Plasmid-Encoded ComI Inhibits Competence in the Ancestral 3610 Strain of Bacillus subtilis

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Natural competence is a process by which bacteria construct a membrane-associated machine for the uptake and integration of exogenous DNA. Many bacteria harbor genes for the DNA uptake machinery and yet are recalcitrant to DNA uptake for unknown reasons. For example, domesticated laboratory strains of Bacillus subtilis are renowned for high-frequency natural transformation, but the ancestral B. subtilis strain NCIB3610 is poorly competent. Here we find that endogenous plasmid pBS32 encodes a small protein, ComI, that inhibits transformation in the 3610 strain. ComI is a single-pass trans-membrane protein that appears to functionally inhibit the competence DNA uptake machinery. Functional inhibition of transformation may be common, and abolishing such inhibitors could be the key to permitting convenient genetic manipulation of a variety of industrially and medically relevant bacteria.

Laboratory strains of Bacillus subtilis are powerful model genetic systems due to their rapid growth as dispersed cells and their ability to take up and incorporate exogenous DNA by natural competence (1, 2). The ancestral B. subtilis strain NCIB3610 (also known as 3610), however, retains many biological properties that were genetically bred out of the laboratory derivatives, including but not limited to floating pellicle biofilms, colonies of complex architecture, synthesis of an extracellular polysaccharide capsule, synthesis of a poly-γ-glutamate slime layer, synthesis of polypeptide antimicrobials, synthesis of a nonribosomally synthesized lipopeptide surfactant, swarming and sliding surface motilities, and a large extrachromosomally maintained plasmid (3–9). Unfortunately, studies of the 3610 strain are hampered due to the fact that it is poorly competent, thus making genetic manipulation inconvenient (10).

The induction of natural competence in laboratory strains is complex (11). During the transition to stationary phase, two parallel quorum-sensing systems activate genes that enhance the accumulation of the transcription factor ComK (2, 12–14). ComK becomes active in only a subpopulation of cells and directs expression of a regulon that includes approximately 20 gene products necessary for the uptake of exogenous DNA (11, 15–17). For cells that synthesize the competence machinery, exogenous double-stranded DNA binds to the cell surface, and single-stranded DNA (ssDNA) is then actively imported and recombined into the chromosome (1, 18–20). Why ancestral B. subtilis strain 3610 is poorly transformable is unknown.

Here we determine that curing the 84-kb endogenous plasmid, here named pBS32, from the ancestral B. subtilis strain results in a 100-fold increase in transformability. We find that pBS32 encodes a small protein called ComI that appears to antagonize transformation by interfering with the competence machinery within the membrane. Functional inhibition of the competence machinery may be a confounding factor that prevents many industrially and medically relevant bacteria from importing DNA, a trait that would be an asset to molecular genetic manipulation. Finally, we note that the competent comI mutant combines the convenience of rapid transformation with a diverse array of ancestral phenotypes and should substantially reduce the barrier to widespread utilization of strain 3610 in B. subtilis research.

MATERIALS AND METHODS

Strains and growth conditions. B. subtilis strains were grown in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) or on LB plates fortified with 1.5% Bacto agar at 37°C. Modified competence (MC) medium (10×) was made with a solution containing 10.7 g K2HPO4, 5.2 g KH2PO4, 20 g dextrose, 0.88 g sodium citrate dehydrate, 2.2 g L-glutamic acid monopotassium salt, 1 ml 1,000× ferric ammonium citrate, and 1 g casein hydrolysate per 100 ml. Competent cultures were grown in diluted 1× MC medium supplemented with 1% 300 mM MgSO4. When appropriate, antibiotics were included at the following concentrations: 10 μg/ml tetracycline, 100 μg/ml spectinomycin, 5 μg/ml chloramphenicol, 5 μg/ml kanamycin, and 1 μg/ml erythromycin plus 25 μg/ml lincomycin (mbl). Isopropyl-β-D-thiogalactopyranoside ( IPTG; Sigma) was added to the medium at the indicated concentration when appropriate.

