**A Cell Engineering Strategy to Enhance Supercoiled Plasmid DNA Production for Gene Therapy**

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**ABSTRACT:** With the recent revival of the promise of plasmid DNA vectors in gene therapy, a novel synthetic biology approach was used to enhance the quantity, (yield), and quality of the plasmid DNA. Quality was measured by percentage supercoiling and supercoiling density, as well as improving segregational stability in fermentation. We examined the hypothesis that adding a Strong Gyrase binding Site (SGS) would increase DNA gyrase-mediated plasmid supercoiling. SGS from three different replicons, (the Mu bacteriophage and two plasmids, pSC101 and pBR322) were inserted into the plasmid, pUC57. Different sizes of these variants were transformed into *E. coli* DH5α, and their supercoiling properties and segregational stability measured. A 36% increase in supercoiling density was found in pUC57-SGS, but only when SGS was derived from the Mu phage and was the larger sized version of this fragment. These results were also confirmed at fermentation scale. Total percentage supercoiled monomer was maintained to 85–90%. A twofold increase in plasmid yield was also observed for pUC57-SGS in comparison to pUC57. pUC57-SGS displayed greater segregational stability than pUC57-cer and pUC57, demonstrating a further potential advantage of the SGS site. These findings should augment the potential of plasmid DNA vectors in plasmid DNA manufacture.

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**KEYWORDS:** plasmid DNA; supercoiling density; segregational stability; cell engineering; fermentation; *E. coli*
catalytic cycle, thereby introducing two negative supercoils. In a prior study, overexpression of DNA gyrase in bacterial strains led to only a very small increase in plasmid supercoiling (Snoep et al., 2002).

Initial research on gyrase binding to DNA indicated that gyrase bound with varying strength to binding sites on DNA molecules of about 140 bp. The plasmid ColEI, and viruses Mu, dX174 and SV40 all contain a range of binding sites (Morrison and Cozzarelli, 1981). Mu phage has a strong gyrase binding site (SGS) for DNA gyrase which forms an essential part of the bacteriophage's replication. Here we test the effect of the addition of SGS sequences from Mu phage, and from plasmids pBR322 (Lockshon and Morris, 1985) and pSC101 (Wahle and Kornberg, 1988), to the multicopy plasmid pUC57. Small and large versions of these fragments were investigated, since prior work has shown that DNA sequences either side of the central 140 bp of the SGS are important in its activity (Oram et al., 2003; Pato and Banerjee, 2000).

Three possible consequences of adding the strong gyrase binding site (SGS) were identified. Firstly, SGS may lead to an increase in the percentage of supercoiled pDNA relative to open-circles. Alternatively, or additionally, SGS may increase plasmid superhelical density, or increase the rate that plasmid molecules achieve their final superhelical density.

Supercoiling density was calculated according to Bowater et al. (1992). Figure 1 shows an example of a two-dimensional chloroquine diphosphate gel used to analyze supercoiling density. Small-scale studies carried out in falcon tubes, all analyses were performed on stationary phase cultures. Supercoiling density measurements for fermentations were taken at the start of the exponential phase to stationary phase. A plasmid preparation for pUC57-SGS (Mu, 398) isolated from E. coli DH5α after an 8 h fermentation is shown in the gel in Figure 1. Linking number difference, ΔLk is determined by (Lk−Lk0), where Lk is linking number or number of superhelical turns for the supercoiled plasmid and Lk0 is linking number for the corresponding relaxed form of plasmid DNA. In order to determine the linking number difference, ΔLk, of each plasmid type from the two-dimensional chloroquine diphosphate gels, all the topoisomers in the left-hand arc, starting with the most relaxed form (ΔLk = 0) were counted. The supercoiling density or specific linking difference of the plasmid, α, was then determined by dividing ΔLk with Lk0, estimated by (N/h0), where N is the plasmid size in number of base pairs and h0 is the number of base pairs per turn of the DNA helix for circular B form of DNA in its relaxed state, known to be 10.5 bp/turn.

