Transformation of the thermophilic bacterium, *Geobacillus debilis*, by conjugation with the mesophilic bacterium, *Escherichia coli*.

by

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Abstract

A method for transformation of *Geobacillus debilis* by conjugation was developed using a recombinant plasmid, pNW33N-pxyl-bs2-mob, derived from pNW33N. The plasmid includes the *mob* region of RP4 for mobilization, is mobilized from *E. coli* S17-1 to *G. debilis*, and can stably propagate in *G. debilis* trans-conjugants grown at 50 ^{o}C and 55 ^{o}C, in the presence of thiamphenicol. Successful conjugation was depended on the cell density and viability of *G. debilis* when harvested for conjugation, as well as the metabolic activity of *E. coli* S17-1 used for conjugation. Substantial reduction in size of the plasmid DNA was observed when *G. debilis* transconjugants were cultured at 60 ^{o}C in the presence of thiamphenicol, and uniform rearrangement of the plasmid DNA was observed after culturing *G. debilis* transconjugants in the presence of spectinomycin, even at 50 ^{o}C.

Acknowledgments

I would like to thank Dr. Sparling and Dr. Levin for giving me the opportunity to work in this project and their supports and also my supervisory committee members, Dr. Oresnik and Dr. Cicek for their supports and assistance in this project. I would also like to say thank to everybody in our group, especially, Scott for providing me the information about *G. debilis*; Dr. Sharma and Marcel for their advices in the cloning experiments; Dr. Schellenberg for the Cpn60 sequencing and Intergenic spacer identification and Warren for assistance in using the HPLC. I would also like to express my appreciation to my family for their encouragement.

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List of Abbreviations

Cm25	Chloramphenicol, 25ug/ml
Tap6.5	Thiamphenicol, 6.5ug/ml
Tap25	Thiamphenicol, 25ug/ml
Tap125	Thiamphenicol, 125ug/ml
Spc50	Spectinomycin, 50ug/ml
Spc60	Spectinomycin, 60ug/ml
Dt/Mob	DNA transfer system, protein complex, consists of *mob* gene products, forming the relaxasosome, recognizes the *oriT* site and processes of the DNA to the “transfer form” in the conjugation process.
MPF	Mating pair formation, physical apparatus for stabilizing the conjugative junction or providing the conduct for the transfer DNA.
tra	Genes encode the Dt/Mob and MPF

List of strains and plasmids

Strains/ Plasmids	Source
E. coli DH5α	Invitrogen
E. coli S17-1	Tmp^R, Spc^R, Str^R, RP4-2-Tc::Mu-Km::Tn7.Simon et al., 1983.
G. debilis wild-type	From the Lab
pSup202	pMB1 replicon, Ap^R, Tc^R, Cm^R, mob^+, Simon, 1984
pNW33N	pBC1 replicon, Gram-ve (*E. coli*) replicon, Cm^R. *E. coli/Bacillus/Geobacillus* shuttle vector, *Bacillus* Genetics Stock Centre, (BGSC).
pSG1190	$Ap^R Spc^R$ColE1 replicon, *Bacillus* Genetics Stock Centre, (BGSC).
pGrowbs2	pMB1 replicon, Ap^R.evocatal GmbH.

G. debilis(pNW33N-pxyl-bs2-mob, Spc)	*G. debilis* trans-conjugant obtained from conjugation with *E. coli* S17-1 carrying the pNW33N-pxyl-bs2-mob plasmid, selected with Spc60.
G. debilis(pNW33N-pxyl-bs2-mob, Tap)	*G. debilis* trans-conjugant obtained from conjugation with *E. coli* S17-1 carrying the pNW33N-pxyl-bs2-mob plasmid, selected with Tap6.5.
S1 to 11	Back transformed plasmids derived from 50 ^{o}C grown *G. debilis*(pNW33N-pxyl-bs2-mob, Spc)
Tp55-1 to 19	Back transformed plasmids derived from 50^{o}C grown *G. debilis*(pNW33N-pxyl-bs2-mob, Tap)
Tp55-1 to 19	Back transformed plasmids derived from 55 ^{o}C grown *G. debilis*(pNW33N-pxyl-bs2-mob, Tap)
Tp60-1 to 19	Back transformed plasmids derived from 60 ^{o}C grown *G. debilis*(pNW33N-pxyl-bs2-mob, Tap)

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Chapter 1: Literature review of conjugation using RP4 conjugation machinery

1.1 Introduction

Conjugation is a natural process in which genetic material is transferred between bacteria and across species. Plasmids encoding all necessary gene products for conjugation are called “conjugative plasmids”. Many conjugative plasmids have broad-host-ranges and belong to incompatibility groups such as IncP, Q, N and W (Meyeret al.,1991). These plasmids carry antibiotic resistance genes and can effectively distribute antibiotic resistance to a wide variety of bacteria (Garcillan-Barcia et al.,2011). Conjugative plasmids also provide researchers simple and effective tools to perform gene transfer experiments. The conjugation process has been understood by dissecting it into sequential events. Although, there are slight differences in detail for different conjugative plasmids, the overall process is the same. It is divided into two main steps (Smillie et al., 2010). The first, involves the processing of the plasmid deoxyribonucleic acid (DNA). In the DNA transfer system, Dtr/Mob, the relaxase enzyme recognizes a specific *oriT* on the plasmid. The relaxase enzyme then binds to the *oriT* and cleaves the plasmid, and becomes covalently attached to it in the process (Garcill an-Barciaet al.,2009). Auxiliary proteins also bind to form the “relaxosome”. Helicase and topoisomerase-like proteins then unwind the double-stranded DNA plasmid into single-stranded DNA to produce the “transfer-form” of the plasmid (Guglielmini et al.,2012). In the second step, a physical apparatus, called the “mating pair formation” is formed between the ex -conjugant (the donor species or strain) and the trans-conjugant (the recipient species or strain). The MPF stabilizes the conjugative junction, in which the

plasmid DNA is transferred through a tunnel or pilus-like structure (Samuels et al., 2000).

The MPF is thought to have diverged from the type IV secretion system (T4SS), which is present in pathogenic bacteria and exists by itself without Dtr/Mob, it transmits effector proteins to the host (Guglielminiet al., 2012). The coupling protein, T4CP, a component of Dtr/Mob, delivers the relaxosome to the MPF. Relaxase and primase bound to the plasmid DNA are also carried along and they function to regenerate the double strand circular plasmid in the trans-conjugant (Smillieet al., 1990).

Detail studies have identified the genes encoding the Dtr/Mob and MPF in prototypical conjugative plasmids, and their gene products have been characterized.Mobilizable plasmids are transmissible plasmids but only possess the Dtr/Mob genes. They exist naturally and are capable of being transferred by conjugation with the compatible MPF genes provided*in trans*. These genes were stably inserted into *Escherichia coli*, Theresulting S17-1 strain encoded MPF of the RP4 plasmid in their chromosome, and they are often used to mobilize mobilizable plasmids (Babic et al.,2008). In contrast, conjugative plasmids possess both Dtr/Mob and MPF are also described as self-transmissible (Smillie et al., 2010).

1.2 Classification of the conjugative plasmid and the RP4 plasmid

Conjugative plasmids are classified in two ways (Smillie et al.,2010). In the MPF classification system, conjugative plasmids are classified based on the physical appearance of their MPFs. There are 4 classes of MPF, 1) the short and rigid MPF_T group, containing the prototypical plasmids, RP4 and Ti; 2) the thick and flexible MPF_F group, containing the prototypical Fertility (F) plasmids of *E. coli*; 3) the thin and flexible

MPF_I group, containing the prototypical R64 plasmid; and 4) the MPF_G group(Bradley et al.,1996). MPF_G is evolutionary distinct from other groups and it is related to genomic island (Juhas et al., 2007).

Phylogenetic analyses for the MPF groups utilize the TraD, TraB, and TraC/VirB4 proteins (F-plasmid naming) (Firth et al.,1996; Guglielmini et al.,2012). Measuring evolutionary distance by homology comparison of the *virB4* gene among plasmids in different MPF groups results mono-phylogenetic clades which co-inside with single MPF group (Guglielminiet al.,2012). The RP4 plasmid belongs to the MPF_T group. It encodes short and rigid MPF gene product and the MPF encoded gene organization resembles the prototype Ti plasmid (Farrandet al.,1996).

MPF groups have also been characterized for differences in their conjugation efficiencies on solid and liquid media. Conjugation with plasmids encoding flexible MPF systems, the MPF_I and MPF_F plasmids, can take place in liquid medium. In contrast, rigid MPF systems, MPF_T plasmids (for example, the RP4 plasmid) require solid medium, so that ex-conjugant and trans-conjugant can get very close to each other (Juhas et al., 2007). Another advantage of phylogenetic conjugative plasmid classification is that it can give some indications of how closely or distantly related the donor and recipient bacteria are to each other.

The second method of conjugative plasmid classification is based on phylogenies of their relaxase and coupling protein genes. Phylogenetic analyses of these two genes give very similar results, resolving the plasmids into six clades, called “mobilization families”: MOB_F, MOB_H, MOB_Q, MOB_C, MOB_P and MOB_v (Juhas, et al., 2007). RP4 belongs to the MOB_P family, which includes many diverse plasmids found in both Gram-

positive and Gram-negative bacteria. Phylogenetic trees generated with MOB_P family sequences contain multiple mono-phylogenetic clades, and locates RP4 in the P11 Gram-negative clade. RP4 is closely related to the Gram-negative plasmids, such as those in the IncP incompatibility group (Smillie et al.,2010). Despite the closer relation to the P11 Gram-negative plasmids, RP4 relaxase shares a motif with the MOB_P Gram-positive plasmids (pC221, pC223, pS194 *Staphylococcus aureus* plasmids and the pSK639 *staphylococcus epidermidis* plasmid), and they belong in the P7 clade, which consists entirely of plasmids found in Firimcutes bacteria (Garcill an-Barciaet al.,2009; Franciaet al.,2004).

The transmissibility of RP4 is consistent with the relaxase/coupling protein MOB classification revealed by phylogenetic analysis. The RP4 plasmid is transferable to many of Gram-negative bacteria including *Vibrio*, (Stabb et al., 2002), *Fusobacteria* (Claypoolet al.,2010), *Haemophilus*(Robinson, et al., 2010), *Acidothiobacillus*(Chenaet al.,2011), *Thiobacillus*(Jinet al.,1992), *Sinorhizobium*, *Achromobacter* and *Pseudomonas* species and more, spanning α, β and γ proteobacteria (Musovicet al., 2010). On the other hand, the RP4 plasmid can also self-transmit from *E. coli* to Gram-positive species , such as *Bacillus*(Britskaya et al., 1990; II'iashenko, 2010), *Streptomyces*, *Enterococcus, Streptococcus*, *Listeria*, and *Staphylococcus* species (Giebelhauset al.,1996; Biermanet al., 1992; Mazodieret al., 1989)and *Enterococcus*(Borgoet al.,2009). With RP4's MPF encoded genes, RP4 can also mobilize natural occurring mobilizable plasmids, including the pMV158 (MOB_V plasmid) (Farias et al., 2000), the pIP501 and pAMβ1 streptococcal plasmids (Mazodieret al., 1989), and IncQ mobilizable plasmids prototypical RSF1010/R1162/R300B and other

IncQ like plasmids (Meyer, 2009). The ability of RP4's MPF to facilitate transfer of plasmids which have different Dtr/Mob system of MOB families indicates that the Dtr/Mob system and the MPF system can work independently, it also suggests that the broad host range of RP4 transmissibility might be limited by the ability of its replicon to replicate in different bacteria not the inability of the conjugation machinery to transmit. Also, there is no apparent coherency between the phylogenies of the MOB and the MPF classifications. Plasmids of all MOB families are present in the MPF_T group and at least 3 different MOB families are present in each of the other 3 MPT groups (Smillieet al.,2010). This might also indicate that the Dtr/Mob and MPF are somewhat independent along the evolution process.

All naturally occurring mobilizable plasmids contain *oriT*s and Mob genes (Meyer, 2009). However, only the presence of *oriT* is sufficient for mobilization (Guineyet al., 1988). In RP4 plasmid, there are three regions *Tra1*, *Tra2* and *Tra3* for conjugation, and all three are necessary for self-transmissibility. The Mob genes are mainly located in the *Tra1* region. The TraJ gene product binds to the *nic* site of the *oriT*, the TraI, cleaves the plasmid and produces singles strand and TraH gene product stabilizes the relaxosome (Watermanet al.,1993). However, Guiney, Deiss, & Simnad (1988), showed that mobilization of a plasmid could still occur in the absence of the *Tra* genes if the plasmid contained a 250 bp fragment containing the *oriT* of the *Tra1* region.