For the competence assay, 2-ml cultures were grown for 4.5 h at 37°C in MC medium. Four hundred microliters of the culture was transferred into a new tube, and 500 ng plasmid DNA (pDG364) (21) was added. The culture was grown for an additional 1.5 h at 37°C and plated onto LB plates containing chloramphenicol to determine the number of transformants and onto LB plates to determine total viability. Transformation frequency was calculated by dividing the number of transformants by the number of viable colonies.

PCR confirmation of plasmid curing. The fliG chromosomal locus was PCR amplified from 3610 DNA by using primer pair 748/760. The rapB pBS32-encoded locus was PCR amplified from 3610 DNA by using primer pair 349/350.

Biofilm assay. For pellicle formation experiments, 10 μl of culture grown overnight at room temperature in LB medium was inoculated into 10 ml minimal MSg medium (5 mM potassium phosphate [pH 7], 100

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mM morpholinepropanesulfonic acid [MOPS] [pH 7], 2 mM MgCl₂, 700 μM CaCl₂, 50 μM MnCl₂, 50 μM FeCl₂, 1 μM ZnCl₂, 2 μM thiamine, 0.5% glycerol, 0.5% glutamate, 50 μg/ml tryptophan, 50 μg/ml phenylalanine, and 50 μg/ml threonine) in 6-well microtiter plates and incubated at 25°C (22). For colony architecture analysis, colonies were toothpick inoculated onto minimal M9g medium fortified with 1.5% Bacto agar and incubated for 3 days at 25°C.

**Motility assay.** For the motility assay, swarm agar plates containing 25 ml LB medium fortified with 0.7% Bacto agar were prepared fresh, and on the following day, they were dried for 20 min in a laminar flow hood. Each plate was toothpick inoculated from a colony grown overnight and scored for motility after 18 h of incubation at 37°C (23). Plates were visualized with a Bio-Rad Geldoc system and digitally captured by using Bio-Rad Quantity One software.

**Microscopy.** Fluorescence microscopy was performed with a Nikon 80i microscope with a phase-contrast Nikon Plan Apo 100× objective and an Excite 120 metal halide lamp. mCherry was visualized with a C-FL HYQ Texas Red filter cube (excitation filter, 532 to 587 nm; barrier filter, >590 nm). Cyan fluorescent protein (CFP) fluorescent signals were viewed by using a C-FL HYQ CFP filter cube (excitation filter, 426 to 446 nm; barrier filter, 460 to 500 nm). Yellow fluorescent protein (YFP) was visualized by using a C-FL HYQ YFP filter cube (excitation filter, 490 to 510 nm; barrier filter, 520 to 550 nm). Green fluorescent protein (GFP) was visualized by using a C-FL HYQ fluorescein isothiocyanate (FITC) filter cube (excitation filter, 460 to 500 nm; barrier filter, 515 to 550 nm). TMA-DPH fluorescent signals were visualized by using a UV-2E/C 4’.6-diamidino-2-phenylindole (DAPI) filter cube (excitation filter, 340 to 380 nm; barrier filter, 435 to 485 nm). Images were captured with a Photometrics Coolsnap HQ2 camera in black and white, false colored, and superimposed by using Metamorph image software.

For fluorescence microscopy of transcriptional fusions, cells were grown overnight at 22°C in 1× MC medium. Cultures were subcultured to an optical density at 600 nm (OD₆0₀) of ~0.01 and grown at 37°C until competence. A total of 1.0 ml of each culture was harvested by centrifugation and resuspended in 50 μl phosphate-buffered saline (PBS). Samples were observed by spotting 4 μl of the suspension onto a glass microscope slide and were immobilized with a poly-l-lysine-treated coverslip.