All seven types of pUC57-SGS were prepared as synthetic genes in TOP10 strain of E coli by GenScript Corporation (Piscataway, NJ). The resulting plasmids consisted of a mixture of dimers and monomers. Since, dimers and multimers would be considered as contaminants in the process, plasmids preparations isolated from the TOP10 strain of E. coli that were mainly monomer were propagated. The result of this was an average of 60% supercoiled monomer for all pUC57-SGS regardless of SGS source (Appendix A). As not all dimers and multimers were completely eliminated in these plasmids, the plasmids were digested and re-ligated before being transformed into a Rec− strain of E. coli (that prevents plasmid multimerisation), DH5-α. This was successful in achieving 100% monomer that consisted of more than 90% supercoiled plasmid DNA, with the remainder being open-circles (Fig. 2A). This supports the findings in Yau et al. (2008) where, DH5-α cells transformed with the 5.8 kb plasmid, gWiz had a similarly high supercoiled monomer content.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** (A) Equations and definitions for calculating supercoiling density. (B) Plasmid preparations were run in the first dimension with 0.6 mg/L chloroquine diphosphate, and in the second dimension with 3 mg/L chloroquine diphosphate. The gel is showing an example plasmid preparation after an 8 h fermentation for pUC57-SGS (Mu, 398) isolated from DH5α E. coli strain. (C) Counting method to determine ΔLk in Figure 1B and supercoiling density, α, from ΔLk.
No other sources of SGS led to a change in supercoiling density. This is most likely because the plasmid gyrase site, pBR322 is weaker than Mu SGS (Pato and Banerjee, 1999). On the other hand, pSC101 although it is a strong site that allows cleavage in the presence of gyrase, it is unable to promote Mu replication, indicating that it is a less efficient site (Pato and Banerjee, 1999).

Further studies are required to establish whether this increase in supercoiling density improves plasmid integrity during downstream processing and uptake by cells for greater biological activity or efficiency, but studies demonstrating that percentage supercoiling (Cupillard et al., 2005; Dhanoya et al., 2011, 2012; Pillai et al., 2008) is important for uptake or activity, and for downstream processing (Li et al., 2011) indicate that this might be the case.

To show that the increases in superhelical density can be scaled, batch fermentations using bioreactors were performed. Growth profiles for pUC57 and pUC57-SGS, were similar, reaching a maximal OD at 600 nm of about 20 (Fig. 3A). There was a steady increase in plasmid DNA yield with cell growth up to the maximal at 10–11 h of batch culture (Fig. 3B) and *E. coli* DH5α harboring plasmids with SGS were able to produce almost double the plasmid DNA yield of those harboring pUC57 alone with an average maximum of 9 μg/ml of culture. The reason for the increase in plasmid yield in DH5α cells for pUC57-SGS was not investigated further via experimentation, however, a possible reason is that in Rec− strains, more efficient supercoiling causes more efficient DNA replication hence increasing plasmid yield. A higher superhelical density would potentially lead to easier melting of the DNA duplex and might enable the pRNA II promoter to initiate more often and/or facilitate the RNA/DNA duplex formation that is the precursor to the initiation of RNase III cleavage and DNA replication.

Percentage supercoiled monomer was successfully maintained at about 90% from the beginning of the fermentation to mid-exponential phase in all cases, dropping only slightly to about 85% from mid-exponential phase to the end of the fermentation (Fig. 3C), possibly due to greater energy used by the cells at this later point in the batch fermentation, in cell division and in plasmid DNA replication. Figure 3D and E show that, similarly to the small-scale results in 15 ml cultures grown under less controlled conditions, for pUC57-SGS (Mu, 398) grown in this *E. coli* strain, there was a statistically significant, 36%, increase in supercoiling density relative to the parent plasmid, pUC57, (P = 0.008, t-test, one-tailed distribution, two-samples equal variance, Excel 2013). A prior study (Yau et al., 2008) however, has shown that percentage supercoiling is affected by *E. coli* host strain and our studies have shown that benefits of the SGS (Mu, 398) are not visible in strains allowing plasmid multimerisation (in BL21 DE3 cells, Appendix B), and hence careful choice of *E. coli* host strain for plasmid DNA production is vital.