1.3 Pertinence of developing a plasmid transfer and cloning strategy in *Geobacillus* strain

Members of the genus *Geobacillus* are important industrial thermophilic organisms, especially for the production of thermostable enzymes for deconstruction of complex carbohydrates other than cellulose (Satyanarayana et al., 2012). Some, such as *Geobacillus thermoglucosidasius* are facultative anaerobes capable of ethanol fermentation. Development of genetic tools enabled the generation of a strain with enhanced ethanol production capabilities (Cripps et al., 2009). Indeed there are several publications currently describing the development of shuttle vectors specifically designed for use in various *Geobacillus* sp. (Taylor et al., 2008; Suzuki and Yoshida 2012).

Within the context of consolidated bioprocessing of lignocellulosic materials for biofuels production, the use of members of the genus *Geobacillus* has limitations because of their inability to use cellulose.However,*Geobacillus* species are known to be associated with fermentative cellulolytic organisms such as *Clostridium thermocellum* in some aerobic environments (Ronan et al., 2013; Miyazaki et al., 2008). Ethanol production from a co-culture of *Clostridium thermocellum* and *Geobacillus stearothermophilus* has also been studied previously (Sharma 1991). Co-culturing of *Clostridium butylicum* with the mesophilic, facultative anaerobe *Bacillus subtilis* has also been used to permit solvent production of the *Clostridium* in the presence of air (Tran et al., 2010). The observation that *Geobacillus* strains are naturally found with cellulolytic clostridia combined with reports of strain improvement through molecular engineering provided the impetus for the development of molecular tools for a strain of *Geobacillus*

debilis, an ethanol producing strain that associates with *C. thermocellum* in natural minimal air-tolerant cellulolytic consortia isolated in the laboratory (Wushke, Levin and Sparling, unpublished).

*C. thermocellum*is capable of growing on cellulose and bagasse through the breakdown of plant cell walls (Blume et al., 2013).*G. debilis* as many other *Geobacillus* species are found capable of utilizing oligosaccharides derived from cellulose enzymatic degradation, such as cellobiose. However, it is likely that oligosaccharide utilizationsis controlled by inducible operons and catabolic repression. In particular, the I-Arabinose Utilization System and the ABC Transporter for Xylo-Oligosaccharides in *G. stearothemophilus* have been characterized (S. Shulami et al., 2011and 2007). Consortia with *C. thermocellum* allow *G. debilis* to utilize cellobiose derived fromliginocellulose biomass.The use of transformation andgenetic recombination to artificially alter inducible operons to be constiutive expressed might help in better substrate ultization in the consortia.As a result, the symbiosis between *G. debilis* and *C. thermocellum* would be strengthened,and presumably *G. debilis* can be better in utilization of substrates produced from *C. thermocellum* and in return providing more oxygen protection for *C. thermocellum*.Thus an efficient aerotolerant consolidated ethanol production might be achievable.

1.4 Objectives of the Thesis

The objective of this thesis was to develop a transformation system for *Geobacillusdebilis*, a facultative anaerobic, thermophilic bacterium that grows in wide range of temperatures up to 60 ^{o}C. A conjugative transformation system was developed using the derived plasmid from pNW33N, a shuttle vector that replicates in both *E. coli*

and *Geobacilius stearothermophilus*. pNW33N, which contains a thermo-stable replicon from plasmid pBC1 originally isolated from *Bacillus coagulans* (De Rossiet al.,1992). Growth of pNW33N-transformed *B. coagulans* at 50 ^{o}C confirmed the thermo-stability of the pNW33N plasmid (Rhee et al., 2007).

An 1800 bp fragment of the *Tra1* region, containing the RP4 *oriT*, *traI,* and *traJ* genes was cloned into pNW33N to create a mobilizable plasmid that can be used for conjugation. The *bs2* fluorescence protein gene from the pEvoGlow plasmid, and the spectinomycin resistant gene under transcriptional control of the *Bacillus subtilis* 168 xylose promotor (*pxyl*) derived from plasmid pSG1190 were also cloned into pNW33N, creating a plasmid called pNW33N-pxyl-bs2-mob.

Using an inter-species bi-parental conjugation method, pNW33N-pxyl-bs2-mob was introduced to *E. coli* S17-1, which contains the RP4 conjugation machinery and can mediate transfer of mobilizable plasmids with compatible *oriT* (Babicet al., 2008). A similar approach was used to successfully introduce plasmids into *Geobacillus kaustophilus* HTA 426, in which the conjugation took place at 37 ^{o}C on a solid matrix (Suzuki et al., 2012). Although 37 ^{o}C is a sub-optimal temperature for the thermophilic *Geobacillus kaustophilus*, and the *G. kaustophilus* cells were not metabolically active, the *E. coli* cells were at their optimal growth temperature and actively driving the conjugation.

We developed an inter-species bi-parental conjugation method of conjugation for *G. debilis* with *E. coli* S17-1, and postulated that such approach would be successful for gene transfer into *G. debilis*. However, since conjugation involves transfer of single-stranded, plasmid DNA, we hypothesize that the success of the conjugation would be

dependent on the metabolic state of *G. debilis* at 37^0C, as well as the metabolic activity of *E. coli* S17-1, also the thermo-stability of the plasmid transfer via conjugation at higher temperatures. Therefore, the specific objectives of this thesis were to:

1) Demonstrate gene transfer via conjugation from *E. coli* S17-1 to *G. debilis*; and

2) Investigate the stability of plasmid transfer in *G. debilis* under thermophilic conditions (50 ^{o}C, 55 ^{o}C, and 60 ^{o}C).

Chapter 2: Construction of the pNW33N-pxyl-bs2-mob plasmid

2.1 Introduction of the pNW33N-pxyl-bs2 and pNW33N-pxyl-bs2-mob plasmids

The pNW33N-pxyl-bs2 and pNW33N-pxyl-bs2-mob plasmids are derivative of the pNW33N plasmid for the purpose of conjugative transformation of *G. debilis* in this thesis. pNW33N is a shuttle vector that contains the pBC1 replicon from the pBC1 plasmid, a wild-type plasmid found in *B. coagulans* (De Rossi et al., 1992). It also has the Gram-negative replicon of pUC19 plasmid, a derivative of pMB1 replicon (GenBank, accession number: AY237122.1). Thus, pNW33N can replicate in both *E. coli* and possibly thermophilic *Geobacillus* species. The pNW33N plasmid has been found capable of replication and propagation in *Bacillus methanolicus* (Nilasari et al, 2012), *G. stearothermophilus* and *B. coagulans* (Rhee et al., 2007).

The purpose to construct the pNW33N-pxyl-bs2 plasmid is to show that *G. debilis* is transformable and the plasmid can replicate and propagate in the bacterium, which furtherallows expression of genes encodedon the plasmid. For using these plasmids for recombination such as gene knockout or chromosomal insertion in future experiments, further knowledge of counter-selection methods against this plasmid will be required.

The pNW33N-pxyl-bs2 plasmid also carries the spectinomycin resistance gene (*spc*) in addition to the chloramphenicol resistant gene of the pNW33N plasmid, and the bs2 gene, encoded a fluorescent protein, is under transcriptional control of the xylose promoter (pxyl). Those components are cloned into the multiple cloning site of pNW33N to create the pNW33N-pxyl-bs2 plasmid. The bs2 gene encodes the FMN flavinmono-nucleotide dependent fluorescent gene from pEvoGlow plasmid (BGSC, Catalog of Strains, 7th edition Vol.3). The bs2 fluorescent protein was chosen because that it can

fluoresce *in vivo* in both aerobic and anaerobic conditions (Choi CH et. al., 2011). It is a truncated protein developed from the Light-Oxygen-Voltage, LOV domain of thenaturally existing *B. subtilis* YtvA blue-light photoreceptor devoid of the effector domain (Drepper T. et. al., 2007). In this project, the purpose of the fluorescent gene is to provide an additional possible marker for successful transformation. It is made under the control of *Bacillus subtilis*168 *pxyl* promoter, from another DNA fragment of the pSG1190 plasmid (BGSC, Catalog Vol.4). However, in the later progress in this project, it is found possible to directly verify the plasmid in the transformed *G. debilis*.

In the clone fragment of the *B. subtilis*168 *pxyl* promoter, the SalI, KpnI fragment of pSG1191 also contains the spectinomycin resistant gene, *spc*, thus the spectinomycin resistant phenotype could be resulted in the subsequent transformants.

In order to make conjugation possible for the transfer of the plasmid from *E. coli* S17-1 to *G. debilis*, the mob region from the pSUP202 plasmid (GenBank, accession number: AY428809.1), containing the *traI*, *traJ* genes and the *nic* sites of the RP4 conjugative plasmid is further cloned into the SacI site of the pNW33N-pxyl-bs2 plasmid. The resulting a mobilizable plasmid is called pNW33N-pxyl-bs2-mob plasmid.

2.2 Construction an verification of the pNW33N-pxyl-bs2 plasmid

The pNW33N-pxyl-bs2 plasmid was constructed by combining key components: 1) the SalI digested pNW33N; 2) the *spc-pxyl* fragment from SalI, KpnI digested pSG1190; and 3) the *bs2* fluorescent gene,amplified from the plasmid pEvoGlow using primers specific for *bs2* with termini KpnI and SalI restriction sites (Table 2.1; Figure 2.1).The 1500 bp *spc-pxyl* fragment was obtained by digestingthe pSG1190 plasmid with KpnI and SalI restriction enzymes. The digestion was subjected to gel

electrophoresis, the 1500 bp fragment on the gel was excised and gel purified(Figure2.2A). The 400 bp*bs2* gene fragment was obtained by PCR amplification from pEvoglow-Bs2 plasmid using the KpnI_Pglow_Forand Pglow_SalI_Revprimers (Figure 2.2B). The PCR product was digested with KpnI and SalI restriction enzymes, then subjected to gel electrophoresis, the 400 bpband on the gel was excised and gel purified. These fragments were cloned into the pNW33N in a single ligation step.The pNW33N plasmid was digested with KpnI and SalI, treated with Calf Intestinal Phosphatase (CIP), and gel purified. Then, it was further ligated with the *spc-pxyl* fragment and the *bs2* fragment using T4 ligase at 4 ^{o}C, overnight. The ligation product was transformed into *E. coli* DH5α using chemical method, the transformed *E. coli* Cm25 LB culture was subjected to plasmid prep (Qiagen) to obtain the pNW33N-pxyl-bs2 plasmid.

The pNW33N-pxyl-bs2 plasmid was verified with KpnI and SalIrestriction enzymes. The presence of the 1500 bp *spc-pxyl* band in the KpnI digestion of the pNW33N-pxyl-bs2 plasmid (Figure 2.2C, lane1), the 1900 bp*spc-pxyl-bs2* band in the SalI digestion (Figure 2.2C, lane2) and the 1500 bp *spc-pxyl* and 400 bp*bs2* band in the KpnI, SalI digestion (Figure 2.2C, lane3) confirmed the presences of the inserts in the pNW33N-pxyl-bs2 plasmid. The orientation of the *pxyl-bs2* fragment was verified by nucleotide sequencing of the PCR product generated by the pNW33Nmcs_For and pNW33Nmcs_Rev primers, and the transcriptional direction of the cloned *bs2* gene was selected to be the same direction with that of the *lac* α-peptide gene. The nucleotide sequence data also confirmed that the *bs2* gene was in-frame with the *pxyl* promoter.

Table 2.1. Primers use for PCR amplification during plasmid construction.

Primer Name	Primer Sequence
PNW33NmcsFor [1]	5'-TCGGGATTCGTTTTACTTTCC-3'
PNW33NmcsRev [2]	5'-CAATTTCACACAGGAAACAGC-3'
KpnI_Pglow_For[3]	5'-TATAGGTACCATGGCGTCGTTCCAGTCG-3'
Pglow_SalI_Rev[4]	5'-TATAGTCGACTCACTCGAGCAGCTTTTCAT-3'
SacI_PSup202_For [5]	5'-TATAGAGCTCCCCCGTGGAGGTAATAATTG-3'
PSup202_SacI_Rev [6]	5'-TATAGAGCTCCTGTCAAACATGGCCTGTCG-3'

[1]Forward primer for PCR amplification of insert in pNW33N multiple cloning site.
[2]Reverse primer for PCR amplification of insert in pNW33N multiple cloning site.
[3]Forward primer for PCR amplification of *bs2* in the pEvoGlow plasmid.
[4] Reverseprimer for PCR amplification of *bs2* in the pEvoGlow plasmid.
[5]Forward primer for PCR amplification of *mob* in the pSUP202 plasmid.
[6] Reverseprimer for PCR amplification of *mob* in the pSUP202 plasmid.