For detection of mCherry-ComI and ComGA-GFP by microscopy, cells were foraged and spotted onto swarm agar plates containing 25 ml LB broth with mls selection at a permissive temperature for plasmid replication (22°C) for 14 h, diluted in fresh LB broth, and incubated for 12 h at a restrictive temperature for plasmid replication (42°C) while maintaining mls selection. To evoke the integrated plasmid, the strain was incubated in 3 ml LB broth at a permissive temperature for plasmid replication (22°C) for 14 h, diluted 30-fold in fresh LB broth, and incubated at 22°C for another 8 h. Dilution and outgrowth were repeated 2 more times. Cells were then serially diluted and plated onto LB agar at 37°C. Individual colonies were patched onto LB plates and LB plates supplemented with erythromycin-lincomycin (MLS) to identify MLS-sensitive colonies that had evicted the plasmid. Chromosomal DNA from colonies that had excised the plasmid was purified and screened by PCR using primer pair 1724/1727 to determine which isolate had retained the ΔQII region.

All of the following in-frame markerless deletion constructs in pBS32 were built similarly to ΔQII: ΔQIII using primer pairs 1728/1729 and 1730/1731 to generate pKB134, ΔQIV using primer pairs 1732/1733 and 1734/1735 to generate pKB135, ΔR1 using primer pairs 1865/1866 and 1867/1868 to generate MP22, ΔR2 using primer pairs 1951/1952 and 1953/1954 to generate pMP23, ΔR3 using primer pairs 1951/1952 and 1953/1954 to generate pMP24, ΔzpcW using primer pairs 1955/1956 to determine which isolate had retained the ΔQII region.

In-frame deletions on pBS32. To generate the quadrants QII of pBS32 (ΔQII) in-frame markerless deletion construct, the region upstream of QII of pBS32 was PCR amplified from 3610 chromosomal DNA by using primer pair 1724/1725 and digested with EcoRI and BamHI, and the region downstream of QII was PCR amplified from 3610 chromosomal DNA by using primer pair 1726/1727 and digested with BamHI and SalI. The two fragments were then simultaneously ligated into the EcoRI and SalI sites of pMiniMAD2, which carries a temperature-sensitive origin of replication and an erythromycin resistance cassette, to generate pKB133 (25).

Plasmid pKB133 was introduced into DS2569 by transformation and maintained as a plasmid at the permissive temperature for plasmid replication (22°C), using mls resistance for selection. The plasmid was then transduced into 3610 at the permissive temperature. To force integration of the plasmid into pBS32, the strain was incubated in 3 ml LB broth with mls selection at a permissive temperature for plasmid replication (22°C) for 14 h, diluted in fresh LB broth, and incubated for 12 h at a restrictive temperature for plasmid replication (42°C) while maintaining mls selection. To evoke the integrated plasmid, the strain was incubated in 3 ml LB broth at a permissive temperature for plasmid replication (22°C) for 14 h, diluted 30-fold in fresh LB broth, and incubated at 22°C for another 8 h. Dilution and outgrowth were repeated 2 more times. Cells were then serially diluted and plated onto LB agar at 37°C. Individual colonies were patched onto LB plates and LB plates supplemented with erythromycin-lincomycin (MLS) to identify MLS-sensitive colonies that had evicted the plasmid. Chromosomal DNA from colonies that had excised the plasmid was purified and screened by PCR using primer pair 1724/1727 to determine which isolate had retained the ΔQII region.