Another important consideration for plasmid DNA manufacture is the current presence of antibiotic in the medium, which is not favored by the regulatory authorities for gene therapy or DNA vaccination due to the possibility of horizontal transfer of antibiotic resistance to the circulating microbial population (Sodoyer et al., 2008) however, has shown that percentage supercoiling is affected by *E. coli* host strain and our studies have shown that benefits of the SGS (Mu, 398) are not visible in strains allowing plasmid multimerisation (in BL21 DE3 cells, Appendix B), and hence careful choice of *E. coli* host strain for plasmid DNA production is vital.
Antibiotics are traditionally added to kill plasmid-free cells that arise due to the segregational instability of the plasmid replicons, thus increasing the segregational stability of plasmids could be beneficial in enabling growth in the absence of antibiotics. Most multicopy plasmids used in molecular biology and gene therapy are derivatives of the Col EI replicon (Prather et al., 2003). This plasmid is naturally occurring, highly stable and gives rise to no plasmid-free cells during replication and cell division. However, during the process of creating the most common high copy number vectors, several DNA regions have been removed. One of these DNA sections, cer, contains a binding site for the recombinase XerC. The recombinase enzyme handles reversing dimer and higher multimer formation by recombination across two cer sites on the same molecule thus maintaining Col EI replicons as monomers. Plasmid multimer formation is segregationally unstable and leads to “dimer catastrophe” (Summers et al., 1993) and segregation of plasmid-free cells. French and Ward (1995) demonstrated that the insertion of a 150 bp DNA fragment containing cer can confer segregational stability to a plasmid such that no antibiotic is needed in fermentation and 100% of the cells can successfully retain plasmids. The addition of the cer fragment to the multicopy plasmid, pUC57 should prevent mutimer formation and increase the segregational stability of these plasmids. In this article, we use synthetic biology to increase the level of plasmid supercoiling and plasmid

Figure 3. Batch fermentation of plasmids pUC57 and pUC57-SGS (with SGS derived from Mu, for 162, 298, and 398 bp of the SGS) in DH5α E. coli cells. Error bars represent standard error of the mean, (SEM). (A) Optical density at 600 nm. (B) DNA concentration per ml of culture. Plasmid DNA measurements were the result of Qiagen Minipreps on triplicate cell culture samples. (C) Total percent supercoiling. (D) Supercoiling density with culture time. Supercoiling density results are shown for three different fermentations performed under the same conditions. (E) Average of supercoiling density shown in D. There was a statistically significant difference (P = 0.008) between pUC57 and pUC57-SGS (Mu, 398), (T-test, one tailed distribution, two samples equal variance, Excel 2013), as indicated by the asterisk above the graph.
segregational stability, to build an improved backbone vector for plasmid DNA bioprocessing.

Plasmid segregational stability was analyzed as described in French and Ward (1995). Figure 4 shows the percentage plasmid retaining cells with increasing numbers of bacterial transfer into fresh media and growth in the absence of antibiotics for 24 h at 37°C for (A) pUC57, (B) pUC57- cer, (C) pUC57-SGS (Mu, 298), (D) pUC57-SGS (Mu, 298)- cer, (E) pUC57-SGS (pBR322, 294), and (F) pUC57-SGS (pSC101, 297). Averages and SEMs are shown for duplicates. Statistically significant decreases (P < 0.05, T-test, one-tailed distribution, two samples equal variance, Excel 2013) between the first transfer and subsequent transfers are indicated by an asterisk above the plots.

Figure 4. Plasmid segregational stability. Percentage of plasmid retaining cells with increasing numbers of bacterial transfer into fresh media in the absence of antibiotics for 24 h at 37°C for (A) pUC57, (B) pUC57- cer, (C) pUC57-SGS (Mu, 298), (D) pUC57-SGS (Mu, 298)- cer, (E) pUC57-SGS (pBR322, 294), and (F) pUC57-SGS (pSC101, 297). Averages and SEMs are shown for duplicates. Statistically significant decreases (P < 0.05, T-test, one-tailed distribution, two samples equal variance, Excel 2013) between the first transfer and subsequent transfers are indicated by an asterisk above the plots.

Figure 4A and B shows that the cer fragment, can partially improve segregational plasmid stability when added to pUC57 and analyzed in the DH5α strain of E. coli, but this construct drops to 30% of plasmid containing cells and stays there for three non-selective growths (Fig. 4B). pUC57-SGS in DH5α displayed even greater segregational stability (Fig. 4C–F). This increase was highest for pUC57-SGS with the Mu, (298) fragment where 100% of the cells retained the plasmid through six cycles of non-selective growth (Fig. 4C). Although the further addition of a cer site to this plasmid (Fig. 4D) seemed to indicate a decrease in plasmid segregational stability of the plasmid with only 55% of cells ultimately retaining the plasmid, there was no statistically significant difference between this and the first transfer (P = 0.065, T-test, one-tailed, two samples equal variance, Excel 2013).

