

pLOSS*, A Powerful Screening Tool for Isolating Synthetic Lethal Mutations[†]

In a landmark 2003 publication, an international consortium of *Bacillus subtilis* researchers published a tour-de-force study in which they systematically inactivated individual genes in the organism (9). They succeeded in identifying 271 genes that are essential for viability in *B. subtilis* under standard laboratory conditions, most of them involved in nucleic acid metabolism, protein production, envelope synthesis, cell shape and division, and respiration. While this type of analysis can provide deep insights into the physiology and development of *B. subtilis*, it suffers from at least one important limitation: it cannot detect essential functions that are provided by a set or two or more redundant genes.

The phenomenon of a gene masking or modulating the effect of a mutation in a second gene is known as "buffering" (7). Buffering can make the identification of essential genes and the analysis of their function difficult. Yet the converse is also true; if it can be shown that a buffering exists for two genes, it can be inferred that their products likely play a role in the same physiological process or biochemical pathway. For this reason, the identification of "synthetically lethal" mutations—those that are lethal in combination but not as single mutations—is a potentially powerful analytical strategy.

Synthetic lethal mutations are known in *B. subtilis*. For example, mutations in *smc*, *spo0J*, and *spoIIIE*, genes involved in chromosome partitioning, have been examined individually and in combination. A knockout mutation in any one of these genes causes significant impairments in growth or sporulation septum formation or both, yet none is absolutely essential for cell viability under normal laboratory conditions (5, 8, 11, 12). The *smc spo0J* double mutant is synthetically sick, however, showing a slower growth rate and a more severe partitioning defect than either single mutant (5). The *smc spoIIIE* double mutation is synthetically lethal, bringing all cell growth to a halt 9-11 generations after *smc* expression is turned off, while the *smc spo0J spoIIIE* triple mutation halts growth even more rapidly (4). These phenotypes provide powerful evidence for the joint participation of these proteins in chromosome partitioning and reveal important clues about the specific role of each.

Typically, synthetic lethal mutations are uncovered when one already suspects that two genes participate in a common function, as with the chromosome partitioning genes discussed above. In such a case, it is a relatively straightforward matter to knock out one gene, place the second one under an inducible promoter, and assay for viability when the inducer is removed. Yet for many applications, such as functional genomics studies, what is really needed is a strategy to allow hundreds or thousands of mutation combinations to be screened for synthetic phenotypes. Pioneering studies in yeast genetics (1, 2) have developed just such a strategy, and it has proved very useful in *Escherichia coli* functional genomics as well (3). The strategy makes use of special segregationally unstable vectors. One such vector, pLOSS*, has now been developed to facilitate screening for synthetic lethal or sick mutations in *B. subtilis* and other gram-positive organisms (6). The features of pLOSS* (which stands for Lethal Or Synthetic Sick) are detailed in Figure 1 and Table 1 (next page).