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### Table 1 Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotypea</th>
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<tr>
<td>3610</td>
<td>pBS32-containing wild type (ancestral strain)</td>
</tr>
<tr>
<td>PY79</td>
<td>swrA::spf” (laboratory strain)</td>
</tr>
<tr>
<td>DK69</td>
<td>amyE::comIQ12L::spec [PY79]</td>
</tr>
<tr>
<td>DK315</td>
<td>Δrok ΔcomIQ12L amyE::PcomIQ12L-comGA-GFP cat thrC::ProK-mCherry linker-comIQ12L mls [DS2569]</td>
</tr>
<tr>
<td>DK1042</td>
<td>comIK::Tn10 [3610]</td>
</tr>
<tr>
<td>DS2569</td>
<td>Cured strain [3610 lacking pBS32]</td>
</tr>
</tbody>
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a Strains in brackets indicate the parental background.
primer pair and 2682/2683 and digested with Xhol and BamHI. The two fragments were then simultaneously ligated into the EcoRI and BamHI sites of pMiniMAD2, which carries a temperature-sensitive origin of replication and an erythromycin resistance cassette, to generate pMP81. Plasmid pMP81 was introduced into PY79 by single-crossover integration by transformation and maintained as a plasmid at the permissive temperature (37°C), using mls resistance as a selection. The integrated plasmid was then transduced into DS2569. To evoke the plasmid, the strain was incubated in 3 ml LB broth at a permissive temperature for plasmid replication (22°C) for 14 h, diluted 30-fold in fresh LB broth, and incubated at 22°C for another 8 h. Dilution and outgrowth were repeated two more times. Cells were then serially diluted and plated onto LB agar at 37°C. Individual colonies were patched onto LB plates and LB plates containing mls to identify mls-sensitive colonies that had evicted the plasmid. Chromosomal DNA from colonies that had excised the plasmid was purified and screened by PCR using primer pair 2680/2683 to determine which isolate had retained the Δrok allele.

The following in-frame markerless deletion construct was built similarly to Δrok: ΔcomGA using primer pairs 2686/2687 and 2688/2689 to generate pMP83.

**Inducible coml construct.** To generate the inducible anyE::P<sub>any hypA</sub>-coml spec construct pMP76, a PCR product containing coml was amplified from 3610 chromosomal DNA by using primer pair 2276/2273, digested with Nhel and SphI, and cloned into the Nhel and SphI sites of pDR111 containing a spectinomycin resistance cassette, a polylinker downstream of the P<sub>any hypA</sub> promoter, and the gene encoding the LacI repressor between the two arms of the anyE gene (26).

**P<sup>comK</sup>-YFP transcriptional reporter.** To generate the transcriptional fusion of P<sup>comK</sup> to YFP, a fragment containing the comK promoter was amplified by using 3610 as a template and primer pair 1213/1214 and was digested with EcoRI and Nhel. The digested fragment was ligated into the EcoRI and Nhel sites of pLC19 (a gift from Loralyn Cozy, University of Hawaii) containing an erythromycin resistance cassette, a polylinker downstream of the P<sub>any hypA</sub> promoter, and the gene encoding the LacI repressor between the two arms of the anyE gene (26).

**P<sup>comGA</sup>-mCherry transcriptional reporter.** To generate the transcriptional fusion of P<sup>comGA</sup> to mCherry, a fragment containing the comGA promoter was amplified by using 3610 as a template and primer pair 1124/1514 and was digested with EcoRI and Nhel. The digested fragment was ligated into the EcoRI and Nhel sites of pDR111 containing a spectinomycin resistance cassette to create pKB88, pKB89 was integrated into DS2569 at the ectopic site lacA.

**P<sup>thrC</sup>G-mCherry translational fusions.** To generate the inducible translational fusion of Coml to mCherry, a fragment containing the mCherry gene was amplified using pDP195 as a template and primer pair 2516/2517 and was digested with Nhel and Xhol. A fragment containing the coml gene was amplified by using 3610 chromosomal DNA as a template and primer pair 2518/2273 and was digested with Nhel and Sphl. The two fragments were then simultaneously ligated into the Nhel and Sphl sites of pDP150 containing an erythromycin (mls) resistance cassette, a polylinker downstream of the P<sub>any hypA</sub> promoter, and the gene encoding the LacI repressor between the two arms of the anyE gene (26).

