Supplemental Figure 1
Figure S1. *sinl* expression is sustained in biofilm but not sporulation medium. Shown are luciferase activities from a 3610 derivative (ALM66) harboring a P_{sinl}-lux fusion and cultured in either DS (A) or MSgg (B) medium. Growth curves of the cells were shown in open squares (□) and luciferase activities were shown in filled diamonds (♦) and in arbitrary units (AU).
Supplemental Figure 2
Figure S2. (A) A schematic drawing shows insertion of kan selective marker to a chromosomal locus between sinR and tasA. (B) Pellicle formation in DS medium by 3610 derivatives that contain mutant alleles in the putative 0A~P operators in the native sinI promoter. (C) β-Galactosidase activities of 3610 derivatives that contain either PepsA-lacZ (upper panel) or PyqxM-lacZ (lower panel) at the amyE locus on the chromosome. Cells were grown in DS medium. Each reporter strain also contains either wild type (♦), M1 allele (O), M2 allele (•), M3, 4 allele (□), or Δ(2-4) (Δ) in the native sinI promoter.
Supplemental Figure 3
Figure S3. Block of transcription read-through into sinI from upstream yqhHG operon impairs biofilm formation. (A) Colony and pellicle formation by 3610 (left-hand panel) and its derivatives (middle and right-hand panels) in which the spec gene was inserted upstream of sinI in two different loci. (B) Colony and pellicle formation by a ∆sinI sinR mutant complemented with a DNA fragment containing either yqhHG sinI sinR (left-hand panels) or sinI sinR (right-hand panels) and integrated at the bkdB gene locus.
Supplemental Figure 4
Figure S4. Matrix genes were shut off in early sporulating cells. In the left-hand panels, green fluorescence represents YC161 cells expressing GFP from a constitutive promoter ($P_{spank}$-$gfp$). Green fluorescence in the middle and right-hand panels represents YC716 (middle panels) and YC717 (right-hand panels, $\Delta2-4$) cells, respectively, expressing proteolytically unstable GFP proteins (GFP-LCN) from the promoter for the $yqxM$ operon. The asymmetric division septums in early sporulating cells were indicated by arrows. In all panels, red fluorescence represents cell membrane staining using the dye FM4-64.
Figure S5. β-Galactosidase activities of 3610 containing either a wild type $P_{sin}$-lacZ fusion or $P_{sin}$-lacZ with mutations in the imperfect 0A-box. Strains used were described as follows: wild type (■), or M1 allele (●), or M2 allele (♦), or M3-4 allele (▲) or Δ(2-4) allele (Δ), or random sequence shuffle of O(2-4) (★) in the promoter region of $P_{sin}$-lacZ. Reporter fusions were integrated at amyE on the chromosome of 3610 and cells were grown in MSgg medium.
**Table S1. Strains used in the study.**

<table>
<thead>
<tr>
<th>E. coli</th>
<th>DH5α</th>
<th>an E. coli strain used for molecular cloning</th>
<th>Invitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)</td>
<td>E. coli B F' dcm ompT hsdS(rB-) mB gal λ(DE3)</td>
<td>Stratagene</td>
<td></td>
</tr>
<tr>
<td>RL3514</td>
<td>a BL21/DE3 derivative for overexpression of Spo0A-CTD CmR, AmpR</td>
<td>(Molle et al, 2003)</td>
<td></td>
</tr>
</tbody>
</table>

**B. subtilis**

3610: wild B. subtilis strain capable of forming robust biofilms (Branda et al, 2001)

PY79: laboratory strain used as a host for transformation

AHB277: Tn917@140(yinF/yloA):amyE::Pspo0a-sigG::spec, SpecR gift of Camp A

AHB289: Tn917@317(ywrfK):amyE::Pspo0a-sigG::spec, SpecR gift of Camp A

AHB290: Tn917@213(bkdB):amyE::Pspo0a-sigG::spec, SpecR gift of Camp A

ALM66: Pα-lux at sacA, ΔpshH::tet, SpecR, TetR gift of McLoon A

DL841: lacA::Pγlac-lacZ, MlsR gift of Lopez D

RL3618: spoOAspo0a-sad67::spec, SpecR (Ireton et al, 1993)