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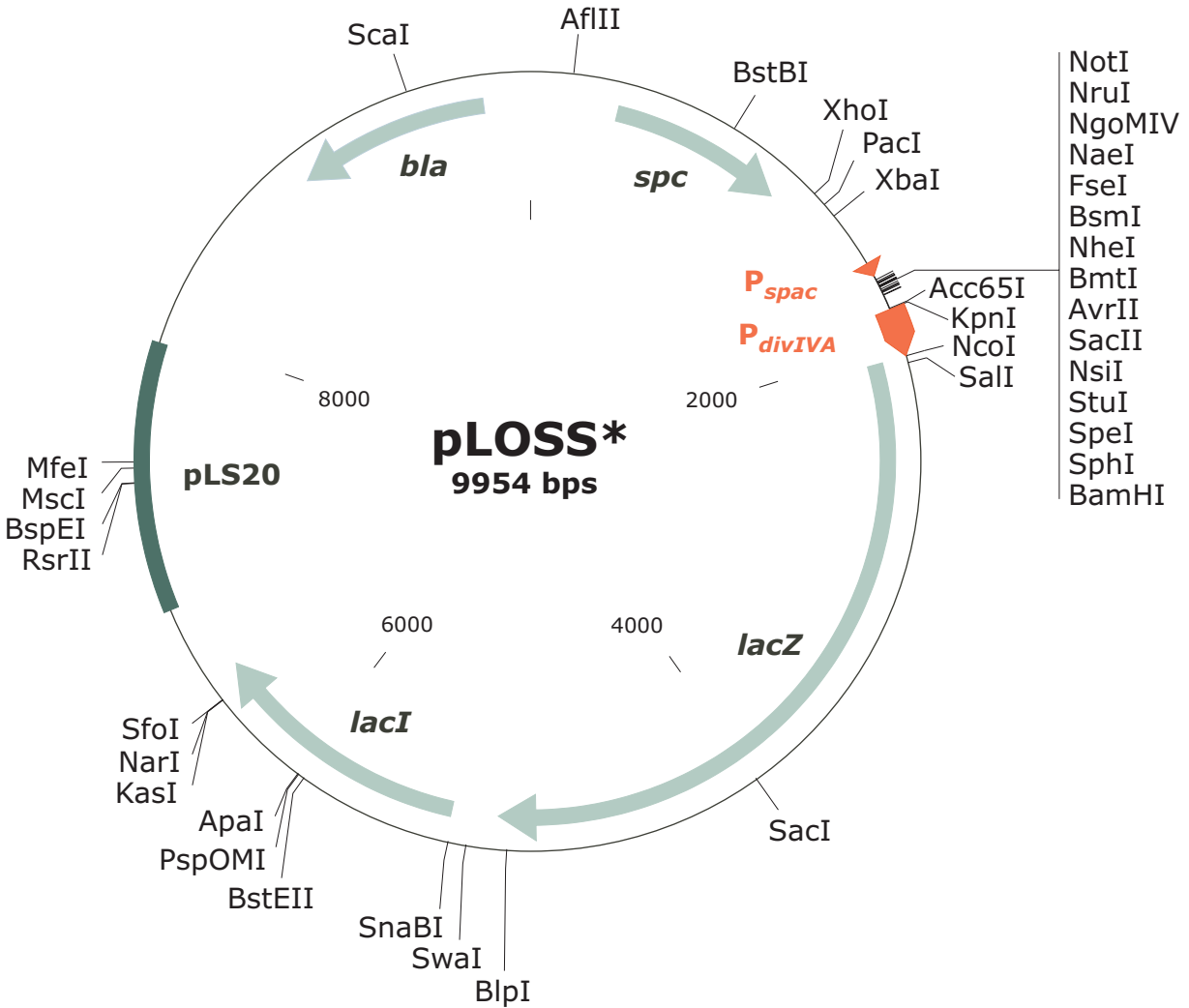


Figure 1. Genetic and physical map of the synthetic lethal mutaiton screening vector, pLOSS* (Claessen *et al.*, 2008). Gray arrows denote coding sequences, boxes denote replication functions, and orange arrows denote promoters and other regulatory regions. Single-cutting restriction enzyme sites are indicated. See text for details.

Table 1. Features of the pLOSS* plasmid

Function	Product	Coordinates
P _{spac}	IPTG-inducible promoter for <i>Bacillus</i>	1651..1709
P _{divIVA}	constitutive promoter driving <i>lacZ</i>	1859..2059
<i>lacZ</i>	reporter gene (blue/white on X-gal)	β -galactosidase
<i>lacI</i>	inducer of the P _{spac} promoter	<i>lac</i> operon repressor
<i>bla</i>	ampicillin resistance (in <i>E. coli</i> only)	β -lactamase
<i>spc</i>	spectinomycin resistance	O-nucleotidyltransferase
pLS20	gram-positive replication functions	