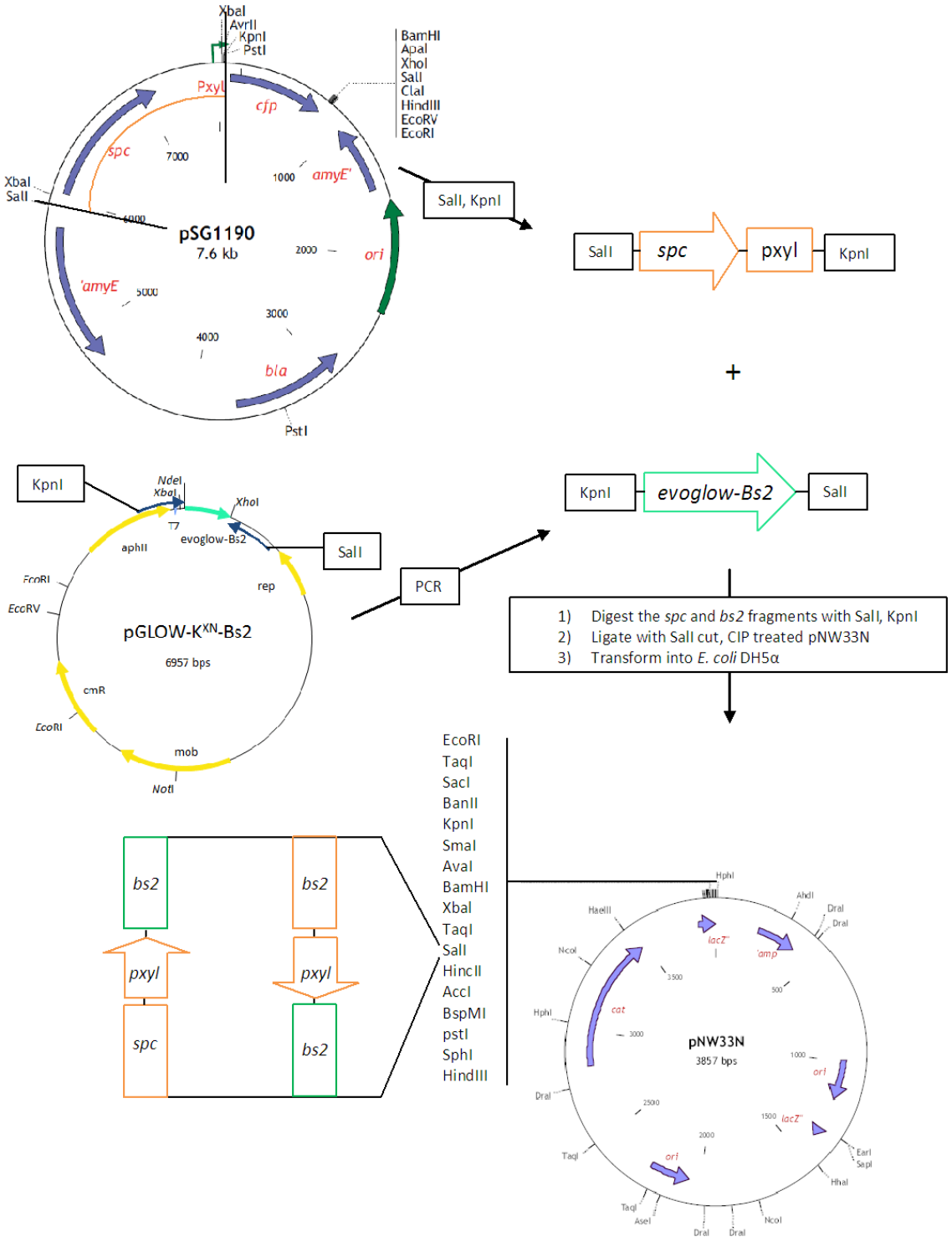

Figure2.1. Construction of the pNW33N-pxyl-bs2 plasmid.

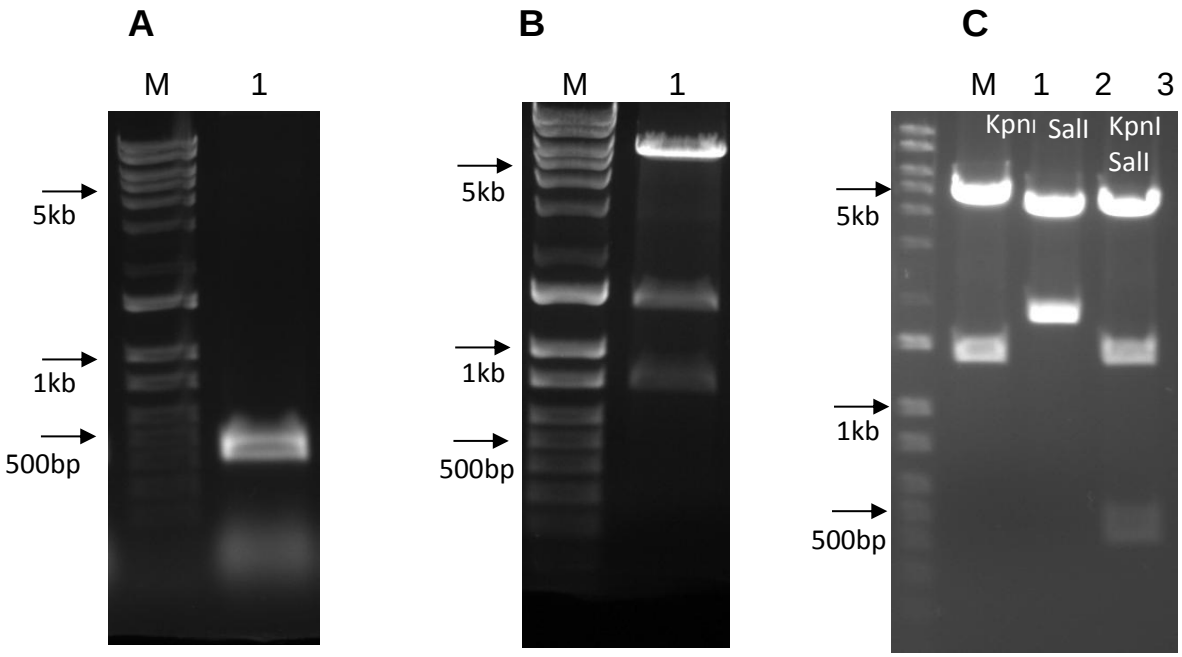

Figure 2.2. Verification of the pNW33N-pxyl-bs2 plasmid construct. A) KpnI, SalI digested pSG1190; B) PCR amplification of the 400 bp fragment containing the Bs2 gene; C) Analysis of pNW33N-pxyl-bs2: Lane 1) KpnI digest; lane 2)SalI digest; lane 3) KpnI + SalI digests. M: Molecular size marker, 100bp-10kp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are pointed with arrows.

2.3 Construction and verification of the pNW33N-pxyl-bs2-mob plasmid

The pNW33N-pxyl-bs2-mob plasmid was constructed with the procedure as shown in Figure 2.3. An 1800 bp plasmid mobilization (*mob*) fragment containing the *traI* and *traJ* genes, and the *nic* element from plasmid pSUP202 was cloned into the SacI site of thepNW33N-pxyl-bs2 plasmid. The *mob* fragment was obtained from PCR reaction of pSUP202 as the template using the SacI_PSup202_For and PSup202_SacI_Rev primers. The PCR product was further digested with SacI restriction enzyme and gel purified. The pNW33N-pxl-bs2plasmid was also digested with SacI, treated with CIP and gel purified, it was further ligated with the *mob* fragment using T4 ligase, 4 ^{o}C, overnight. The ligation product was transformed into *E. coli* DH5α using chemical method, and single colonies were picked for the transformed *E. coli* into Cm25 LB broth.

The pNW33N-pxyl-bs2-mob plasmids were obtained by screening using PCR amplification of the *mob* in each of the plasmid preps of the recombinant *E. coli* transformed with the ligation product of the *mob* fragment and the linearized pNW33N-pxyl-bs2.The presence of the 1800 bp band indicated the presence of *mob* in the plasmid (Figure 2.4). Plasmids from *mob*$^{+}$ strains were further characterized by REN digestion using PvuI and BstAPI restriction enzymes to determine the insert orientation, as well as the orientation of the bs2 gene relative to lac α-peptide gene (Figure 2.5, lanes 1, 3, 4, and 5). Only the pNW33N-pxyl-bs2-mob plasmid 5 had the transcriptional direction of the *traI* and *traJ* genes anti to that of the lac α-peptide gene, and the pNW33N-pxyl-bs2-mob plasmid 1 was chosen for the rest of the experiments in this project.

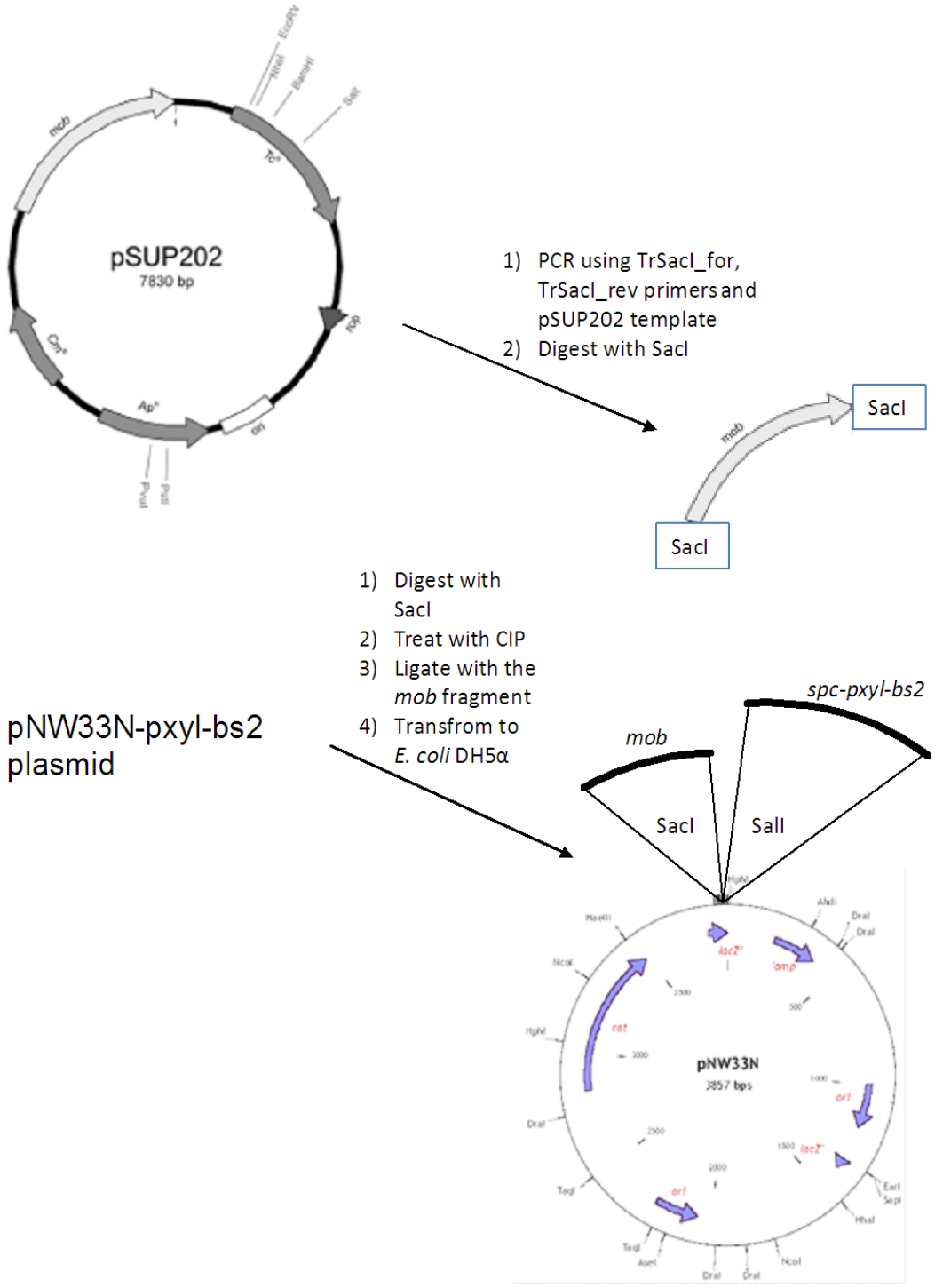

Figure 2.3. Construction of the pNW33N-pxyl-bs2-mob plasmid.