**P<sup>comK</sup>-YFP transcriptional reporter.** To generate the transcriptional fusion of P<sup>comK</sup> to YFP, a fragment containing the comK promoter was amplified by using 3610 as a template and primer pair 1213/1214 and was digested with EcoRI and Nhel. The digested fragment was ligated into the EcoRI and Nhel sites of pLC19 (a gift from Loralyn Cozy, University of Hawaii) containing an erythromycin resistance cassette, a polylinker downstream of the P<sub>any hypA</sub> promoter, and the gene encoding the LacI repressor between the two arms of the anyE gene (26).

**P<sup>comGA</sup>-mCherry transcriptional reporter.** To generate the transcriptional fusion of P<sup>comGA</sup> to mCherry, a fragment containing the comGA promoter was amplified by using 3610 as a template and primer pair 1124/1514 and was digested with EcoRI and Nhel. The digested fragment was ligated into the EcoRI and Nhel sites of pDR111 containing a spectinomycin resistance cassette to create pKB88, pKB89 was integrated into DS2569 at the ectopic site lacA.

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**P<sup>comGA</sup>-mCherry transcriptional reporter.** To generate the transcriptional fusion of P<sup>comGA</sup> to mCherry, a fragment containing the comGA promoter was amplified by using 3610 as a template and primer pair 1124/1514 and was digested with EcoRI and Nhel. The digested fragment was ligated into the EcoRI and Nhel sites of pDR111 containing a spectinomycin resistance cassette to create pKB88, pKB89 was integrated into DS2569 at the ectopic site lacA.

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centrifuged at 5,000 \times g for 10 min, the supernatant was discarded, and the pellet was resuspended in the remaining volume. One hundred microliters of cell suspension was then plated onto TY broth fortified with 1.5% agar, the appropriate antibiotic, and 10 mM sodium citrate.

RESULTS

Endogenous plasmid pBS32 inhibits competence in the ancestral strain of B. subtilis. The ancestral B. subtilis strain 3610 contains a large extrachromosomally maintained endogenous plasmid (8, 31). Illumina sequencing and assembly of the 3610 genome suggested that the plasmid was 84 kb in size and was 97% identical to a plasmid found within Bacillus subtilis subsp. natto called pLS32 (32) (see Fig. S1A in the supplemental material). Here we name the plasmid found in strain 3610 pBS32, for B. subtilis plasmid 32, to emphasize the overall similarity (despite three regions of insertion/deletion) with the plasmid of the same number from the B. subtilis subsp. natto strain (GenBank accession number for pBS32 is KF365913). We note that a plasmid from B. subtilis strain 3610 was reported and previously named pAS32, but we could not be certain that pAS32 and pBS32 were synonymous because pAS32 was not assembled or annotated, was reported to be \( \sim 6 \) kb smaller than pBS32, and was described as being similar to pLS32 over only a 7-kb origin region rather than over the entire length (31). We have annotated and submitted to GenBank 102 genes carried by pBS32, named by using the following nomenclature: zpxX, where the x’s are sequential letters clockwise from the repN gene encoding an essential replication protein (see GenBank accession number KF365913 for individual gene annotations) (33). Whereas genes of unknown function in the chromosome begin with the letter “y,” and genes carried by chromosomal prophages begin with the letter “x,” we chose the letter “z” to indicate genes of unknown function carried on the endogenous plasmid.

Plasmid pBS32 is absent from domesticated descendants of strain 3610, such as strains 168, PY79, and JH642 (9). To simulate the process of domestication, the B. subtilis ancestral strain was cured of the endogenous plasmid by introducing a second plasmid (pJSAS) with an origin of replication highly similar to that of pBS32 (33, 34) (see Fig. S1B in the supplemental material). The resulting transductant could not maintain both plasmids simultaneously, and selection for chloramphenicol resistance ensured that pJSAS was preferentially retained (35). Growth in the absence of chloramphenicol selection resulted in rapid loss of pJSAS, as indicated by chloramphenicol sensitivity in individual isolates. To construct the data for the black bars: “lab” (PY79), “ancestral” (3610), and “cured” (DS2569). Green bars represent the frequency of cells expressing \( P_{\text{comGA}-\text{mCherry}} \), and the pink bars represent the frequency of cells expressing \( P_{\text{comK}-\text{YFP}} \) (Fig. 1B).