RL3852: ΔpshH::tet, TetR (Kearns et al, 2005)

RL3855: ΔsinI sinR::spec, SpecR (Kearns et al, 2005)

YC161: amyE::Pγlac-gfp, CmR this study

YC256: amyE::Pγlac-atg, CmR this study

YC256(M1): amyE::Pγlac-atg, amyE operator mutation in O1, CmR this study

YC256(M2): amyE::Pγlac-atg, amyE operator mutation in O2, CmR this study

YC256(M3, 4): amyE::Pγlac-atg, amyE operator mutations in O3 and O4, CmR this study

YC256(s2-4): amyE::Pγlac-atg, deletions from O2 to O4, CmR this study

YC256(RS2-4): amyE::Pγlac-atg, scrambled sequence from O2 to O4, CmR this study

YC264: spec gene inserted downstream of yqhG, SpecR this study

YC270: ΔsinI sinR::spec, yqhH-sinI-sinR at amyE, CmR, SpecR this study

YC285: kan gene inserted between sinR and tasA, KanR this study

YC285(M1): mutations in 0A-P operator 1 in YC285, KanR this study

YC285(M2): mutations in 0A-P operator 2 in YC285, KanR this study

YC285(M3, 4): mutations in 0A-P operators 3 and 4 in YC285, KanR this study

YC285(M1-4): mutations in 0A-P operators 1, 2, 3 and 4 in YC285, KanR this study

YC285(s2-4): deletions from 0A-P operators 2 to 4 in YC285, KanR this study

YC290: amyE::Pγlac-atg, CmR this study

YC290(M1): mutations in 0A-P operator 1 in YC290, CmR, KanR this study

YC290(M2): mutations in 0A-P operator 2 in YC290, CmR, KanR this study

YC290(M3, 4): mutations in 0A-P operators 3 and 4 in YC290, CmR, KanR this study

YC290(s): deletions in 0A-P operators 2-4 in YC290, CmR, KanR this study

YC291: amyE::Pγlac-atg in YC285, CmR, KanR this study

YC291(M1): mutations in 0A-P operator 1 in YC291, CmR, KanR this study

YC291(M2): mutations in 0A-P operator 2 in YC291, CmR, KanR this study

YC291(M3, 4): mutations in 0A-P operators 3 and 4 in YC291, CmR, KanR this study

YC291(s): deletions in 0A-P operators 2-4 in YC291, CmR, KanR this study

YC550: ΔsinI sinR::spec, amyE::yqhH-sinI-sinR at 140(yinF/yloA), CmR, SpecR this study

YC551: ΔsinI sinR::spec, amyE::yqhH-sinI-sinR at 317(ywrfK), CmR, SpecR this study

YC552: ΔsinI sinR::spec, amyE::yqhH-sinI-sinR at 213(bkdB), CmR, SpecR this study

YC553: spec gene inserted immediately upstream of yqhH, SpecR this study

YC561: amyE::Pγlac-atg (0A-4), CmR this study

YC562: amyE::yqhH-sinI-sinR at 213(bkdB), CmR this study

YC686: thrC::Pγlac-atg in 3610, MlsR this study

YC687: ΔsinI sinR::spec, amyE::yqhH-sinI-sinR, thrC::Pγlac-atg, SpecR, CmR, MlsR this study

YC688: amyE::yqhH-sinI-sinR at 213(bkdB), thrC::Pγlac-atg, CmR, SpecR, MlsR this study

YC716: ΔpshH::tet, amyE::Pγlac-gfp(LCN), kan gene inserted between sinR and tasA TetR, CmR, KanR this study

YC717: ΔpshH::tet, amyE::Pγlac-gfp(LCN), kan gene inserted between sinR and tasA TetR, CmR, KanR this study

YC718: spoOA spo0A-sad67, kan gene inserted between sinR and tasA, deletions in 0A-P operators 2-4, TetR, CmR, KanR this study

YC719: spoOA spo0A-sad67, ΔsinI-sinR:kan, amyE::yqhH-sinI-sinR, deletions in 0A-P operators 2-4, lacA::Pγlac-yfp, SpecR, CmR, CmR MlsR this study

YC734: amyE::Pγlac-gfp, sacA::Pγlac-hux, SpecR, CmR this study

YC750: ΔsinI sinR::spec, amyE::c3-sinI-sinR at 213(bkdB), CmR, SpecR this study
Table S2. Plasmids and primers used in the study.