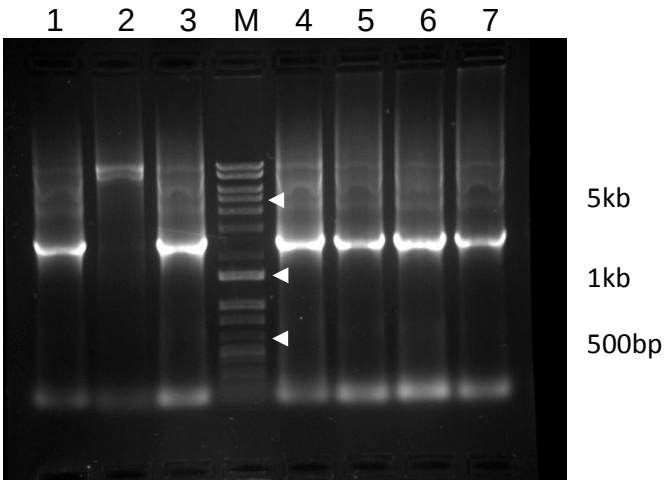

Figure 2.4. Screening for *mob*$^{+}$ plasmids by PCR amplification of the 1800 bp *mob* fragment. The ligation product of *mob* and pNW33N-pxyl-bs2 was transformed into *E. coli* DH5α, 7 colonies were picked and plasmid was extracted. Lanes 1 to 7 show the bands resulting form PCR amplification of the 1800 bp *mob* region. M: molecular size marker100bp-10kp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

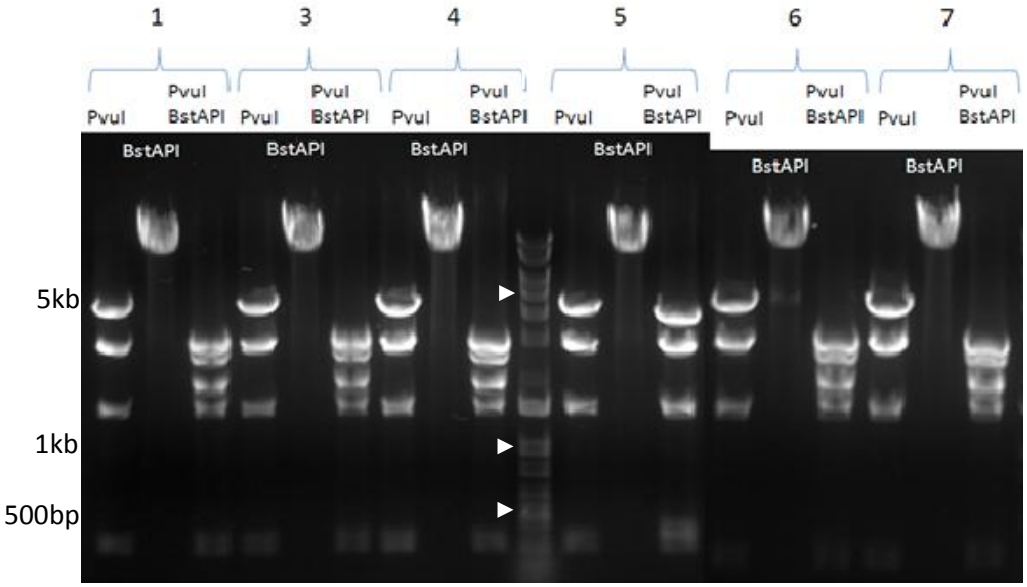

Figure 2.5. Restriction Endonuclease (REN) analyses of pNW33N-pxyl-bs2-mob plasmids by PvuI and BstAP1 digestion. REN digests 1, 3, 4, 6, and 7: pNW33N-*pxyl-bs2* plasmids in which the *bs2* gene has the same transcriptional direction as the *traI*, *traJ* and *lac* α-peptide genes. REN digest 15: pNW33N-*pg3pdH-bs2* plasmid in which the *bs2* gene has the same transcriptional direction as the *traI*, *traJ* and *lac* α-peptide genes; REN digest 5: pNW33N-*pxyl-bs2* in which the bs2 gene is in the opposite orientation with respected to the *traI*, *traJ* and *lac* α-peptide genes. M: Molecular size marker, 100bp-10kp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp, 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

Chapter 3: Conjugation of *G. debilis* with *E. coli* S17-1(pNW33N-pxyl-bs2-mob)

3.1 Introduction

The conjugation of *G. debilis* is done using an inter-species bi-parental conjugation method with *E. coli* S17-1, which encoded the RP4 conjugation machinery. The procedure for the conjugation is similar to the conjugation of *G. kaustophilus* in which the conjugation took place at 37 ^{o}C (Suzuki et al., 2012). However, based on experience in the course of the attempts in conjugation of *G. debilis* with *E. coli* S17-1 strain, the following considerations were found relevant.

Firstly, conjugation involves transfer of genes from one bacterium and to another, and requires expression of conjugation-specific transfer proteins by the donor bacterium. Thus, the metabolic activity of the donor bacterium is critical for successful conjugation, as conjugation process demands cellular expression of the Dtr/Mob system and the MPF in the donor bacterium to facilitate the transfer. Following this logic, donor bacteria are better donors if they are in a more metabolically active state as long as the recipient bacteria are viable.

Secondly, oxygen availability might be one of the important factors in conjugation. Kro´l et al. (2011) studied conjugation between *E. coli* stains in biofilms using an IncP1 conjugative plasmid and found that the trans-conjugants were formed on the pellicle of the biofilm, not in the interior. In the same paper, it was also indicated that the disparity in conjugation taking place in biofilm with respect to its depth might also be of consequence to the difference of the biofilm forming ability populated on the surface and beneath the biofilm. However, using the *E. coli* MG1655 *csrA* mutant strain, which

penetrated deeper into the vertical cross-section of the biofilm, as a recipient, it was found that the highest number of all trans-conjugants, donors, and recipients were all on the pellicle (Kro´l et al., 2011). Thus, it is likely that a difference in oxygen levels can influence conjugation efficiency directly and indirectly, and higher oxygen flavors conjugation. In this project, two methods of dispensing the mating mixture were attempted.The first one was using minimal suspension volume, so that the mixture of cells is put as a concentrated droplet on the filter paper. And, the second one was using larger suspension volumes, so that the mixture of cells is put as a thin layer on the filter paper. It is believed that the use of these two methods may result in different oxygen availabilities to the *E. coli* ex-conjugants. In the first method, less oxygen may be available to the *E. coli* ex-conjugants and in the second method, more oxygen may be available to the *E. coli* ex-conjugants.

3.2 Methods

3.2.1 Conjugation of *G. debilis* with *E. coli* S17-1 (pNW33N-pxyl-bs2-mob)

E. coli S17-1, containing pNW33N-pxyl-bs2-mob was conjugated with *G. debilis.* A 100-200 mL culture of *G. debilis* was grown to mid-log phase (OD_{600} = 0.2-0.3) and then centrifuged (Sorvall RC-6 Plus, SH-3000 rotor) for 15mins, 4500 rpm at 4^0Cand re-suspended in 2-3 mL 2% Luria Bertini (LB) medium. The suspension was divided into 2 or 3 portions and a 50 µL of freshly grown culture (OD_{600} =0.5) of *E. coli* S17-1 carrying pNW33N-pxyl-bs2-mob was added to the *G.debilis* suspensions. One portion was always left without the *E. coli* S17-1 to serve as a control. The mixture of cells and the control were washed once with 1.2 mL 2% LB, then re-suspended in LB and each was

dispensed on a 0.2 μm pore size filter paper on top of a 2% agar plate with the same LB medium. The two methods of suspending and dispensing of the mating mixture on the filter paper were attempted. In the first type of experiment, the *E. coli* and *G. debilis* were suspended with a minimal amount (about 20μl) of conjugation medium. The cells formed a thick dome-shape droplet on the filter paper. In the second type of experiment, the mating mixture was suspended with larger amount (about 100ul) of conjugation medium so that the cells were spread out as a thin layer on the filter paper.

The conjugation LB plate was incubated at overnight (16-18 hours) at 37 ^{0}C. After incubation, the filter paper was placed into a 1.5 mL eppordorf tube with 1 mL TGP broth (contained tryptone (17g/L); soy peptone (3g/L); NaCl (5g/L); K2HPO4 (2.5g/L), post-autoclaved addition of filter sterilized pyruvate and glycerol to final concentrations of 4 g/L and 4ml/L). It was vortexed thoroughly. The suspension was further incubated at 55 ^{o}C for 3 hours. Then, spectinomycin (1 ng), or thiamphenicol (0.1 ng), was added, and the cells were incubated for an additional 1 hour at 55 ^{o}C. Conjugants were selected on TGP agar plates containing 60 g/mL spectinomycin (Sp60) or 6.5 μg/mL thiamphenicol (Tap6.5) and incubated at 55 ^{o}C overnight. Conjugant colonies were observed on the selective plate within 48 hours.

A small aliquot (5 μL) of the mating mixture was sampled, stained, and observed by light microscopy using Nikon eclipse T*i*-U microscope, before and after the conjugation reaction at 37 ^{o}C to check for spores of *G. debilis*. The Gram stain procedure was conducted following standard procedures (http://www.microbelibrary.org/component/resource/gram-stain/2886-gram-stain-protocols).

The viability of the *G. debilis* control was also checked after conjugation at 37 ^{o}C by serial dilution and a plate count method.

3.2.2 Confirmation of *G. debilis* trans-conjugants by back transformation

Plasmid DNA (pNW33-pxyl-bs2-mob) was extracted from *G. debilis*trans-conjugants was prepared as follows. *G. debilis* trans-conjugant cultures (10 mL) were pelleted by centrifugation at 4,000 x g. The supernatant was discarded and the cell pellets were resuspended in 700 µl of Tris-EDTA buffer (10mM Tris, 1mM EDTA, pH 8.0) and 200 µl of lysozyme (10mg/mL in Tris-EDTA) and incubated at 37 ^{o}C for 15 minutes. The plasmid DNA was then extracted using the Qiagen Mini Prep kit.

The plasmid extract was back transformed into *E. coli* DH5α as follows. *E. coli* DH5α was inoculated from overnight culture into 100 mL LB medium, incubated at 37 ^{o}C with shaking (180 rpm) and grown to an OD_{600} of 0.5 to 0.7. The culture was cooled on ice for 30 minutes, and harvested by centrifugation at 4500 rpm (Sorvall RC-6 Plus, SH-3000 rotor) for 15 minutes at 4^{0}C. The pellet was re-suspended in 50 mL 100mM $CaCl_2$ solution, and cooled on ice for another 30 minutes, harvested by the same centrifugation procedure, and re-suspended in 1 mL 100mM $CaCl_2$ solution. Plasmid DNA was mixed with 200 µl of competent cells in a 1.5 mL eppendorf tube, incubated on ice for 30 minutes, subjected to heat shock at 42 ^{o}C for 90 seconds, and then incubated on ice for 5 minutes. Then, 800 µl of LB broth was added to the eppendorf, incubated at 37 ^{o}C for 1 hour. Finally, 250 µl of the mixture was plated on the LB agar (1.5%) plates containing 25 mg/mL chloramphenicol (Cm25).Plasmid

DNA extracted from the *E. coli* DH5α back transformants (Qiagen), was digested with PvuI, SacI, and RsaI restriction endonucleases, and subjected to gel electrophoresis.

3.2.3 Direct confirmation of the *G. debilis* trans-conjugant plasmid extract

The plasmid DNA extracts from trans-conjugant *G. debilis*(pNW33-pxyl-bs2-mob) was digested with PvuI. The digested plasmid DNA were subjected to electrophoresis on 0.8% agarose gels, and their digested patterns were compared with that of thepNW33N-pxyl-bs2-mob parent plasmid to identify and confirm stability of the *in vivo* plasmids.

3.2.4 Identification of *G. debilis* by 16S intergenic spacer region (IGSR) PCR amplification

*G. debilis*trans-conjugants were confirmed as *G. debilis* species by the use of 16S intergenic spacer region (IGSR) PCR amplification with the Isr_for(5'-GTCGTAACAAGGTAGCCGTA-3') and Isr_rev(5'-GCCAAGGCATCCACC-3') primers (Cardinale, M. et. al., 2004). The PCR products were resolved by gel electrophoresis and produced a diagnostic pattern identical to the known and expected IGSR pattern of *G. debilis*.

3.2.5 Confirmation of *G. debilis* trans-conjugants by fermentation end-product analysis

In 1191 medium (contains KH_2PO_4(1.5g/L), Na_2HPO_4(3.35g/L), NH_4Cl (0.5g/L), $MgCl_2$ (0.18g/L), yeast (2g/L))with reduced yeast,*G. debilis* synthesizes acetate, formate, and ethanol as fermentation end-products, but does not synthesize lactate.

Since this trait is rather unusual, this phenotype is used for monitoring against possible thermophilic contaminants in addition to the intergenic space identification.