Plasmid pLOSS* is a shuttle vector. In *E. coli*, it replicates as a high copy number plasmid, allowing for easy isolation and purification. In *B. subtilis*, replication depends on a 1.1 kb fragment from the large "*B. subtilis* var. *natto*" plasmid pLS20. Replication is unstable, however, due to point mutations engineered in the DnaA box of this fragment. A spectinomycin resistance gene is included on pLOSS* so that the user can apply selective pressure to maintain the plasmid. The presence of the plasmid can also be verified visually due to the constitutively expressed β -galactosidase gene, *lacZ*, providing for the familiar "blue-white" colony screening on X-gal media. Finally, pLOSS* includes the well-characterized P_{spac} promoter with an adjacent multiple cloning site, so that the expression of a cloned gene can be controlled by IPTG induction. In short, pLOSS* is an unstable expression vector, the presence of which can be ensured by spectinomycin selection and monitored by the blue-white phenotype on indicator media. These features facilitate a powerful yet elegantly simple screening strategy for synthetic lethal mutations.

This first step is to construct a library of double knockout mutants in which the function of one inactivated gene is supplied *in trans* by a copy cloned on pLOSS*. Next, a second plasmid, this one a transposon delivery vector, is introduced into the cell. The *mariner* transposon plasmid pMarA (10) has been shown to work well in conjunction with pLOSS* (6), but in principle other transposon delivery systems developed for use in *Bacillus* should work also. A transposon mutant library is prepared in this background, with continued selection for pLOSS*. There should exist in this library virtually every combination of double mutant, with the first mutation in the gene of interest (still complemented by the plasmid) and the second gene being any of the nearly 4000 non-essential genes of *B. subtilis*.

Aliquots of the library are now plated on indicator media *without* antibiotic selection. Under these conditions, the pLOSS* vector should rapidly be lost, unless another selective pressure exists—the requirement for the functional cloned gene supplied by the plasmid. It is a simple matter, then to screen for potential synthetic lethal mutations—they should be solid blue on the indicator, showing that all viable cells have retained the plasmid (see Figure 3, next page). White or highly sectored colonies show that the two mutations are not

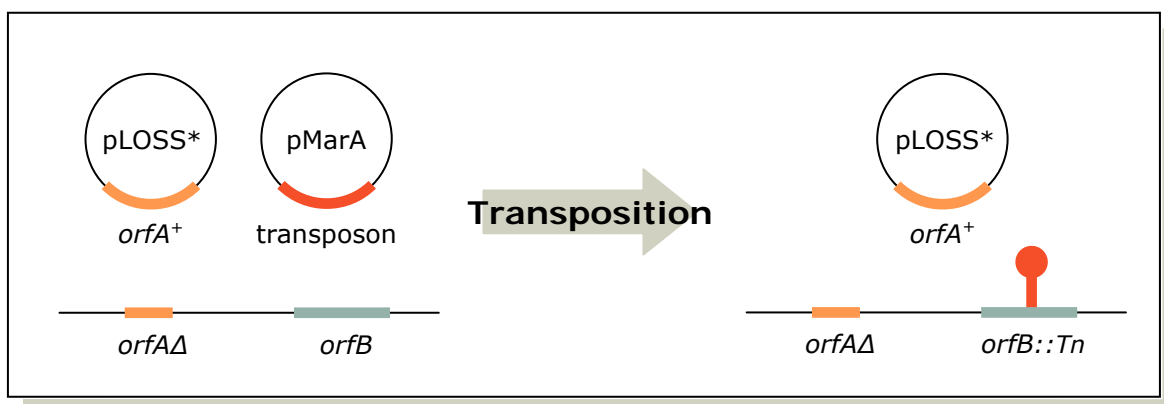


Figure 2. Construction of library to for isolation of synthetic mutations. The gene of interest, *orfA*, is inactivated; a functional copy is supplied on pLOSS*. A transposon delivery vector, such as pMarA, is introduced and insertion events are then selected. The result is a library of double mutants, with one mutation (*orfA*Δ) complemented by the pLOSS* vector and a second (*orfB*) knocked out by transposon insertion.

synthetically lethal, since plasmid loss is tolerated. Two mutations that are synthetically "sick" but still viable should form healthy blue colonies, but upon replating may segregate both blue and smaller, less robust white colonies.