<table>
<thead>
<tr>
<th>Plasmids:</th>
<th>Primer:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pAH49</strong> pBSK(+) derivative, KanR, AmpR (gift of Camp A)</td>
<td>gfp-F1: 5'-gacaagctttcggtaccgggccccccctcgaggt-3'</td>
</tr>
<tr>
<td><strong>pAH54</strong> pBSK(+) derivative, SpecR, AmpR (gift of Camp A)</td>
<td>gfp-R1: 5'-gacggatccgagctcctatttgtatagttcatctcatgcc-3'</td>
</tr>
<tr>
<td><strong>pDG268</strong> amyE integration vector, promoter-less lacZ, CmR</td>
<td>gfp-R2 (LCN): 5'-gacggatccctagttgcacagtttgtatagttcatctcatgcc-3'</td>
</tr>
<tr>
<td><strong>pDG1662</strong> amyE integration vector, SpecR (Guérout-Fleury et al, 1996)</td>
<td>Peasp-F1: 5'-gtcgaattcctagaaattctcctctattcctgtcg-3'</td>
</tr>
<tr>
<td><strong>pDG1730</strong> amyE integration vector, SpecR (Guérout-Fleury et al, 1995)</td>
<td>Peasp-R1: 5'-gtcaagcttctagacaatcgcatataattctttg–3'</td>
</tr>
<tr>
<td><strong>pNGFP</strong> vector with a promoter-less gfp for transcriptional fusion</td>
<td>PepsA-F1: 5'-gtcgaattcctagaaattctcctctattcctgtcg-3'</td>
</tr>
<tr>
<td><strong>pYC166</strong> amyE::PsinI-lacZ, CmR (this study)</td>
<td>PepsA-R1: 5'-gtcaagcttctagacaatcgcatataattctttg–3'</td>
</tr>
<tr>
<td><strong>pYC166(Δ2-4)</strong> pYC166 derivative, deletion of 0A–P operator sites 2-4</td>
<td>PsinI-F1: 5'-gacgaattcactgacgtctcaaatatgtg-3'</td>
</tr>
<tr>
<td><strong>pYC171</strong> 1-kb DNA upstream and downstream of the stop of yqhG cloned into <strong>pAH54</strong> to flank the spec gene, SpecR</td>
<td>PsinI-R1: 5'-gtcggatcccttcatgcagtttctcctccta-3'</td>
</tr>
<tr>
<td><strong>pYC191</strong> a pDG1662 derivative, amyE::yqhHG sinI sinR, CmR (this study)</td>
<td>PsinI-MF1: 5'-attctcgtttttggtgtgaaaatacgattat-3'</td>
</tr>
<tr>
<td><strong>pYC202</strong> 1-kb DNA upstream and downstream of the start of yqhH cloned into <strong>pAH54</strong> to flank the spec gene, SpecR</td>
<td>PsinI-MR1: 5'-ataaatcgtattttcacaccaaaaacaggaat-3'</td>
</tr>
<tr>
<td><strong>pYC213</strong> a pDG1662 derivative, amyE::PqxM-LCN, CmR (this study)</td>
<td>PsinI-AR1: 5'-aaaaaacgagaatgagggttaatggtaaaaaagtt-3'</td>
</tr>
<tr>
<td><strong>pYC237</strong> a pDG1730 derivative, amyE::PspoIIA-gfp, SpecR (this study)</td>
<td>PsinI-∆R1: 5'-gacaagctttatacctttattataatcgt-3'</td>
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<tr>
<td><strong>pYC213</strong> a pDG1662 derivative, amyE::yqhHG sinI sinR, CmR (this study)</td>
<td>PsinI-MF2: 5'-aataaaggtataatgcaataaaattctgg-3'</td>
</tr>
<tr>
<td><strong>pYC166</strong> pAH54 to flank the spec gene, SpecR (this study)</td>
<td>PsinI-MR2: 5'-ccagaattttattgcattatacctttatt-3'</td>
</tr>
<tr>