Consistent with previous observations, ancestral strain 3610 was 1,000-fold less transformable than laboratory strain PY79 (Fig. 1B) (10). In comparison, the cured strain that lacked pBS32 was 100-fold more transformable than the ancestral strain (Fig. 1B). We infer that the inability to incorporate antibiotic resistance was due to a failure in transformation rather than a defect in recombination, as a similar pattern of transformation frequencies was observed when laboratory, ancestral, and cured strains were transformed with an extrachromosomally maintained plasmid (see Fig. S3 in the supplemental material) (37). Furthermore, all three strains were similarly proficient for recombining antibiotic resistance genes into the chromosome when delivered by SPP1 phage-mediated generalized transduction (data not shown). We conclude that pBS32 was at least one reason why transformation was inhibited in the ancestral strain. We note that transformability was reduced 10-fold in the cured strain relative to the laboratory strain, which we presume is likely due to a chromosomal single-nucleotide polymorphism(s) (9).

pBS32 competence inhibition acts posttranscriptionally. One way in which pBS32 might inhibit competence is by the in-
hition of competence gene expression. In laboratory strains, competence genes are expressed in only a subpopulation (16, 17, 38–40). To examine gene expression of a heterogeneous population, the ancestral, laboratory, and cured strains were fluorescently labeled such that the PcomK promoter that directs expression of the ComK transcription factor was fused to YFP (lacA::PcomK-YFP), while the ComK-dependent PcomGA promoter that directs expression of ComGA, an ATPase required for DNA uptake, was fused to mCherry (thrC::PcomGA-mCherry) (41). Consistent with the regulatory hierarchy, in all strains, cells that expressed PcomGA-mCherry were a subset of those that expressed PcomK-YFP (see Fig. S3 in the supplemental material). The frequency of PcomK- and PcomGA-directed gene expression in the ancestral strain was only 10-fold lower than that in the laboratory strain (Fig. 1B). Furthermore, curing pBS32 did not increase the frequency of gene expression in the cured strain relative to the ancestral strain (Fig. 1B). We conclude that the 10-fold reduction in comK and comGA expression levels cannot account for either the 1,000-fold reduction in the ancestral strain transformation frequency or the rescue of competence in the cured strain. We infer that competence in the ancestral strain is likely inhibited at the posttranscriptional level.

**Plasmid-encoded ComI is necessary and sufficient to inhibit competence.** pBS32 encodes the RapP protein, which, when ectopically expressed from an IPTG-inducible promoter, inhibited competence in a domesticated laboratory strain (36). The gene encoding RapP did not appear to be responsible for competence inhibition by pBS32, however, as deletion of rapP did not restore transformability to the 3610 strain (36). To identify a gene(s) encoding a competence inhibitor, pBS32 was divided into roughly four quadrants, and in-frame markerless deletions were generated for each quadrant save quadrant QI because QI encoded the plasmid origin of replication (Fig. 2A; Table 2) (33). Deletion of quadrants QII and QIII resulted in modest increases in transformation frequency, but deletion of quadrant QIV restored the transformation frequency to a level comparable to that of the cured strain (Fig. 2B). Three subdeletions of quadrant QIV (R1, R2, and R3) were generated, and deletion of region R1 restored transformability to a level comparable to that observed in the cured strain (Fig. 2A and B). Individual deletion of four of the genes, zpcW, zpcX, zpcY, and zpcZ, in region R1 resulted in modest increases in transformability, but deletion of the fifth gene, here named comI, restored transformability to a level comparable to that of the cured strain (Fig. 2B and C). We conclude that the comI gene is necessary for competence inhibition.