Trans-conjugants were cultured in 1191 0.2% cellobiose with reduced yeast extract 0.67%(instead of the normal 0.2%) under aerobic conditions at 60^0C for four days. The supernatants from these cultures were analysed by HPLC. Organic acids (lactate, acetate, and formate) were measured with a high performance anion-exchange column (AS11-HP) and conductivity detector installed in an ion-chromatography system (Dionex ICS-3000). Mobile phase of the liquid chromatography was 0.004 N H_2SO_4 and the flow rate was maintained at 0.75 mL/min. Different concentrations of acetate,formate, and lactate were run on the HPLC to establish standard curves (Figure 3.1).

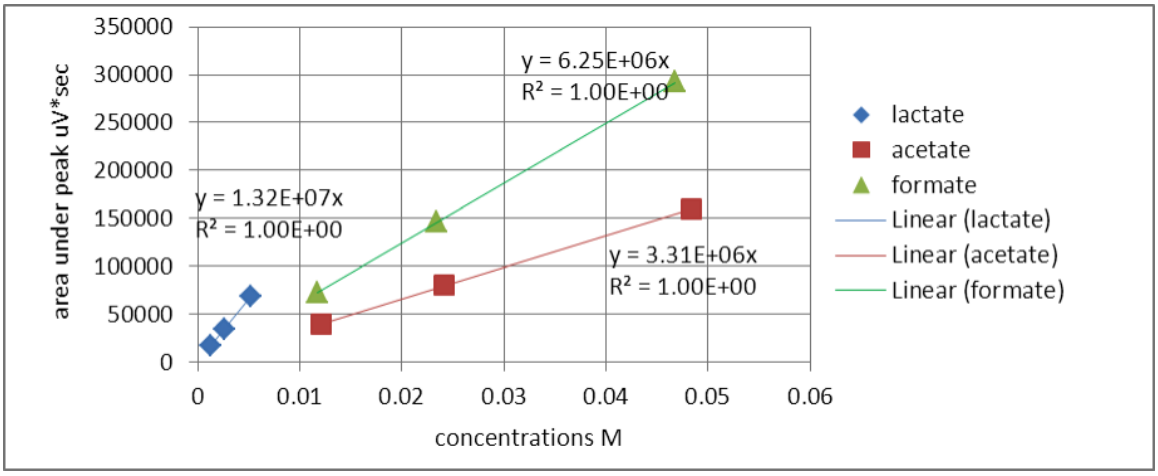

Figure 3.1. Standard curves foracetate, formate, and lactate.

3.3 Results and Discussion

Table3.1 summarizes the various conditions used during the*G. debilis*X*E. coli*conjugation experiments.Plating of *G. debilis* indicated that *G. debilis* was viable after the conjugation incubation at 37^{o}C. The highest plating efficiency was 2.0 x 10^{7} CFU/mL. In the course of the conjugation experiments, it was noticed that sporulation would occur after the conjugation incubation at 37 ^{o}C, if *G. debilis* cells were grown to stationary phase (overnight 18 hours in TGP; OD_{600} = 1.7). Thus, it was hypothesized that successful conjugation is highly depended on the age of the culture (as reflected by the cell density), as older cultures formed spores more readily. However, microscopy of the mating mixtures after conjugation at 37 ^{o}C revealed *G. debilis* cultures grown to cell densities of 0.1 to 0.3 OD_{600}did not contain spores (Figure 3.2A). *G. debilis* was negative for the Gram stain (Figure 3.2B), which is consistent with previous observations (Banat et al., 2004). However, some cells stained faintly compared with the Gram staining of *E. coli* (Figure 3.2B and 3.2C).

In addition to the viability of *G. debilis*, the metabolic activity of *E. coli* S17-1 was also found to be important for successful conjugation. Abundant *E. coli* S17-1 growth could be observed in the mating mixture on the filter paper suspension compared to the control filter paper, which had only a suspension of *G. debilis*. It was found that abundant *E. coli* S17-1 growth occurred in all successful conjugations. The volume of the mating mixture suspension could also influence the outcome of the conjugation experiment. A small volume of mating mixture resulted in a thick suspension with minimal spreading, and had

a dome-shape appearance. In contrast, larger volumes resulted in a thin layer on the filter paper. In conjugation experiments 1, 2 and 3 (Table 3.1), a thin layer of mating mixture was spread on 2 filter papers, and both resulted in overnight colonies on the Tap 6.5 TGP 1.5% agar selective plates. It is speculated that thin layer suspension allowed better oxygen diffusion, resulting in better metabolic activity of the *E. coli* S17-1.This strategy was found successful in contrast with the thick suspension, which never produced colonies on the selective plate. However, putting the mating mixture suspension on 5 filter papers (experiment 5, Table 3.1) versus 3 filter papers (experiment 1,2 and 3) with the same quantity of *G. debilis* bacteria might have made *G. debili s*ex-conjugants less viable, resulting in unsuccessful conjugation.

Also, with the use of a slightly higher amount of LB for the conjugation medium, 2.5% versus 2.0%, the conjugation efficiency was improved several folds. The conjugation experiment repeated twice (experiments 1 and 2, Table.3.1) with the use of 2.0% LB for the conjugation medium resulted in efficiencies about 1 x 10^{-7}number of *G. debilis* trans-conjugants per colony forming unit of the *G. debilis* control. Using the same protocol as experiments 1 and 2, except higher amount of LB 2.5% instead of 2.0%, the conjugation efficiency was significantly higher, 8 x 10^{-7} number of *G. debilis* trans-conjugants per colony forming unit of the *G. debilis* control, performed in a single trial. Thus, these results supported the overall strategy of increasing of *E. coli* S17-1 metabolic activity while maintaining the viability of the *G. debilis*

recipient. Conjugation experiment 3 was the most successful. It resulted in a total of 20 selected trans-conjugant colonies in two days. However, more convincing evidence would require repeated trials with side by side experiments showing the difference of the conjugation efficiencies with the use of 2.5% LB verses 2.0% LB in a systematic way. Also, a systematic approach for optimization of the conjugation efficiency subjected to various conditions had not done.

Table 3.1. Summary of conditions used during the *G. debilis*X *E. coli* conjugation experiments.

Conjugation Experiment	OD_{600} of *G. debilis* culture	Viability of*G. debilis* after conjugation (CFU/ml)	Conjugation medium	Number of filter papers used	Appearance of the suspension of the mating mixture	Efficiency (number of conjugants/CFU)
1,2	0.190, 0.212	2.0×10^{7}, 3.2×10^{7}	2.0% LB Miller formulation (Sigma) / 1.5% agar plate	2	Thin layer	1.0×10^{-7}, 9.5×10^{-8} Avg.=9.8×10^{-8}(a)
3	0.118	5.2×10^{6}	2.5% LB Miller formulation (Sigma) / 1.5% R2A agar plate	3	Thin layer	8×10^{-7}
4	0.204	6.0×10^{8}	2.0% LB Miller formulation (Sigma)/ 1.5% agar plate	2	Thick dome-shape	---
5	0.212	5.6×10^{5}	2.5% LB Miller formulation (Sigma) / 1.5% R2A agar plate	5	Thin layer	---

Notes for (a): the average 9.8×10^{-8} number of conjugants per CFU was obtained from the duplicate experiments of 1 and 2, experiments 3,4 and 5 were performed in a single trial.

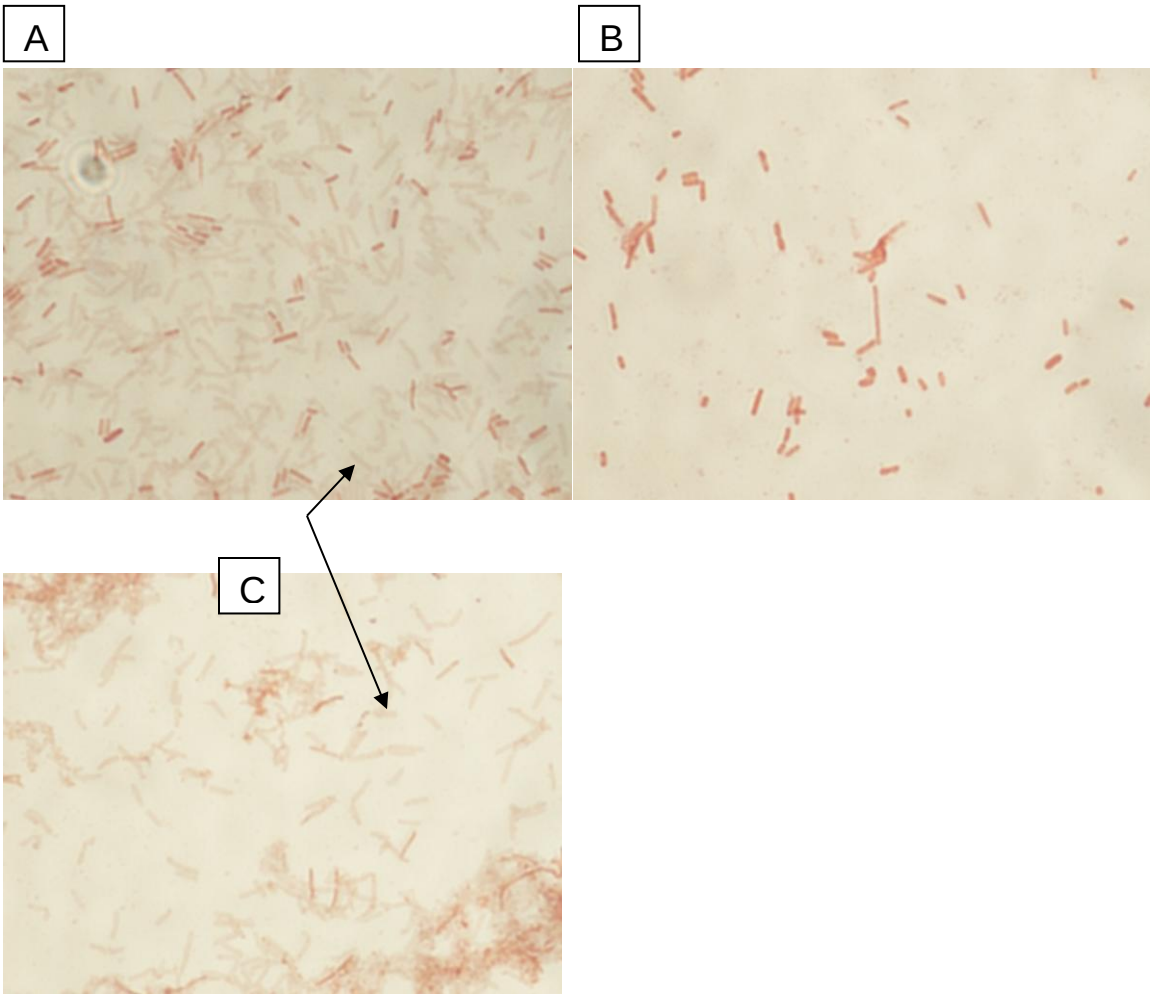

Figure 3.2. Gram straining of the *E. coli* and *G. debilis* mating mixture. A) Gram straining of the mating mixture. The mating mixture was incubated on filter paper for 18 hours 37^{o}C, and then vortexed with 1 mL TGP medium. Two (2) µl of the mating mixture was subjected to the Gram stain; B) Gram staining of *E. coli*; C) *G. debilis* control. The blackarrows point to *G. debilis* faintly stained with the Gram stain.

Plasmids extracted from the trans-conjugants were confirmed to be pNW33N-pxyl-bs2-mob by PvuI digestion (Figure 3.3). Confirmation that the trans-conjugants were indeed *G. debilis* was obtained in two ways. First, intergenic spacer region PCR amplification was used to confirm the trans-conjugants were *G. debilis* species which shown the trans-conjugants had the same intergenic PCR amplified fragment pattern same as the reference *G. debilis*wild-type (Figure 3.4). Second, three trans-conjugants were cultured under aerobic conditions and the resulting media were analysed by HPLC for the presence of organic acids (acetate, formate, and lactate),.HPLC analysis revealed that lactate was not significant, a non-quantifiable trace amount below the detection limit of 0.2mM or absence in their end-product metabolites (Figure 3.5).The overnight cell densities of these cultures were all approximately 0.1 at OD_{600}(Table 3.2). The low OD_{600} and the absence of lactate with the retention time 13.1 ± 0.4 minutes in the HPLC chromatograms of the trans-conjugants end-products (Figure 3.2) supported the assumption that the conjugants were *G.debilis*. Furthermore, the mean acetate: formate molar ratios were 0.45 ± 0.10, which is in agreement with the theoretical *G.debilis* acetate : formate molar ratio of 0.5 (Table 3.2). The absence of lactate and the intergenic spacer region (IGSR) identification data confirmed that the trans-conjugants were indeed *G. debilis*.