In hindsight, it would have been better to test pUC57-SGS (Mu, 398) with cer rather than pUC57-SGS (Mu, 298) as this showed the best supercoiling density, however, this experiment was conducted to test whether segregational stability could be increased using the cer fragment, regardless of whether there was an SGS site present. To this effect, pUC57-SGS (Mu, 298), and pUC57 alone were tested, with the addition of a cer site. Although it was an expected result that cer would increase segregational stability, we did not expect the SGS site also to increase plasmid segregational stability. After the results with pUC57-SGS (Mu, 298), we also tested pUC57-SGS (pBR322, 294) and pUC57-SGS (pSC101, 297) to see whether, the benefits of SGS on segregational stability were specific to the source of the SGS site. Both pUC57-SGS (pBR322, 294) and pUC57-SGS (pSC101, 297) increased segregational stability of the DH5–α E. coli cells to a level where on average 70% of the cells retained the
plasmid (Fig. 4E and F), with no statistical difference between the first and last transfers for the former but a statistically significant drop between the first and second transfers in the latter \( (P = 0.003) \). This indicates that in RecA strains of \( E. \) coli, the SGS has an unforeseen benefit of improving the segregational stability of the plasmids, demonstrating a further advantage of this site. The increase in plasmid segregational stability with the strong gyrase binding site may be due to having gyrase bound and/or an increased superhelical density; however, a mechanism for how this leads to an increase in segregational stability awaits further study.

These results demonstrate that alongside the conventional methods of optimizing fermentation conditions, synthetic biology can be used to optimize plasmid DNA yield, supercoiling density, and segregational stability. SGS \((\sim 296 \text{ bp})\) sourced from the Mu phage, and plasmids pBR322 and pSC101 was found to enhance plasmid segregational stability. Mu phage-sourced SGS was also found to enhance pUC57 yield, and the larger fragment of this site \((398 \text{ bp})\) increased supercoiling density. Further work is required to demonstrate whether increased plasmid supercoiling density by the addition of this SGS site leads to the anticipated increase in transfection efficiency. Nonetheless, applying supercoiling density measurements together with traditional percentage supercoiling analysis could be a useful analytical tool or product characterization method in the plasmid DNA production process.

**Materials and Methods**

**Construction of Plasmids**

The strong gyrase binding sites (SGS) from three different replicons were designed and synthesized as synthetic genes from GenScript Corporation. The regions of SGS from each of these replicons was designed based on the data in Oram et al. (2003) and were reduced or extended for desired lengths from the sequences of Mu (Morgan et al., 2002), pBR322 (Lockshon and Morris, 1985), and pSC101 (Wählé and Kornberg, 1988). Three lengths of the Mu fragment \((162, 298, \text{ and } 398 \text{ bp})\) and two fragments of pBR322 \((294 \text{ and } 394 \text{ bp})\) and pSC101 \((297 \text{ and } 397 \text{ bp})\) were obtained. These fragments were inserted into the XbaI site in the 2.7 kb plasmid, pUC57 by GenScript Corporation.

The \( \text{cer} \) sequence from CoEl1, described by Summers and Sherratt (1988), Summers (1989), and Balding et al. (2006), was extended beyond its Rcd transcript and was designed to have restriction sites of KsaI and PfoI at both ends. This 309 bp sequence was ordered as a synthetic gene from Eurofins MWG operon, (Ebersberg, Germany) and was then inserted into the PfoI site of pUC57 and pUC57-SGS (Mu, 298) and transformed into \( E. \) coli DH5α using standard cloning methods (Sambrook and Russell, 2001).

**Culture and Plasmid Isolation for 15 ml, Bench Scale Experiments**

A single colony from DH5α \( E. \) coli transformed cells was grown overnight at 37°C in 15 ml Luria-Bertani (LB) media with 100 \( \mu \)g/ml Ampicillin. Overnight culture was pelleted and the plasmid constructs isolated using standard protocols in the QIAprep miniprep kit (Qiagen, Crawley), with elution in 30 \( \mu \)L of elution buffer.

**Parallel Fermentation in Semi-Defined Media**

Fermentations were performed using four parallel 1 L fermenters, Multifors, (Infors-HT, Bottmingen, Switzerland), using 700 ml of the semi-defined medium containing casamino acids, SDCAS (O’Kennedy et al., 2000), with 100 \( \mu \)g/ml Ampicillin, and inoculated with 20 h shake flask culture, so that the starting OD at 600 nm was 0.4. To prepare the inoculum, a single colony was grown in 5 ml of Luria-Bertani (LB) containing 100 \( \mu \)g/ml ampicillin for 20 h and this was used to inoculate 100 ml of fresh LB media containing ampicillin in a 250 ml shake flask for a further 20 h. Fifty milliliters of the second inoculum was then used to inoculate 100 ml of SDCAS medium at pH 7 for 20 h, and this was used to inoculate the fermenters as described above. Fermentation conditions were maintained at pH 7 and 37°C and air flow of 1.4 vvm, with dissolved oxygen tension being maintained at 30% or above with a stirrer speed of 500–1,100 rpm. A few drops of 100% polyethylene glycol (PEG) were added as required, to control foaming at the surface of the culture.