<td><strong>pYC171</strong> 1-kb DNA upstream and downstream of the stop of yqhG cloned into <strong>pAH54</strong> to flank the spec gene, SpecR</td>
<td>PsinI-MF3: 5'-ggtgttatttaacgggaataatgcacctcag-3'</td>
</tr>
<tr>
<td><strong>pYC191</strong> a pDG1662 derivative, amyE::yqhHG sinI sinR, CmR (this study)</td>
<td>PsinI-MR3: 5'-ctggaagtcatttccgtttaaatcacc-3'</td>
</tr>
<tr>
<td><strong>pYC202</strong> 1-kb DNA upstream and downstream of the start of yqhH cloned into <strong>pAH54</strong> to flank the spec gene, SpecR</td>
<td>PsinI-MF4: 5'-ggcaaatgacatcctgtgactaatgaag-3'</td>
</tr>
<tr>
<td><strong>pYC213</strong> a pDG1662 derivative, amyE::yqhHG sinI sinR, CmR (this study)</td>
<td>PsinI-MR4: 5'-cttcattagtcacaggatgtcatttgcc-3'</td>
</tr>
<tr>
<td><strong>pYC237</strong> a pDG1730 derivative, amyE::PspoIIA-gfp, SpecR (this study)</td>
<td>PsinI-RS-F: 5'-gctaagcttttaacagtttctgtgtatagttcatctcatgcc-3'</td>
</tr>
<tr>
<td><strong>pYC213</strong> a pDG1662 derivative, amyE::yqhHG sinI sinR, CmR (this study)</td>
<td>PsinI-RS-R: 5'-agcaagctttttcattatgcaactttatgctattagacaca-3'</td>
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<tr>
<td><strong>PsinI</strong> 1-kb DNA upstream and downstream of the stop of yqhG cloned into <strong>pAH54</strong> to flank the spec gene, SpecR</td>
<td>PsinI-FP-F1: 5'-gctagaattctttatctgcaaaataatatttc-3'</td>
</tr>
<tr>
<td><strong>PsinI</strong> 1-kb DNA upstream and downstream of the stop of yqhG cloned into <strong>pAH54</strong> to flank the spec gene, SpecR</td>
<td>PsinI-FP-R1: 5'-gctaggatcccctaaaatacttgtttatgtat-3'</td>
</tr>
<tr>
<td><strong>PsinI</strong> 1-kb DNA upstream and downstream of the stop of yqhG cloned into <strong>pAH54</strong> to flank the spec gene, SpecR</td>
<td>PsinI-KO-P1: 5'-gactctcgaggaattccgtgaaataagtgcctttgcg-3'</td>
</tr>
<tr>
<td><strong>PsinI</strong> 1-kb DNA upstream and downstream of the stop of yqhG cloned into <strong>pAH54</strong> to flank the spec gene, SpecR</td>
<td>PsinI-KO-P2: 5'-gactctcgaggaattccgtgaaataagtgcctttgcg-3'</td>
</tr>
<tr>
<td><strong>PsinI</strong> 1-kb DNA upstream and downstream of the stop of yqhG cloned into <strong>pAH54</strong> to flank the spec gene, SpecR</td>
<td>PsinI-KO-P3: 5'-gactctcgaggaattccgtgaaataagtgcctttgcg-3'</td>
</tr>
<tr>
<td><strong>PsinI</strong> 1-kb DNA upstream and downstream of the stop of yqhG cloned into <strong>pAH54</strong> to flank the spec gene, SpecR</td>
<td>PsinI-KO-P4: 5'-gactctcgaggaattccgtgaaataagtgcctttgcg-3'</td>
</tr>
<tr>
<td><strong>PsinI</strong> 1-kb DNA upstream and downstream of the stop of yqhG cloned into <strong>pAH54</strong> to flank the spec gene, SpecR</td>
<td>PsinI-KO-P5: 5'-gactctcgaggaattccgtgaaataagtgcctttgcg-3'</td>
</tr>
</tbody>
</table>
Supplemental Materials and Methods