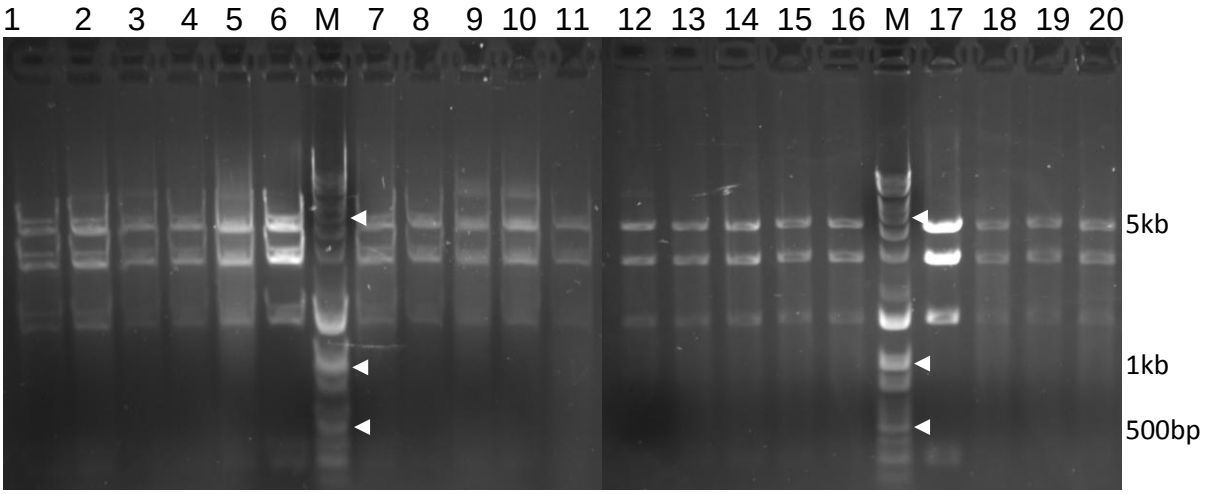

Figure 3.3. Direct confirmation of *G. debilis*(pNW33N-pxyl-bs2-mob) conjugants with PvuI digest of the plasmid preps; 6) and 17) are pNW33N-pxyl-bs2-mob plasmid positive references; 1-5), 7-16) and 18-20) are from *G. debilis* (pNW33N-pxyl-bs2-mob, Tap) single colonies on the selective plates. M, Molecular size marker, 10kb-100bp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

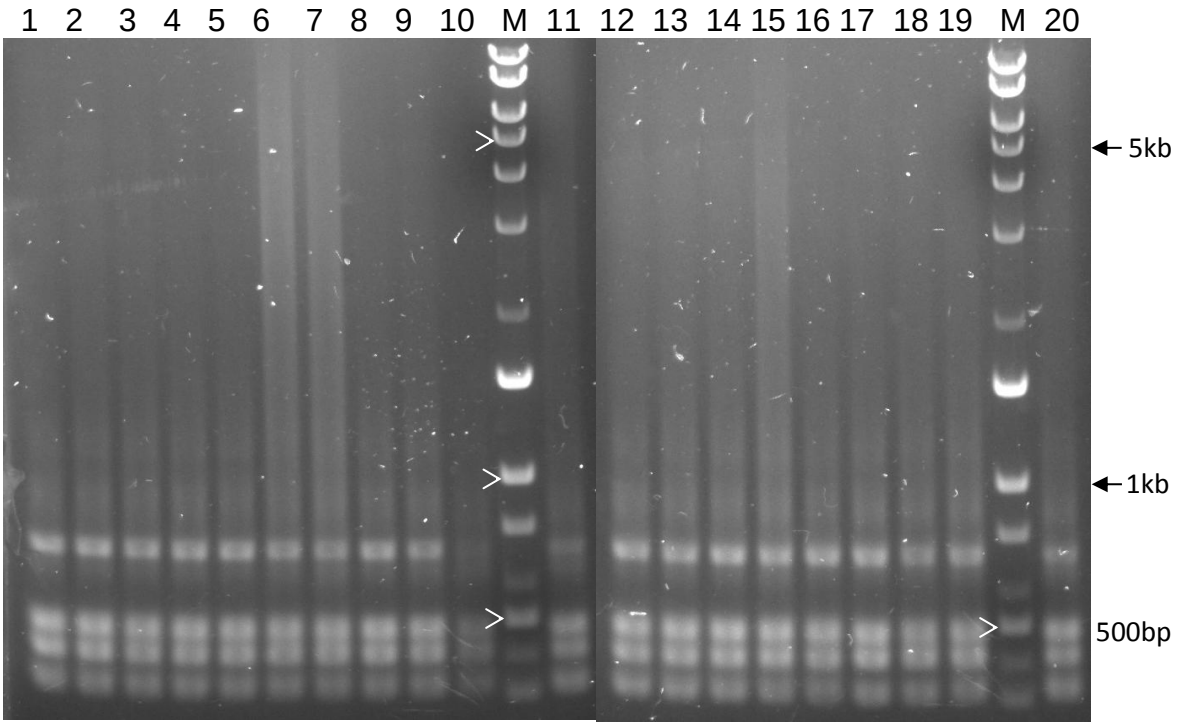

Figure 3.4. 16S Intergenic Spacer Region PCR amplification of *G. debilis*(pNW33N-pxyl-bs2-mob) conjugant genomic preps with the Isr_for and Isr_rev primers.11)and20) are pNW33N-pxyl-bs2-mob plasmid positive references; 1-10) and12-19)are from *G. debilis*(pNW33N-pxyl-bs2-mob, Tap) single colonies on the selective plates. M, Molecular size marker, 10kb-100bp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

Table 3.2. Analysis of the 60 ^{o}C grown *G. debilis* trans-conjugant fermentation end-products by HPLC

Trans-conjugant	Cell density (OD_{600}) at 18 hr	[Acetate] (M)	[Formate] (M)	Acetate :Formate ratio
G. debilis (pNW33N-pxyl-bs2-mob,Spc)_1	0.098	0.01081	0.01946	0.5557
G. debilis (pNW33N-pxyl-bs2-mob, Tap)_3	0.096	0.00651	0.01855	0.3511
G. debilis (pNW33N-pxyl-bs2-mob,Spc)_1 sub-cultured	0.092	0.00752	0.01653	0.4550
			Avg.± dev.	0.45±0.10

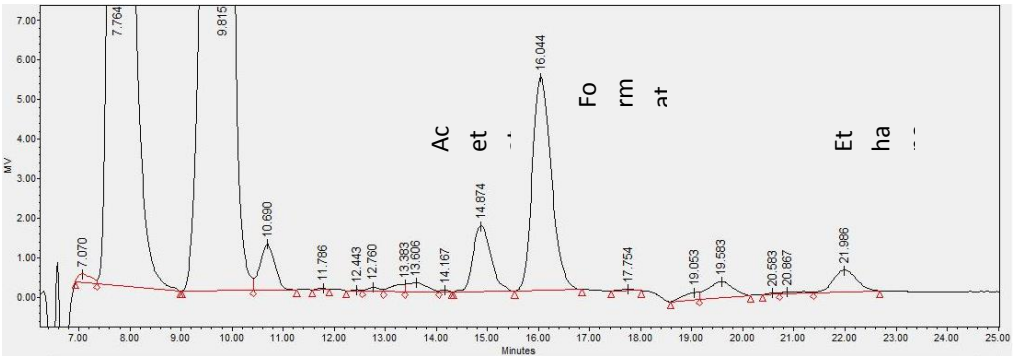

Figure 3.5. HPLC analysis of fermentation end-products synthesized by *G.debilis* X *E.coli*S17-1 (pNW33N-mob-pxyl-bs2, Tap) trans-conjugant 1. These results were representative of all other *G.debilis* conjugants

Chapter 4: *In vivo* stability of pNW33-mob-pxyl-bs2 in the presence of spectinomycin versus thiamphenicol

4.1 Introduction

Thermostable antibiotics and their resistant gene products are essential for transformation selection of thermophiles, since thermophiles live in high temperature environments and selection of recombinant bacteria often requires 1 to 3 days of incubation. Thermo-unstable antibiotics can cause false positive apparent trans-conjugants in the selection process, as they can lose their effectiveness over time. Peteranderl et al.(1990) tested the thermo-stabilities of ampicillin, kanamycin, streptomycin, and neomycin antibiotics using thermophilic clostridia and other indicator strains and found that the effectiveness of ampicillin only lasts 3.3 hours. Antibiotics stabilities were also related to the pH of the medium. Kanamycin and neomycin were stable at 72 ^{o}C and pH 7.3. Also, thiamphenicol is a heat-stable derivative of chloramphenicol (Franciaet al., 2004).

The kanamycin resistant gene from a *Thermus* species, which encodes a thermostable kanamycin nucleotidyl transferase, was utilized to create the shuttle vector, with the *Geobacillus* wild-type pBST1 plasmid, for *Geobacillus* species (Tayloret al.,2008). Other methods such as mutagenesis of antibiotic resistance genes that encode thermo-labile enzymes had been also used to create thermostable antibiotic gene products (Liao et al., 1986).

In this thesis, spectinomycin and thiamphenicol antibiotics are used to select for the *G. debilis* conjugants. The derived pNW33N-pxyl-bs2-mob encodes both the

spectinomycin and thiamphenicol antibiotic resistant genes. The spectinomycin resistance gene, encoding the spectinomycin adenyltransferase, was derived from pSG1190, which originated from pJS2 (Lewis et al, 1999). The thiamphenicol resistant gene encodes the chloramphenicol acetyl transferase, which originated from plasmid pC194 derived from the mesophilic bacterium *Staphylococcus aureus* (Bacillus Genetic Stock Center Catalog of Strains 7th Edition, Volume 3).

In this chapter, the stability of pNW33N-pxyl-bs2-mobin *G. debilis*, subjected to selection with either spectinomycin or thiamphenicol, was tested at 50 ^{o}C.Trans-conjugants selected with spectinomycin are referred to as *G. debilis* (pNW33-mob-pxyl-bs2, Spc), whereas trans-conjugants selected with thiamphenicol are referred to as *G. debilis* (pNW33mob-pxyl-bs2, Tap).

4.2 Methods

4.2.1 Testing the *in vivo*stability of the pNW33N-pxyl-bs2-mob plasmid

E. coli S17-1, containing pNW33N-pxyl-bs2-mob was conjugated with *G. debilis,* as described in Section 3.2.1.Conjugants were selected on TGP agar plates containing 60 g/mL spectinomycin (Sp60) or 6.5 μg/mL thiamphenicol (Tap6.5) and incubated at 50^{o}C. Conjugant colonies were observed on the selective plate within 48 hours. Plasmid DNA were then extracted and further analyzed by back transformation into *E. coli* DH5α, as described in Section 3.2.2, and restriction endonuclease digestion, as described in Section 3.2.3.

4.2.2 Testing the transmissibility of the back transformed plasmids

The transmissibility of the back transformed plasmids were further checked by conjugation of the *E. coli* S17-1 carrying the back transformed plasmid with the *E. coli*

Nx^R strain. Each of the back transformed plasmids was chemically transformed into *E. coli* S17-1. Then, 0.7mL of the overnight *E. coli* S17-1 culture (grown for 18 hours in 2%LB at 37 ^oC) was mixed in a sterile 1.5 mL eppendorf tube, with another 0.7mL of overnight grown *E. coli* Nx^R. Another two portions of each culture (i.e. 1.4mL of the *E. coli* S17-1 culture and 1.4mL of the *E. coli* Nx^R culture) were added to two separate 1.5 mL eppendorf tubes to serve as negative controls. All three eppendorf tubes were centrifuged using a micro-centrifuge at 4,000 x g. The supernatants were discarded and the cell pellets were resuspended in 1.5mL 2%LB, mixed, and centrifuged again. The cell pellets were finally resuspended in 20µl 2%LB, and incubated at 37 ^oC for 4 hours without agitation. After the incubation, 1mL of 2%LB was pipetted into each of the eppendorf tubes and the suspension was mixed. Aliguots (250µl) of each of the bacterial suspensions was plated on a 2%LB 1.5% agar plate containing 25 ug/mL chloramphenicol (Cm25) and 100µg/ml nalidixic acid (Nx100). The plates were then incubated 48 hours at 37^{o}C. Numerous colonies on the plate with both *E. coli* strains and absence of colonies on the plate with the single *E. coli* strain indicated that the back transformed plasmid was transmissible (*mob*$^+$).

