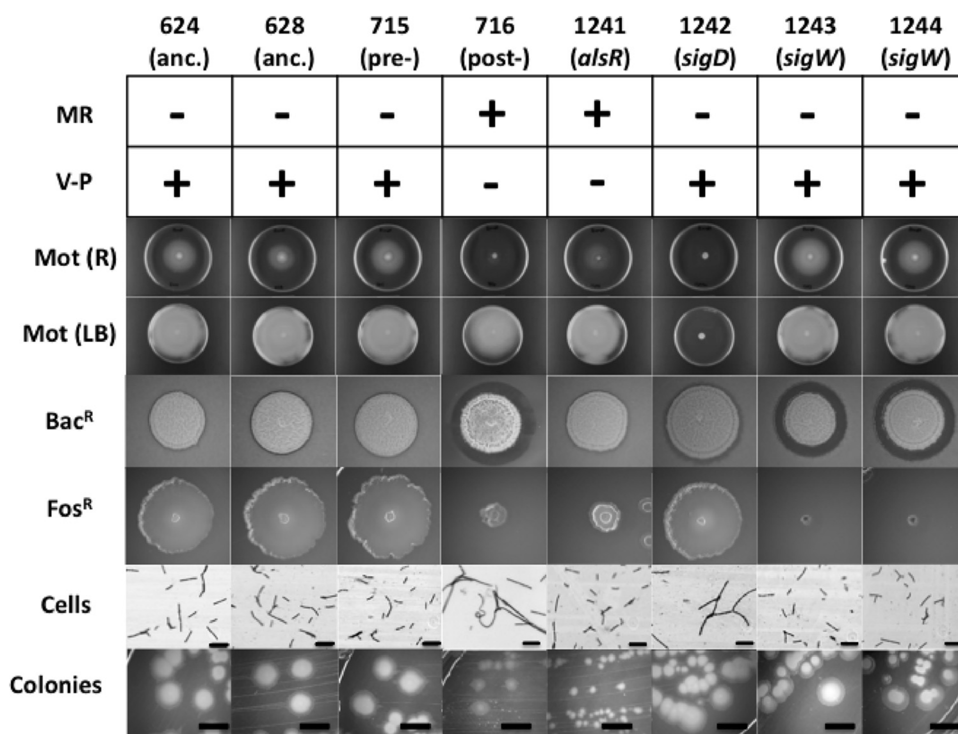


**FIG 1** Phenotypic characterization of ancestral strains WN624 and WN628 (anc. 624 and 628, respectively), presweep strain WN715 (pre-), and postsweep evolved strain WN716 (post-). For comparative purposes, the phenotypes are compared with those of congenic strains carrying knockout mutations *alsR*::*spc* (strain WN1241), *sigD*::*kan* (strain WN1242), *sigW*::*kan* (strain WN1243), and *sigW*::*erm* (strain WN1244). The phenotypes tested were the acidification of medium using the methyl red (MR) test, acetoin production by the Voges-Proskauer (V-P) test, motility (Mot) on R or LB plates, and resistance to the bacteriocin produced by *B. subtilis* subsp. *spizizenii* (Bac$^r$) or to fosfomycin (Fos$^r$). All photos were of typical plates or microscopic fields; all photos in each row were taken of cultures at the same time of incubation. Size bars, 10 $\mu$m for cells and 5 mm for colonies.