Strain and plasmid construction

1) Plasmid construction

pYC166 contains a $P_{sinI}$-lacZ transcriptional fusion in pDG268. pDG268 is an amyE locus integration vector which also contains a promoter-less lacZ gene (Antoniewski et al, 1990). To construct pYC166, the promoter region of $sinI$ was amplified by PCR using 3610 chromosomal DNA as the template and primers $P_{sinI-F1}$ and $P_{sinI-R1}$. PCR products were gel-purified and cloned into the EcoRI and BamHI sites of pDG268, resulting in pYC166. Introduction of mutations to the regulatory region of $sinI$ in pYC166 was achieved by site-directed mutagenesis as described above. To construct a 43 base-pair deletion spanning from the operators 2 to 4 in the regulatory region of $sinI$, two DNA fragments were amplified by PCR using primers $P_{sinI-F1}$ and $P_{sinI-MR1}$, and primers $P_{sinI-MF1}$ and $P_{sinI-R1}$, respectively. The first DNA fragment starts from the 5' end of the $sinI$ promoter region and ends at the 5' end of operator site 2, and the second DNA fragment covers from 3' end of the operator site 4 to the immediate upstream of $sinI$. These two DNA fragments were cloned into pDG268 sequentially between the EcoRI and HindIII, and the HindIII and BamHI sites, and that resulted in a recombinant plasmid pYC166(Δ2-4). To make a scrambled sequence for the operators 2 to 4, two overlapping oligonucleotides, $P_{sinI-RS-F1}$ and $P_{sinI-RS-R1}$, were used to generate a double strand DNA by overlapping extension in polymerization reaction. The DNA fragment was then cloned into the HindIII site of pYC166(Δ2-4).
pYC191 contains a 3.5-kb *yqhHG* *sinI sinR* fragment cloned into pDG1662 (Guérout-Fleury et al, 1996). To construct pYC191, the above 3.5-kb DNA sequence was amplified by PCR using primers P\(_{yqhHG}\)-F1 and *sinIR*-R1. The PCR products were gel-purified and co-digested with EcoRI and BamHI. After co-digestion, the PCR products were turned into two smaller fragments. One starts from the promoter of *yqhH* and ends at an internal EcoRI site in *yqhH*, and the other starts from the internal EcoRI site to the end of *sinR*. Both DNA fragments were cloned into pDG1662 by three-fragment ligation, resulting in pYC191.

To construct pYC213, the promoter sequence of the *yqxM* matrix operon (P\(_{yqxM}\)) was amplified by PCR using primers P\(_{yqxM}\)-F1 and P\(_{yqxM}\)-R1 and 3610 chromosomal DNA as the template. The gene encoding a proteolytically unstable GFP protein (*gfp-LCN*) was also amplified by PCR using primers *gfp*-F1 and *gfp*-R2(LCN) and the plasmid pNGFP (Arnaud et al, 2004) as the template. Both PCR products were then digested and cloned into the plasmid pDG1662 sequentially, resulting in pYC213.

To construct pYC237, the promoter sequence of the *spoIIA* operon was amplified by PCR using primers P\(_{spoIIA}\)-F1 and P\(_{spoIIA}\)-R1 and 3610 chromosomal DNA as the template. The *gfp* gene was amplified by PCR using primers *gfp*-F1 and *gfp*-R2, and the plasmid pNGFP as the template. Both PCR products were then cloned into pDG1730 (Guérout-Fleury et al, 1995) sequentially, resulting in pYC237.

2) Construction of YC256 and its derivatives
YC256 is a 3610 derivative that contains a transcriptional fusion of \( P_{sin}^{r} \) lacZ integrated into the amyE locus on the chromosome. To construct YC256, the recombinant plasmid pYC166 was first introduced into PY79 by transformation (Gryczan et al, 1978). Transformants were selected for integration of \( P_{sin}^{r} \) lacZ to the amyE locus in the chromosome of PY79 by double crossover recombination. The DNA fragment containing the \( P_{sin}^{r} \) lacZ transcriptional fusion was then introduced into 3610 by SSP1 phage-mediated transduction (Yasbin & Young, 1974). Introduction of \( P_{sin}^{r} \) lacZ with mutant alleles into 3610 chromosome was performed similarly as described above by using derivatives of the plasmid pYC166 that contain corresponding mutations. Derivatives of YC256 were named as YC256(M1), YC256(M2), YC256(M3, 4), YC256(\( \Delta \)2-4), and YC256(RS2-4). The corresponding operator mutations in those derivative strains were described in Figure 2A.