4.3 Results and Discussions

4.3.1 Conjugation of *G. debilis* X *E. coli* S17-1 (pNW33-mob-pxyl-bs2) using thiamphenicol selectionselection

G. debilis trans-conjugants selected with thiamphenicol (pNW33N-mob-pxyl-bs2, Tap) and maintained with 6.5 mg/mL thiamphenicol(Tap6.5) at 50 ^oC, yielded plasmid DNA identical to the parental pNW33N-pxyl-bs2-mob plasmid in direct confirmation and in back transformation of the conjugant plasmid. The PvuI digestion patterns of the *G.*

debilis trans-conjugants plasmid extract DNA were identical to pNW33N-pxyl-bs2-mob (Figure 4.1). Back transformation of plasmid DNA extracted from *G. debilis* (pNW33N-pxyl-bs2-mob, Tap) yielded *E. coli* DH5α strains that were all able to grow in LB medium containing 50 mg/mL spectinomycin (Spc50). These results indicated that the pNW33N-mob-pxyl-bs2 plasmid was stable in *G. debilis*cultured at 50 ^{o}C in the presence Tap6.5.

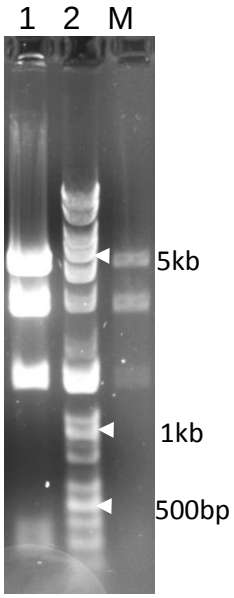

Figure 4.1. PvuI digest of plasmid DNA extracted form *G. debilis* (pNW33N-mob-pxyl-bs2, Tap) conjugants cultured at 50 ^{o}C;1) pNW33N-pxyl-bs2-mob positive reference, PvuI; 2)*G. debilis* (pNW33N-mob-pxyl-bs2, Tap) conjugants. M, molecular size marker, 10kb-100bp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

4.3.2 Conjugation of *G. debilis* X *E. coli* S17-1 (pNW33-mob-pxyl-bs2) using spectinomycin selection

In contrast to the back transformed *E. coli*DH5α derived from *G.debilis*(pNW33N-mob-pxyl-bs2, Tap) trans-conjugants, plasmidDNA recovered from *E. coli* DH5α back transformed with plasmids derived from *G. debilis*(pNW33N-mob-pxyl-bs2, Spc) trans-conjugants, did not produce the same REN patterns as the original pNW33N-pxyl-bs2-mob when cut with PvuI or SacI. PCR amplification with the SacI_PSup202_For and PSup202_SacI_Rev primers, which were used in the original cloning of the *mob* insert, also did not result in amplification of the expected fragment (Figure 4.2). The expected PCR fragment was observed for only one plasmid (Figure 4.2, lane 10). Moreover, all the back transformed *E. coli* DH5α (Figure 4.2, lanes 1 to 9, and 11, derived from isolates S1 to S11), did not grow in LB Sp50 medium. Therefore, the plasmids recovered from the trans-conjugants were not the same as the original pNW33N-pxyl-bs2-mob plasmid used in the transformations.

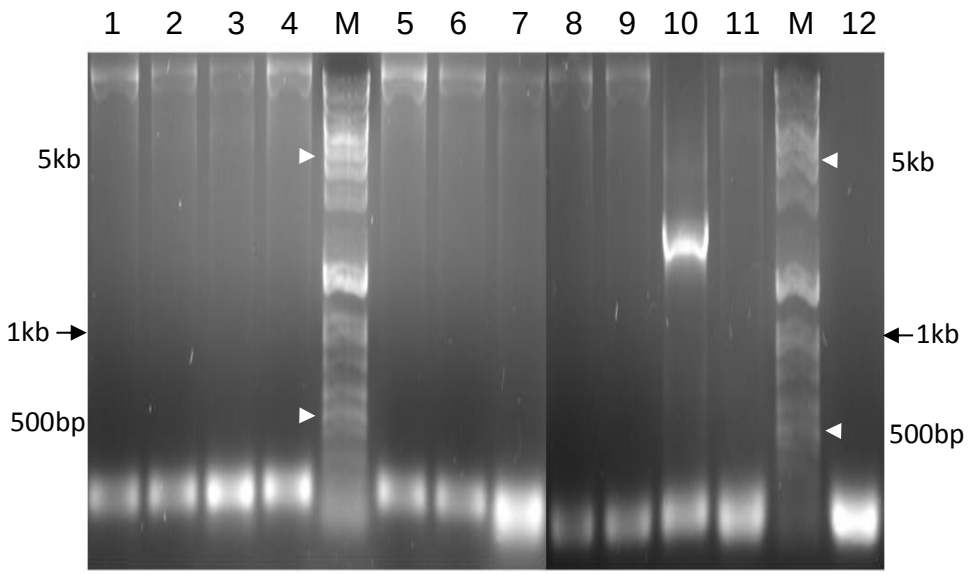

Figure 4.2. PCR amplification with the SacI_PSup202_For and PSup202_SacI_Rev primers using plasmid DNA extracted from *E. coli* DH5α that had been back transformed with *G. debilis*(pNW33-mob-pxyl-bs2, Spc) trans-conjugants. Lanes 1-11) are the back transformed *E. coli* DH5α colonies S1 to S11; lane 12is the PCR positive control. M, Molecular size marker, 10kb-100bp; 10kb, 8kb,6kb,5kb, 4kb,3kb,2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

The plasmid DNA extracted from back transformed *E. coli* DH5α were also analysed by REN digestion with RsaI to determine if they were still intact after conjugation (Figure 4.3). Their REN patterns suggested that there was little variation among them. Only the plasmid S10 had a different pattern, and this plasmid was also the only one that produced a PCR product of the expected size when amplification with the TrSacI_for and TrSacI_rev primers. All REN patterns were different from that of the pNW33N-pxyl-bs2-mob plasmid (Figure 4.3, lane 12).

Despite the difference in the structure of plasmid DNA recovered from *E. coli* DH5α that had been back transformed with *G. debilis* trans-conjugants selected with spectinomycin (pNW33-mob-pxyl-bs2, Spc), the transmissibility and chloramphenicol resistance phenotypes were still present in these plasmids. Selection with spectinomycin resulted in what appears to be either a uniform rearrangement and/or partial loss of the plasmid DNA in the bacteria.

The inability of *E. coli* DH5α that had been back transformed with *G. debilis* (pNW33-mob-pxyl-bs2, Spc) plasmids to grow in Spc50 may be due to the loss or disabling of spectinomycin gene in the plasmid. Thus, plasmid stability can be affected by the use of different antibiotic in the selection process. In contrary, the loss of spectinomycin resistance in the *in vivo G. debilis* (pNW33-mob-pxyl-bs2, Spc) plasmids, supported by theloss of spectinomycin resistance of the S1 to 11 plasmids scavanged by back transformation,did not lead to the cease of growth in further sub-cultures of the *G. debilis*(pNW33N-pxyl-bs2-mob, Spc) trans-conjugant in presence of spectinomycin. The ability of the trans-conjugant to grow in spectinomycin indicated that it was possessing a functional spectinomycin resistant gene.

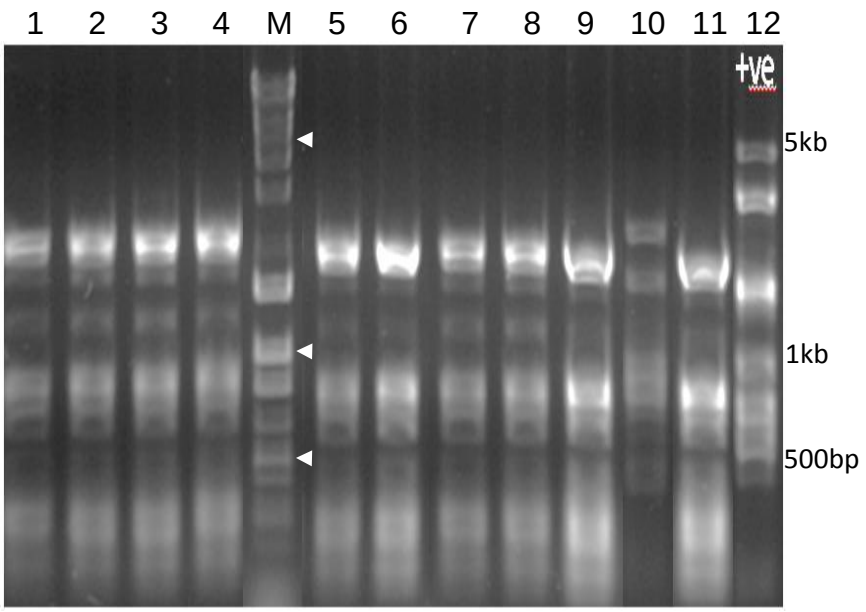

Figure 4.3. RsaI digests of plasmid DNA extracted from *E. coli* DH5α that had been back transformed with *G. debilis* trans-conjugants selected with spectinomycin (pNW33-mob-pxyl-bs2, Spc).Lanes 1-11) are the back transformed *E. coli* DH5α 1 to 11; lane 12 is the pNW33N-pxyl-bs2-mob positive control. M, Molecular size marker, 10kp-100bp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

4.3.3 Conjugation of *G. debilis* X *E.coli* S17-1 (back transformed plasmid) from *G. debilis*(pNW33N-mob-pxyl-bs2, spc)

Plasmids from *E. coli* DH5α back transformantsS1 to S11, derived from *G.debilis* (pNW33N-mob-pxyl-bs2, Spc) trans-conjugants were used in further conjugation experiments with *G. debilis*. They were transformed into *E. coli* S17-1. All *E. coli* S17-1 carrying the back transformed plasmids were able to grow in the presence of chloramphenicol, indicating that they carried the mobilizable back transformed plasmids S1 to 11.The transmissibility of these plasmids was tested by the conjugations with the *E. coli* DH5α Nx^R strain, as described in Section 4.2.2.

E. coli S17-1 carrying the black transformed plasmid S1 (see lane 1 in Figure 4.3) and *E. coli* S17-1 carrying the black transformed plasmid S10 (lane 10 in Figure 4.3) were mated with *G. debilis* in two separate conjugation experiments. Plasmid DNA extracted from the resulting *G. debilis* trans-conjugants were again back transformed into *E. coli* DH5α.

Conjugation of *G. debilis* with *E. coli* S17-1carrying the back transformed plasmid S10, which has a distinct RsaI digest pattern, resulted in *G. debilis* trans-conjugant colonies. Subsequent back transformation to *E. coli* DH5α with Cm25 selection yielded plasmids with the same RsaI digestion pattern as all the other back transformed plasmids (Figure 4.3, lanes 1 to 9, 11), and was different from that of the original S10 plasmid (Figure 4.3, lane 10). In other words, passage of the plasmid through another round of conjugation into *G. debilis*, with subsequent growth at 50 ^oC, resulted in further rearrangement of the plasmid.

Chapter 5: *In vivo* stability of pNW33-mob-pxyl-bs2 at 55 ^{o}C and 60 ^{o}C

5.1 Introduction

Successful culturing of *G. debilis* trans-conjugants at thermophilic temperatures requires expression of functional thermostable antibiotic resistant enzymes. Data presented in Chapter 4 suggest that rearrangements of the pNW33-mob-pxyl-bs2 plasmid occurred in *G. debilis* trans-conjugants cultured at 50 ^{o}C in the presence of spectinomycin, but not in the presence of thiamphenicol. In this chapter, the stability of the pNW33-mob-pxyl-bs2 plasmid DNA in in *G. debilis* trans-conjugants cultured at 55 ^{o}C and 60 ^{o}C in the presence of thiamphenicol was investigated.

5.2 Methods

5.2.1 Testing the *in vivo* stability of the pNW33N-pxyl-bs2-mob plasmid

To investigate the stability of pNW33N-pxyl-bs2-mob, *G. debilis* conjugants selected with thiamphenicol, termed "*G.debilis*(pNW33N-pxyl-bs2-mob, Tap)", that had never been exposed to temperatures greater than 50 ^{o}C, were sub-cultured once, twice, and three times consecutively in the same temperature at 50 ^{o}C, 55 ^{o}C, or 60 ^{o}C. To ensure complete elimination of growth of the *G. debilis* wild-type controls in presence of thiamphenicol in the 1191 medium at elevated temperatures, increasing thiamphenicol concentrations were used at higher temperatures. 50 ^{o}Cgrown *G. debilis* trans-conjugants were cultured in 1191 medium containing 6.5 ug/mL thiamphenicol (Tap6.5); 55 ^{o}C grown *G. debilis* trans-conjugants were cultured in 1191 medium containing

25 ug/mL thiamphenicol (Tap25); and 60 ^{o}Cgrown *G. debilis* trans-conjugants were cultured in 1191 medium containing 125 ug/mL thiamphenicol(Tap125). Then, each of the once, twice, and third sub-cultures grown at 55 ^{o}C or 60 ^{o}C were once again sub-cultured at 50 ^{o}C prior to plasmid extraction and restriction endonuclease (REN) digestion with PvuI, as described in Section 3.2.3. In addition, the third sub-culture at 55 ^{o}C and the third sub-culture at 60 ^{o}C were harvested without the additional sub-culturing at 50 ^{o}C, and plasmid DNA were extracted and subjected to back transformation analysis. Each of the resulting plasmid DNA extracts was back transformed into *E. coli* DH5αas described in Section 3.2.2, and plasmid DNA recovered from the back transformed *E. coli*DH5α were subjected to REN digestion, as described in Section 3.2.3. Unless otherwise stated, the cultures were grown with agitation (shaking) at 70 rpm.