**Agarose Gel Electrophoresis for Percentage Supercoiling Determination**

This was determined as described by Yau et al. (2008) by comparing the percentage of open-circular bands with supercoiled bands using densitometer scanning of the agarose gels.

**Two-Dimensional Chloroquine Diphosphate Agarose Gels for Supercoiling Density Determination**

Two samples were loaded 8–10 cm apart and electrophoresed in the first dimension at a chloroquine diphosphate concentration of 0.6 mg/L, at 10 V and 24–26 mA for 17 h. The gel was then allowed soak for equilibration in the second electrophoresis buffer (3 mg/L chloroquine diphosphate in 1XTBE) for 3 h. Electrophoresis in the second dimension was performed at 90° to the first dimension at 10 V and 24–26 mA for 20.5 h.

Gels were then rinsed thrice in \( 1 \times \) TBE for an hour each, and were then stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Invitrogen, Paisley, UK) according to the manufacturer’s instructions for 2 h before visualisation under UV light. Gels were scanned with Gene Snap version 7.07.01 (Syngene, Synoptics Ltd., Cambridge, UK). Supercoiling density was determined according to Bowater et al. (1992). For small-scale experiments in 50 ml tubes, average supercoiling density was a result of at least three separate plasmid preparations.

**Plasmid Segregational Stability**

This was carried out as described by French and Ward (1995). In brief, glycerol stocks from DH5α pUC57, pUC57-cer, pUC57-SGS (Mu, 298), pUC57-SGS Mu, (298)-cer, DH5α pUC57-SGS (pBR322) and pUC57-SGS (pSC101) were streaked out onto selective LB agar plates (containing 100 \( \mu \)g/ml ampicillin) and incubated at 37°C for
16 h. A single colony from each was subsequently inoculated into 50 ml of non-selective LB media and incubated at 37°C, 200 rpm for 24 h. The resulting stationary phase culture was then diluted 10<sup>6</sup>-fold into fresh LB without antibiotics and grown for a further 24 h. This process of dilution and non-selective growth was repeated for 5 days. Serial dilutions were made from each 24 h culture just before dilution for the repeated growth, and plated out as duplicates onto selective and non-selective agar plates. The percentage of plasmid retaining cells was determined from the ratio of colonies on selective plates over those on non-selective plates.

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References

Balding C, Blaby I, Summers D. 2006. A mutational analysis of the ColE1-encoded cell cycle regulator Rcd confirms its role in plasmid stability. Plasmid 56(1):88-73.

Bowater R, Aboul-Ela F, Lilley DMJ. 1992. Two-dimensional gel electrophoresis of circular DNA topoisomerons. Methods Enzymol 212:105–120.

Cupillard L, Juillard V, Latour S, Colombet G, Cachet N, Richard S, Blanchard S, Fischer L. 2005. Impact of plasmid supercoiling on the efficacy of a rabies DNA vaccine to protect cats. Vaccine 23:1910–1916.

Dhanoya A, Chain BM, Keshavarz-Moore E. 2011. The impact of DNA topology on polypeptide uptake and transfection efficiency in mammalian cells. J Biotechnol 159(4):377–386.

Dhanoya A, Chain BM, Keshavarz-Moore E. 2012. Role of DNA topology in uptake of polypeptide molecules by dendritic cells. Vaccine 30(9):1675–1681.

French C, Ward JM. 1995. Improved production and stability of E. coli recombinants expressing transketolase for large scale biotransformation. Biotechnol Lett 17(3):247–252.

Li H, Bo H, Wang J, Shao H, Huang S. 2011. Separation of supercoiled from open circular forms of plasmid DNA, and biological activity detection. Cytotechnology 63(1):7–12.

Lockshon D, Morris DR. 1985. Sites of reaction of Escherichia coli DNA gyrase on pBR322 in vivo as revealed by oxolinic acid-induced plasmid linearization. J Mol Biol 181:63–74.

Morgan GJ, Hartfull GF, Casjens S, Hendrix RW. 2002. Bacteriophage Mu genome sequence: Analysis and comparison with Mu-like prophages in Haemophilus, Neisseria, and Deinococcus. J Mol Biol 317:337–359.