3) Construction of YC285 and its derivatives

To construct YC285, we applied a method similar to long-flanking PCR mutagenesis (Wach, 1996). In the first round PCR reaction, a 1.2-kb DNA fragment starting from the middle of the yqhH gene to the downstream of sinR was amplified by PCR using primers yqhH-F1 and yqhH-R1 and 3610 chromosomal DNA as the template. A second DNA fragment starting from the end of the tasA gene to the middle of tasA was also amplified by PCR using primers tasA-F1 and tasA-R1. These two PCR products were gel-purified and used as primers in the second round PCR reaction, in which the kan drug
resistance gene from linealized plasmid pAH49 was used as the template. The products from the second round PCR reaction were introduced into PY79 by transformation and selected for kanamycin resistance. The resulting transformants contained a \textit{kan} gene inserted to the integenic region between \textit{sinR} and \textit{tasA}. The DNA fragment containing the \textit{kan} gene and the flanking sequences were then introduced into 3610 by transduction. The resulting strain was named YC285. YC285 is otherwise the same as 3610 except for the \textit{kan} gene insertion.

To construct derivatives of YC285 that contained various operator mutations and deletions, we applied a slightly different strategy. First, in the first round PCR reaction, operator mutations or deletions were generated by using the method that was described previously and incorporated into the first PCR products starting from the middle of \textit{yqhH} to the end of \textit{sinR}. After the second round PCR reaction, the PCR products were introduced by transformation into a PY79 derivative mutant for $\Delta$\textit{sinI} $\Delta$\textit{sinR}. The resulting transformants were verified for the \textit{kan} gene insertion between \textit{sinR} and \textit{tasA} and introduction of operator mutations or deletions by DNA sequencing. The DNA fragment containing \textit{kan} and operator mutations was then introduced into 3610 by transduction.

To construct strains YC716 and YC717, the plasmid pYC213, which contains the reporter \textit{amyE}::$P_{yqhM}$\textit{gfp-LCN}, was first introduced into PY79 by transformation. The DNA containing the reporter fusion, as well as the DNA containing the \textit{epsH} null mutation ($\Delta$\textit{epsH}, from RL3852), was introduced into
both YC285 and YC285(Δ2-4) by transduction, resulting in strains YC716 and YC717, respectively.

4) Construction of \( \Delta \text{sinl sinR} \) complementation strains

To construct \( \Delta \text{sinl sinR} \) complementation strains at \( \text{amyE}(28^\circ), \text{ylnF}(140^\circ), \text{bkdB}(213^\circ), \) and \( \text{ywrK}(317^\circ) \) loci on the chromosome, the plasmid pYC191, which contains \( \text{yqhHG sinl sinR} \) fragment flanked by \( \text{amyE} \) fragments was first introduced by transformation into AHB277, AHB289, and AHB290 (Table S1), respectively. The transformants were selected for Cm\(^R\) and Spec\(^S\) on the selective plates. In those transformants, \( \text{amyE}::\text{yqhHG sinl sinR} \) (from pYC191) was integrated at the \( \text{amyE} \) site in \( \text{ylnF}(140^\circ), \text{bkdB}(213^\circ), \) and \( \text{ywrK}(317^\circ) \) loci, instead of at the native \( \text{amyE} \) locus. \( \text{amyE}::\text{yqhHG sinl sinR} \) fragments were then introduced to a \( \Delta \text{sinl sinR} \) strain of 3610 by SSP1-phage mediated transduction.

To construct a strain with two copies of \( \text{yqhHG sinl sinR} \) (YC562), one at the native locus, and the other integrated at an origin-distal site \( \text{bkdB}(213^\circ) \), the \( \text{amyE}::\text{yqhHG sinl sinR} \) fragment integrated at \( \text{bkdB}(213^\circ) \) that was constructed was simply introduced into 3610.