The comI gene is predicted to encode ComI, a 30-amino-acid protein (Fig. 3A). To determine whether comI was sufficient for competence inhibition, the comI gene was cloned downstream of an IPTG-inducible Phypa promoter and inserted at the ectopic amyE locus (amyE::Phypa-comI) of the laboratory strain. At levels of IPTG induction ≤60 μM, the comI-expressing strain remained fully transformable, but at levels of IPTG induction of >70 μM, transformability was inhibited 100-fold or more (Fig. 3B). At levels of IPTG induction of >80 μM, cell viability decreased considerably (Fig. 3B). We conclude that ComI is sufficient to inhibit competence when solely expressed in laboratory strains but becomes toxic when present in excess.

**ComI colocalizes with a component of the competence machinery.** The comI gene is predicted to contain a single-pass transmembrane alpha helix (Fig. 3A). To cytologically determine whether ComI localizes to the cell membrane, the gene encoding mCherry was first translationally fused to the N terminus of the ComI gene expressed under the control of the IPTG-inducible promoter Phypa and inserted at the ectopic thrC locus (thrC::Phypa-mCherry-comI) in the laboratory strain. We note that inducible mCherry-ComI inhibited transformability at a lower level of induction than inducible ComI (Fig. 3C). However, the mCherry-ComI fusion protein was diminished in its ability to cause a loss of viability upon induction (Fig. 3C). We conclude that the mCherry-ComI fusion protein was functional for transformation inhibition and that the transformation inhibition and toxicity functions of artificially expressed ComI were genetically separable.
To determine the localization pattern of mCherry-ComI, mCherry-ComI was expressed from an IPTG-inducible P\textsubscript{hypM} promoter in cells that also expressed a ComGA-GFP fusion expressed from the native P\textsubscript{comGA} promoter and mutated for the repressor of ComK, Rok (42). The Rok protein was disrupted to increase the frequency of cells that express ComK and therefore to increase the frequency of cells that express competence machinery proteins such as ComGA (16, 42). In the absence of IPTG, only a subset of cells grown for 4.5 h in competence medium expressed mCherry-ComI, a subset of cells
ComI Inhibits Ancestral Strain Competence

FIG 3 Expression of comI is sufficient to inhibit competence in laboratory strains. (A) Primary sequence of the ComI protein. Amino acids are indicated by their single-letter code within the boundary. N and C termini are indicated outside the boundary. The gray-shaded area indicates the putative transmembrane alpha helix. The black arrowhead indicates glutamine residue 12 that was mutated to a leucine in ComIQ12L. (B to D) Transformation frequency, indicated by black dots, and cell viability, indicated by white dots, at various IPTG concentrations for a strain expressing a P<sub>amyE</sub>-comI construct integrated at amyE (DS8036) (B), a strain expressing a P<sub>hyppunk</sub>-mCherry-comI construct integrated at thrC (DS8482) (C), and a strain expressing a P<sub>hyppunk</sub>-mCherry-comIQ12L construct integrated at amyE (DK69) (D).

ComI punctate localization did not appear to require competence gene expression, as puncta were present either in a comK mutant or in the subpopulation of wild-type cells that did not express ComGA (Fig. 4B; see also Fig. S4 in the supplemental material). To determine the relevance of ComI localization, an intragenic mutation was constructed that changed hydrophilic glutamine residue 12 within the predicted transmembrane helix to a hydrophobic leucine residue (mCherry-ComIQ12L) (Fig. 3A). Mutation of glutamine 12 abolished ComI activity, as expression of an mCherry-ComIQ12L construct failed to inhibit transformation at any level of inducer tested (Fig. 3D). Furthermore, mCherry-ComIQ12L displayed aberrant diffuse localization, and while puncta were occasionally present, mCherry-ComI and ComGA-GFP puncta were often close to one another but did not appear to overlap (Fig. 4D). Finally, allelic replacement of the ComIQ12L mutant into the native site in pBS32 was sufficient to restore the transformation frequency to levels similar to those found in the cured strain (Fig. 2B). We conclude that ComI glutamine 12 is required for competence inhibition, that glutamine 12 is required for punctate colocalization with ComGA, and that mutation of a single base that disrupts glutamine 12 is sufficient to restore high-level competence to the ancestral strain of B. subtilis.

