Genomic-Based Identification of the Sporulation Restoring Gene in Degenerate *Clostridium acetobutylicum* Strains

There is a renewed interest in the study of *Clostridium acetobutylicum* due to its applicability in renewable and "greener" production methods for alternative fuels and industrial solvents. Furthermore, due to significant advances in genetic technologies, *C. acetobutylicum* has ostensibly become a model clostridia for studying other solventogenic and pathogenic clostridia. Of considerable interest are the solventogenesis and sporulation differentiation programs, both of which are abolished in degenerate strains (lacking the 192-kb, 178-gene megaplasmid "pSOL1"). The operon and small genetic locus necessary for solvent formation has been identified and characterized, but the exact gene or operon necessary for sporulation remains a mystery. Knowledge of this gene or operon can be utilized for bioengineering a non-spore forming, solvent producing strain, ideal for industrial continuous fermentations.

To identify this gene or operon, we developed a functional pSOL1 library and selection assay for spore-forming cells. Our approach utilizes multiple libraries of all pSOL1 genes/operons under constitutive promotion in degenerate strains. Our experience suggests that *C. acetobutylicum* is efficiently transformed by plasmids <10 kb in size, thus library inserts are restricted to 5–6 kb. We created two distinct libraries; a specific PCR generated library and a sheared pSOL1 DNA generated library. To increase cloning efficiency and library versatility, we employed Invitrogen TOPO-TA® cloning and Gateway Technology®. We developed two assays for screening spore-forming cells, one based on chloroform chemical treatment and another by flow-cytometric analysis. We show that chloroform treatment disrupts all *C. acetobutylicum* cells that have not significantly advanced into endospore formation, such that they are unable to yield colony-forming units when spread onto nutrient plates. Although not yet widely used for prokaryotic analysis, we are developing high-throughput flow cytometry assays capable of discerning single-cell morphology (specifically vegetative versus endospore) based upon membrane potential, DNA content, membrane integrity, and forward/side scatter characteristics. Plasmid DNA is isolated from colonies that survive chloroform treatment and sequenced for the identification of the specific gene/operon.