5) Construction of YC264 and YC553

Strain YC264 contains a spectinomycin resistance gene inserted between the 3’ end of the \( \text{yqhG} \) gene and the promoter region of \( \text{sinl} \). To construct YC264, a DNA fragment (~900-bp) starting from the 5’ end of \( \text{yqhG} \) to the stop codon of
the *yqhG* gene was amplified by PCR using 3610 chromosomal DNA as template and primers *P* *sin*-*KO*-P1 and *P* *sin*-*KO*-P2. Similarly, a second DNA fragment (~900-bp) starting from the stop codon of the *yqhG* gene to the end of *sinR* was amplified by PCR using primers *P* *sin*-*KO*-P3 and *P* *sin*-*KO*-P4. PCR products were gel-purified. The first PCR products were cloned into the XhoI site of pAH54 (gift of Camp A), and the second PCR products were cloned sequentially into the BamHI and ScaI sites of pAH54. The resulting plasmid (pYC171) contains the *spec* gene flanked by the above two DNA fragments. The plasmid was then introduced into PY79 by transformation. The *spec* resistance gene containing DNA fragment was then introduced into 3610 by transduction.

Strain YC553 was constructed very similarly to that of YC264. A few differences were described below. First, the first PCR product starts from 5’ upstream of the *yqhH* promoter to the start codon of the *yqhH* gene, and was amplified by PCR using primers *P* *yqhH*-KO-P1 and *P* *yqhH*-KO-P2, the second PCR products starts from the start codon of *yqhH* to an internal site of *yqhH*. These two PCR products were cloned into pAH54 sequentially and generated a recombinant plasmid pYC202. The rest of the steps were the same as described above.

6) Construction of YC718 and YC719

To construct YC718 and YC719, the strain RL3618 (ref) in which the *spo0A* gene was replaced by an IPTG-inducible copy of *spo0A-sad67* (*spo0A*Ω*P* *hyspank*- *spo0A-sad67*) was infected with SSP1 phage. The *sad67*
construct, as well as the reporter fusion lacA::P_yqxM+yfp (made from DL841, a gift of Lopez, D), was then introduced into YC285(Δ2-4) and a derivative of YC285(Δ2-4), in which the yqhHG sinI sinR fragment was transplanted to the origin-proximal amyE locus by transduction, resulting in strains YC718 and YC719.

7) Construction of YC734

Strain YC734 was constructed as follows. The recombinant plasmid pYC237 was first introduced into PY79 by transformation. The DNA containing the reporter fusion was then introduced into ALM91 (gift of McLoon A), a 3610 derivative that contains a luciferase reporter (Stewart & Williams, 1992) fused to the promoter of the yqxM operon (P_yqxM-lux) by transduction, resulting in strain YC734. The construction of the luxABCDE reporter was described previously (Schmalisch et al, 2010).

**Fluorescent microscopic analysis of YC716 and YC717**

YC716 and YC717 cells were grown in MSgg medium to stationary phase. 1-ml cells were harvested, washed twice with PBS buffer, and suspended in 50-μl of PBS buffer. Samples were mixed with FM4-64, and 3-μl of cells were applied to the center of an agar-coated microscopy slide. Cover slides were pretreated with poly-L-lysine (Sigma). Samples were analyzed using 100x oil merged lens on an Olympus workstation BX61. Images were taken using an
automated software program SimplePCI and analyzed with programs MetaMorph (Universal Imaging Corporation) and Image J (http://rsbweb.nih.gov/ij/).


Copy number control

1 A model for matrix gene expression

We consider the activity of a matrix promoter as some function $f(I, R)$ of SinI and SinR concentration. The SinR population is divided into two pools: $[IR]$ is the part bound by SinI and held inactive, and $R^*$ is the free SinR that regulates target promoters. We denote the total SinR population by $R$, so that $R = [IR] + R^*.$

As SinR regulation of target promoters is cooperative, it is reasonable to model the activity of a regulated promoter as a Hill function in $R^*$. We will for the moment leave the precise mechanism of SinI titration of SinR unspecified, instead assuming just that $[IR] = Rg(I)$ for some decreasing function $g$ of SinI concentration. These two assumptions yield the following form for $f$ (with normalized units of activity):

$$f(I, R) = \frac{K^n}{K^n + (Rg(I))^n},$$

where $K$ is the apparent dissociation constant and $n$ is the Hill coefficient.