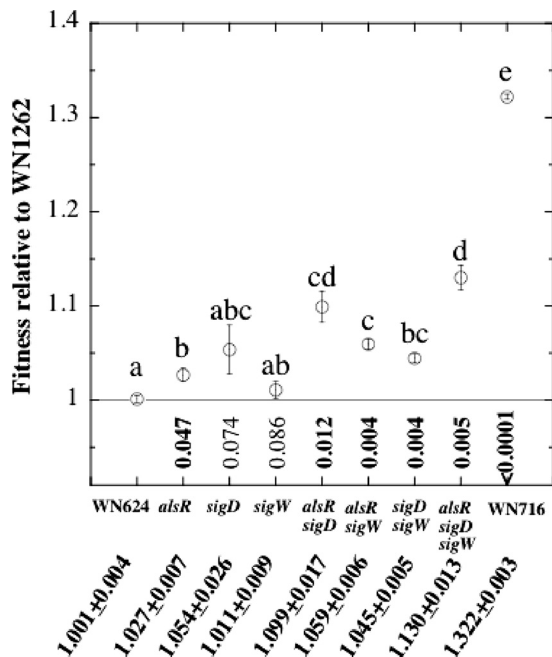


**FIG 2** Results from pairwise competition experiments. Data points are presented as the averages ± standard deviations of duplicate experiments, with each consisting of 7 daily fitness determinations and performed as described previously (14). Below the strain designations are the actual numerical relative fitness values. Relative fitness is defined as $1 + s$, where $s$ is the selection coefficient, calculated as described previously (7). Lowercase letters directly above the data points denote groups of strains whose relative fitnesses were not significantly different from one another at the $P$ level of $\leq$0.05 (by an analysis of variance [ANOVA]). Numbers below the data points on the $x$ axis denote $P$ values (ANOVA) of pairwise comparisons of the relative fitness of the indicated strain and that of the differentially marked ancestral strain WN1262; boldface numbers denote significant differences ($P \leq 0.05$).

*alsR*::*spc sigW*::*erm*, and *sigD*::*kan sigW*::*erm*) were significantly more fit than the ancestor and, for the most part, significantly more fit than the single mutants (Fig. 2). The triple-knockout strain WN1268 (*alsR*::*spc sigD*::*kan sigW*::*erm*) was significantly more fit than all the single mutants and two of the double mutant strains (Fig. 2). However, the triple-knockout strain WN1268 (relative fitness of 1.130 ± 0.013) fell short of the high level of competitive fitness exhibited by evolved strain WN716 (relative fitness of 1.322 ± 0.0003) (Fig. 2).

In summary, because evolved strain WN716 (i) exhibited higher fitness in R medium than its ancestral strain, (ii) carried mutations in *alsR* and *sigW*, as revealed by whole-genome sequencing, and (iii) demonstrated phenotypes consistent with mutations in *alsR*, *sigD*, and *sigW* (Fig. 1), I hypothesized that inactivation of the *alsR*, *sigD*, and/or *sigW* gene would lead to increased fitness of the resulting mutant strains over the ancestral strain in R medium. The data from pairwise competition experiments using engineered congenic strains carrying knockout mutations in *alsR*, *sigD*, and/or *sigW* supported this hypothesis; the single *alsR* knockout mutant, all three double-knockout mutants, and the *alsR*::*spc sigD*::*kan sigW*::*erm* triple-knockout mutant all exhibited significantly increased fitnesses relative to that of the ancestral strain (Fig. 2). These observations support and extend observations from large-scale flux analyses demonstrating that metabolic efficiency of *B. subtilis* cells was improved in *sigD* or *sigW* knockout mutants (8). However, no combination of knockout mutations was able to increase competitive fitness to the level seen in evolved strain WN716. I thus conclude that while inactivation of the pleiotropic regulators AlsR, SigD, and/or SigW does improve *B. subtilis* fitness in R medium, additional genetic factors are likely involved in the dramatic fitness increase of evolved strain WN716 over that of its ancestral strain. In the search for possible additional candidate genes, it should be noted that a whole-genome sequence analysis of WN716 uncovered, in addition to the mutations in *alsR* and *sigW*, a total of 43 other mutations within coding sequences (33 amino acid-changing single nucleotide polymorphisms [SNPs] and 10 +1 frameshift mutations) as well as 11 SNPs located within intergenic regions (3). These additional mutations will be targets for further exploration.

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