Morrison A, Cozzarelli NR. 1981. Contacts between DNA gyrase and its binding site on DNA: Features of symmetry and asymmetry revealed by protection from nuclease. Proc Natl Acad Sci USA 78:1416–1420.

O’Kennedy RD, Baldwin C, Keshavarz-Moore E. 2000. Effects of growth medium selection on plasmid DNA production and initial processing steps. J Biotechnol 76(2):175–183.

O’Kennedy RD, Ward JM, Keshavarz-Moore E. 2003. Effects of fermentation strategy on the characteristics of plasmid DNA production. Biotechnol Biochem 37:83–90.

Oram M, Howells AJ, Maxwell A, Pato ML. 2003. A biochemical analysis of the interaction of DNA gyrase with the bacteriophage Mu, pSC101, and pBR322 strong gyrase sites: The role of DNA sequence in modulating gyrase supercoiling and biological activity. Mol Microbiol 50(1):333–347.

Pato M, Banerjee M. 1999. Replacement of the Bacteriophage Mu strong gyrase site and effect on Mu DNA replication. J Bacteriol 181(18):5783–5789.

Pato ML, Banerjee M. 2000. Genetic analysis of the strong gyrase site (SGS) of bacteriophage Mu: Localization of determinants required for promoting Mu replication. Mol Microbiol 37(4):800–810.

Pillai VB, Hellerstein M, Tuf A, Amara RR, Robinson HL. 2008. Comparative studies on in vitro expression and in vivo immunogenicity of supercoiled and open circular forms of plasmid DNA vaccines. Vaccine 26(8):1136–1141.

Prather KJ, Sagar S, Murphy J, Chartrain M. 2003. Industrial scale production of plasmid DNA for vaccine and gene therapy: Plasmid design, production, and purification. Enzyme Microb Technol 33:865–883.

Sambrook J, Russell DW. 2001. Molecular cloning: A laboratory manual, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Snoep JL, van Der Weijden CC, Anderson HW, Westerhoff HV, Jensen PR. 2002. DNA supercoiling in Escherichia coli is under tight and subtle homeostatic control, involving gene-expression and metabolic regulation of both topoisomerase I and DNA gyrase. Eur J Biochem 269:1662–1666.

Sodoyer R, Courtouis V, Peubez I and Mignon C. 2012. Antibiotic-free selection for bio-production: Moving towards a new "Gold Standard" In: Pana M, editor. Antibiotic-resistant bacteria - A continuous challenge in the new millennium. InTech. p 531–549. Available from: http://www.intechopen.com/books/antibiotic-resistant-bacteria-a-continuous-challenge-in-the-newmillennium/antibiotic-free-selection-for-bio-production-moving-towards-a-new-gold-standard

Summers DK. 1989. Derivatives of ColE1 cer show altered topological specificity in site-specific recombination. EMBO J 8(1):309–315.

Summers DK, Beton CW, Withers HL. 1993. Multicopy plasmid instability: The dimer catastrophe hypothesis. Mol Microbiol 8:1031–1038.

Summers DK, Sherratt DJ. 1988. Resolution of ColE1 dimers requires a DNA sequence implicated in the three-dimensional organization of the cer site. EMBO J 7(3):851–858.

Wahle E, Kornberg A. 1988. The partition locus of plasmid pSC101 is a specific binding site for DNA gyrase. EMBO J 7:1889–1895.

Yau SY, Keshavarz-moore E, Ward J. 2008. Host strain influences on plasmid DNA production in Escherichia coli. Implications for efficient design of large-scale processes. Biotech Bioeng 101(3):529–544.
Appendix A
Percentage supercoiling for pUC57 and the various pUC57-SGS isolated from TOP10 strain of *E. coli*. Averages and SEMs are of 3, 13, 8, 13, 15, 9, 13, and 12 separate plasmid DNA preparations from left to right on the x-axis. All plasmid preparations were derived from a 15 ml overnight cell culture in a 50 ml falcon tube of a single colony in LB media containing 100 mg/ml of ampicillin, at 37°C with horizontal shaking at 170 rpm.

Appendix B
Plasmid supercoiling density of pUC57 and pUC57-SGS in BL21 DE3 *E. coli* cells following a 7 h fermentation. BL21 DE3 are Rec+ cells that were unable to prevent plasmid multimerisation and hence plasmid dimer and multimers were also present. Supercoiling density measurements are only shown for plasmid monomers, with no notable difference between the groups. Averages and SEMs are of duplicates and triplicates.