Claessen *et al.*, who developed the pLOSS* system, used it to screen for mutations that were synthetically lethal or sick in the absence of *ezrA*, a gene believed to participate in both cell division and maintenance of cell shape. They inactivated *ezrA* in the chromosome and supplied a functional copy on pLOSS*. Using transposon mutagenesis and blue-white screening on indicator plate, as outlined above, the authors identified an insertion mutation in *sepF* that was synthetically lethal and a second mutation in a previously uncharacterized gene, *gpsB*, that was synthetically sick in combination with *ezrA*. The analysis provided key insights into both cell division and cell wall synthesis in *B. subtilis* (6).

The development of pLOSS* should facilitate a much more thorough analysis of cell systems that are essential for survival in *B. subtilis* and any number of other gram-positive species.

Availability. The Bacillus Genetic Stock Center is pleased to offer pLOSS* either in *E. coli* (BGSC accession ECE200) or as purified plasmid DNA (accession ECE200P). Please consult our website (www.bgsc.org) to learn about our user fee policy and about the other services we offer.

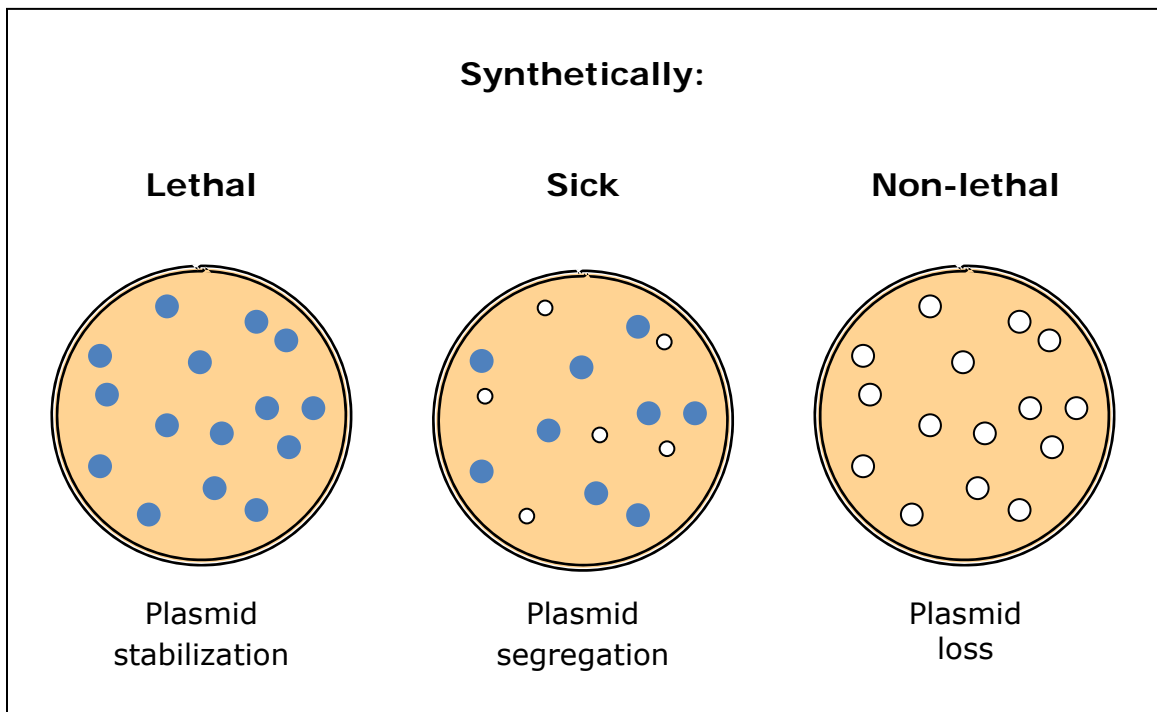


Figure 3. Screen for synthetically lethal mutants. Two genes are inactivated in the host. A functional copy of one is supplied on an unstable vector. Selection for the plasmid is removed and the cells are plated on an indicator. If the double mutation is lethal, all colonies will be blue (left). If the double mutant is sick but viable, healthy blue colonies will remain, but some defective white colonies will also segregate (center). If the double mutant is healthy, the plasmid will be lost, resulting in white or sectored colonies (right).

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