DISCUSSION

Bacillus subtilis laboratory strains were heavily domesticated to improve genetic manipulation at the expense of multicellular, ecologically relevant behaviors such as biofilm formation, surface motility, and antimicrobial synthesis. In contrast, strain 3610, the ancestor of the laboratory strains, retains multicellular phenotypes, but genetic manipulation is inconvenient, as its competence for DNA uptake is reduced 1,000-fold relative to the competence of the domesticated derivatives (10). Here we find that the 84-kb endogenous plasmid pBS32 in strain 3610 encodes an inhibitor of competence, ComI, such that a single-base-pair change in a critical ComI residue is sufficient to restore a level of transformability near to that observed in laboratory strains. We anticipate that the ComI mutant will promote utilization of the powerful 3610 genetic background and will facilitate dissection of many phenotypes that were previously hidden to the B. subtilis community (see Fig. S2 in the supplemental material).

ComI is a 30-amino-acid protein, the sequence of which is dominated by a predicted transmembrane helix. ComI appears to inhibit transformation posttranscriptionally, and puncta of ComI colocalized with the soluble ATPase ComGA that serves as a proxy for the rest of the competence machinery. We speculate that ComI interacts within the membrane via glutamine residue 12 with one of the transmembrane proteins required for competence, ComEA, ComEC, or ComGB (11, 40, 45–47). ComI interaction may disrupt function of the competence machinery, causing ComGA to appear mislocalized from the poles to along the length of the cells or diffuse in the cytoplasm. As such, ComI may directly or indirectly either separate a fueling protein from a mechanical component or lock the two together and may therefore functionally inhibit the competence machinery similar to inhibitors recently reported for another bacterial machine, the flagellum (48, 49, 57–59). ComI also causes a loss of viability when artificially expressed at levels above the amount needed to inhibit competence, but toxicity may be due to overexpression of a membrane...
protein rather than a biologically relevant aspect of ComI function, as no evidence of cell death in wild-type backgrounds was detected. Why pBS32 encodes a functional inhibitor of competence is unknown, but ComI may promote plasmid stability by excluding the uptake of competing plasmids.

Many bacteria, including pathogens like *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Listeria monocytogenes*, are nontransformable and yet carry genes which suggest that they could be capable of natural competence (50–52). Similarly, industrial strains such as *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus subtilis* subsp. *natto* are often poorly competent. For *Bacillus subtilis* subsp. *natto* strains that carry pLS32, we propose that pLS32-encoded ComI is a primary contributor to competence inhibition. Consistent with a general theme of plasmid-encoded competence inhibition, another *B. subtilis* plasmid, pLS20, was found to encode a transcriptional repressor of competence genes but, when mutated, elevated competence to only 1/100 of that of laboratory strains, perhaps suggesting the presence of other inhibitors (53). Finally, while BLAST analysis of short proteins is complicated by the limited sequence, we note that short *trans*-membrane proteins similar to ComI appear to be encoded within a variety of *Enterococcus* species, another example of a group of bacteria that carry competence genes but are poorly competent for unknown reasons (54, 55). Our work raises the possibility that some organisms may lack full competence because they express an as-yet-unidentified functional inhibitor of the transformation machinery. Natural competence greatly enhances molecular genetics, and we suggest that these organisms might be made competent by screening for mutants in a hypothetical inhibitor. Curing endogenous plasmids or other extrachromosomal elements such as prophages may be a good strategy to improve transformability.

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