2 Approximating fold change

We are interested in the relative change in promoter activity when the copy number of SinI and SinR is altered. If copy number increases, we assume each protein increases by some factor $c > 1$. An approximation of the change in promoter activity then follows by Taylor expansion:

$$\frac{f(cI, cR)}{f(I, R)} \approx 1 + (c - 1) \left( \frac{I}{f(I, R)} \frac{\partial f}{\partial I}(I, R) + \frac{R}{f(I, R)} \frac{\partial f}{\partial R}(I, R) \right)$$

$$= 1 + (c - 1)(S_I(I, R) + S_R(I, R)), \tag{1}$$

where we have recognized the logarithmic sensitivities $S_I = \frac{\partial \ln f}{\partial \ln I}$ and $S_R = \frac{\partial \ln f}{\partial \ln R}$. 
In the case of the model described in the previous section, it can be shown by direct computation that

\[ S_I = S_R \cdot \left( 1 + \frac{\partial \ln g}{\partial \ln A} \right) . \]

Christening \( H_R := S_R \) and \( H_I := \frac{\partial \ln g}{\partial \ln I} \), we recognize that each of these is negative. Plugging \(|H_R| = -H_R\) and \(|H_I| = -H_I\) into (1) and picking \( c = 2 \) yields the equation from the main paper:

\[
\frac{f(2I, 2R)}{f(I, R)} \approx 1 + |H_R|(|H_I| - 1) \tag{2}
\]

Having shown the general mechanism, we now specialize slightly. The requirement that activity of SinR-regulated promoters decreases in response to an increase in copy number implies that \(|H_R|(|H_I| - 1) < 0\), which requires that \(|H_I| < 1\). As it is known that SinI and SinR bind 1:1, we approximate the dynamics of the complex as:

\[
\frac{d[IR]}{dt} = k_1[I][R^*] - k_{-1}[IR].
\]

At steady state, together with the condition that \([IR] + R^* = R\), this equation shows that

\[ [IR] = [R] \frac{[I]}{K_1 + [I]} \quad \Rightarrow \quad [R^*] = [R] \frac{K_1}{K_1 + [I]} . \]

Thus the free SinR concentration is given by the total SinR concentration multiplied against a Michaelis-Menten function. (In terms of the notation used previously, \( g(I) = \frac{k_{01}}{K_1 + I} \).) The logarithmic sensitivity of Hill functions varies between 0 and the Hill coefficient, so this simple mechanism, where the Hill coefficient is 1, obviously satisfies the condition \(|H_I| < 1\).

3 A note on the general case

It is clear that the derivations here have nothing to do with SinI or SinR in particular. If we have a gene regulated by a number of activators \( A_1, \ldots, A_n \) and repressors \( R_1, \ldots, R_m \) that undergo changes in copy number \( c_{A_i} \) and \( c_{R_i} \), then it follows from the calculations above that

\[
\frac{f(c_{A_1}, A_1, \ldots, c_{A_n} A_n, c_{R_1} R_1, \ldots, c_{R_m} R_m)}{f(A_1, \ldots, A_n, R_1, \ldots, R_m)} \approx 1 + \sum_{i=1}^{n} (c_{A_i} - 1) \left| \frac{\partial \ln f}{\partial \ln A_i} \right| - \sum_{i=1}^{m} (c_{R_i} - 1) \left| \frac{\partial \ln f}{\partial \ln R_i} \right| .
\]

Thus for a general promoter, the approximate fold change in activity is set by the copy number increase of a given regulator multiplied by the sensitivity with respect to that regulator, where activators cause increases and repressors cause decreases. As in the SinI-SinR case, larger changes follow from high sensitivity, which suggests that promoters where regulators bind with a high degree of cooperativity may be most likely to exhibit copy number control.
Supplemental References


Wach A (1996) PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **12**: 259-265