In a separate experiment, plasmid stability at 60 ^{o}C was again tested but done differently with stepwise increasing the sub-culturing temperature. *G. debilis* trans-conjugants that had never been exposed to temperatures greater than 50 ^{o}C, were sub-cultured once, twice, three times, and four times at 55 ^{o}C, and then continually from the forth 55 ^{o}C grown sub-culture further sub-cultured three times consecutively at 60 ^{o}C. The sub-cultures were grown without shaking in 1191 medium containing 25 ug/mL thiamphenicol (Tap25) at 55 ^{o}C, and 125 ug/mlLthiamphenicol (Tap125) at 60 ^{o}C. Each of the sub-cultures grown at 55 ^{o}C or 60 ^{o}C wassubsequently sub-cultured again at 50 ^{o}C prior to

plasmid extraction and restriction endonuclease (REN) digestion as described in Section 3.2.3.

5.3 Results and Discussions

To test the plasmid stability at 50 ^{o}C, the *G. debilis* trans-conjugants were sub-cultured three times consecutively at 50 ^{o}C, starting at an OD_{600} of 0.02 to a final OD_{600} of approximately 0.7. Three consecutive sub-cultures of the trans-conjugants ensured that the pNW33N-pxyl-bs2-mob plasmid had propagated in the *G. debilis* for at least 15 generations. PvuI digestion patterns of pNW33N-pxyl-bs2-mob extracted from *G. debilis* trans-conjugants after three consecutive sub-cultures at 50 ^{o}C, were the same as the pNW33-pxyl-bs2-mob reference plasmid digested with PvuI (Figure 5.1, lanes 6, 7, 8 compared with lane 5). Thus, pNW33-pxyl-bs2-mob stably propagated in *G. debilis* trans-conjugants at 50 ^{o}C, in the presence of 6.5 ug/mL thiamphenicol.

To test the plasmid stability at 55 ^{o}C,the same sub-culturing was done, except that at the end each of the 55 ^{o}C grown sub-cultures was further sub-cultured once at 50^{o}C. PvuI digestion of plasmid DNA extracted from *G. debilis* trans-conjugants that had been sub-cultured one, two and three times at 55 ^{o}C, resulted an REN digestion pattern that was identical to the PvuI digestion pattern of the reference plasmid extracted from *G. debilis* trans-conjugants grown at 50 ^{o}C (Figure 5.2, lanes 2, 3 and 4 compared with lane 1). Thus, pNW33-pxyl-bs2-mob also stably propagated in *G. debilis* trans-conjugants at 55^{o}C, in the presence of 25 ug/mL thiamphenicol.

In contrast to 50^{o}Cand 55 ^{o}C, at 60 ^{o}C temperature, PvuI digestion of plasmid DNA extracted from *G. debilis* trans-conjugants that had been exposed to 60 ^{o}C for one, two and three sub-cultures following the procedure in Section 5.2.1, generated only a single band (Figure 5.3, lanes 6, 7, 8) of approximately 3 Kb. These results suggested that there was only a single PvuI site in these plasmids, resulting in a linearized DNA molecule. The pNW33N-pxyl-bs2-mob plasmid has 8692bp. Thus, the plasmids extracted from*G. debilis* trans-conjugantscultured at 60 ^{o}C appeared to have lost approximately 5.6 kb of DNA.

The plasmids extracted from *G. debilis* trans-conjugants after the third sub-culture at 60 ^{o}C, were back transformed into *E. coli* DH5α,as described in Section 5.2.1. Twenty (20) of these back transformed *E. coli* DH5α colonies (designated pT60-1 to pT60-19) were selected at random and cultured in LB broth containing 25 ug/mL chloramphenicol or 50 ug/mL spectinomycin. None of the back transformed *E. coli* DH5α strains (pT60-1 to pT60-19) were able to grow in LB broth containing 50 ug/mL spectinomycin (Table 5.1). Moreover, plasmids from back transformed *E. coli* DH5α pT60-1, pT60-2, pT60-8 and pT60-19 were found to be non-mobilizable.

PvuI digestions of plasmid DNA extracted from isolatespT60-1 to pT60-19 resulted in REN patterns those were very different from the PvuI digestion pattern of the reference plasmid, and most of them generated a single band (Figure 5.4, lanes 4, 6, 10, 12, 14, 15, 18 and 19) of approximately 3 Kb. The PvuI digestions of the pT60-1, pT60-2, pT60-8, and pT60-19 plasmids had another bandof approximately 1 kb (Figure 5.4, lanes 1, 2, 9, 20) and PvuI digestion of pT60-12produced a band of approximately

1.5 Kb(Figure 5.4, lane 13), this indicated that variation among the rearranged *in vivo* plasmids resulted from growing of the *G. debilis* trans-conjugants at 60 ^{o}C.

In another experiment, the growth temperature of the *G. debilis* trans-conjugants was increased from 50 ^{o}C to 55 ^{o}C and then from 55 ^{o}C to 60^{o}C. This stepwise increase in culturing temperature to 60 ^{o}C also resulted in plasmid rearrangements and PvuI digestions of the plasmid DNA also generated a 3 Kb band (Figure 5.5, lanes 9, 11, 13), as well as an additional band of approximately 4 kb (Figure 5.5, lanes 9, 11, 13) which was absent in PvuI digests of plasmid DNA extracted from *G. debilis* trans-conjugants that were cultured first at 50 ^{o}C and then at 60 ^{o}C (Figure 5.3, lanes 6, 7, 8). The result suggested that there might be a larger size *in vivo* intermediate plasmid involved in the plasmid size reduction process when *G. debilis* trans-conjugants were grown at 60 ^{o}C. In other words, it might take more than a single step to reduce size of the *in vivo* plasmid at 60 ^{o}C.

Table 5.1. Summary of the phenotypes of *E. coli* DH5α back transformed with pNW33N-mob-pxyl-bs2 extracted from *G. debilis* trans-conjugants.

Back transformed plasmids	Phenotypes
S1 to S11	*Cm^+, Spc^-, mob^+*
Tp50-1 to Tp50-19	*Cm^+, Spc^+, mob^+*
Tp60-3 to Tp60-7 **Tp60-1, Tp60-2, Tp60-8, Tp60-19**	*Cm^+, Spc^-, mob^+* *Cm^+, Spc^-, mob^-*

Note: The S1 to S11, Tp50-1 to Tp50-19, and Tp60-1 to Tp60-19 back transformed plasmids were tested for their transmissibility.

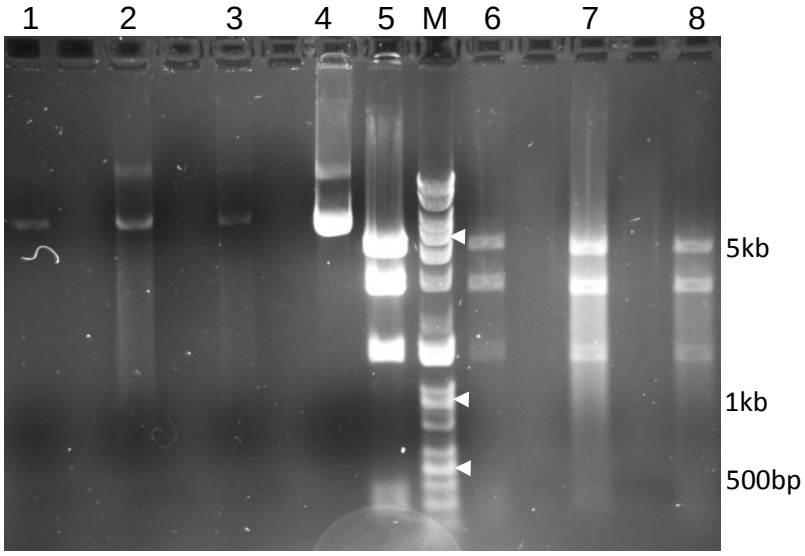

Figure 5.1. PvuI digestion of plasmid DNA extracted from *G. debilis* trans-conjugants containing pNW33N-pxyl-bs2-mob after three consecutive subcultures at 50 ^{o}C. 1) Plasmid DNA extracted after the first sub-culture, undigested (86.28 ng loaded); 2) Plasmid DNA extracted after the second sub-culture, undigested (291.7 ng loaded); 3) Plasmid DNA extracted after the third sub-culture, undigested (122.94 ng loaded); 4) pNW33N-pxyl-bs2-mobDNA, undigested (615.42 ng loaded); 5) pNW33N-pxyl-bs2-mobDNA, PvuI digested (1230.84 ng loaded); 6) Plasmid DNA extracted after first sub-culture, PvuI digested (488.92 ng loaded); 7) Plasmid DNA extracted after second sub-culture, PvuI digested (1020.95 ng loaded); 8) Plasmid DNA extracted after the third sub-culture, PvuI digested (680.0 ng loaded); M, Molecular size marker 10kb-100bp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

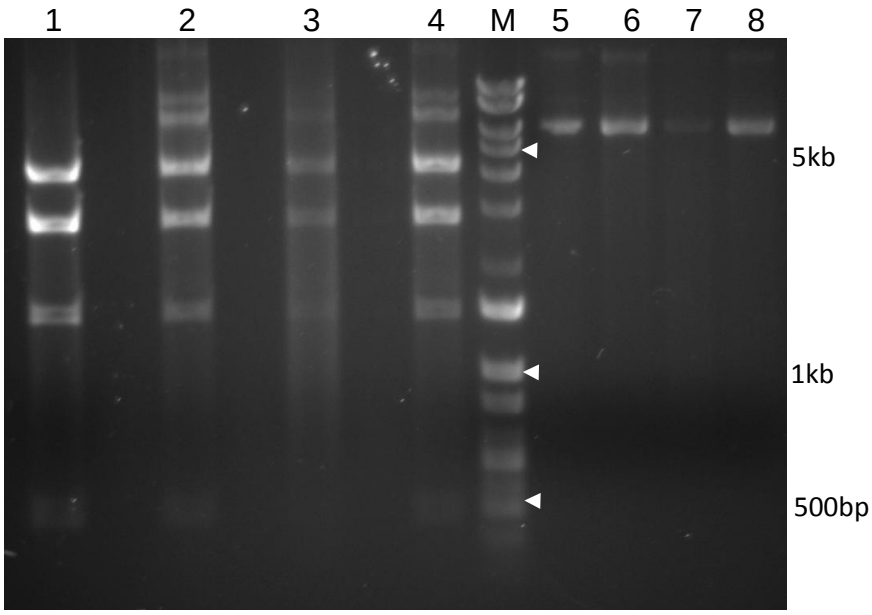

Figure 5.2. PvuI digestion of plasmid DNA extracted from *G. debilis* trans-conjugants containing pNW33N-pxyl-bs2-mob after three consecutive subcultures at 55^{o}C.1) Plasmid DNA extracted of sub-culture at 50 ^{o}C, PvuI digest;2) Plasmid DNA extracted after the one sub-culture at 55 ^{o}C then 50 ^{o}C, PvuI digested; 3) Plasmid DNA extracted after the two sub-culture at 55 ^{o}C then 50 ^{o}C, PvuI digested; 4) Plasmid DNA extracted after three sub-cultures at 55 ^{o}C then 50 ^{o}C, PvuI digested; 5) Plasmid DNA extracted of sub-culture at 50 ^{o}C, undigested; 6)Plasmid DNA extracted after one sub-culture at 50 ^{o}C, then 50 ^{o}C, undigested;7) Plasmid DNA extracted after the two sub-cultures at 55 ^{o}C then 50 ^{o}C, undigested; 8) Plasmid DNA extracted after the three sub-cultures at 55 ^{o}C then 50 ^{o}C undigested; quantities of plasmid DNA extract are normalized to 1200ng, M, Molecular size marker, 10kb-100bp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

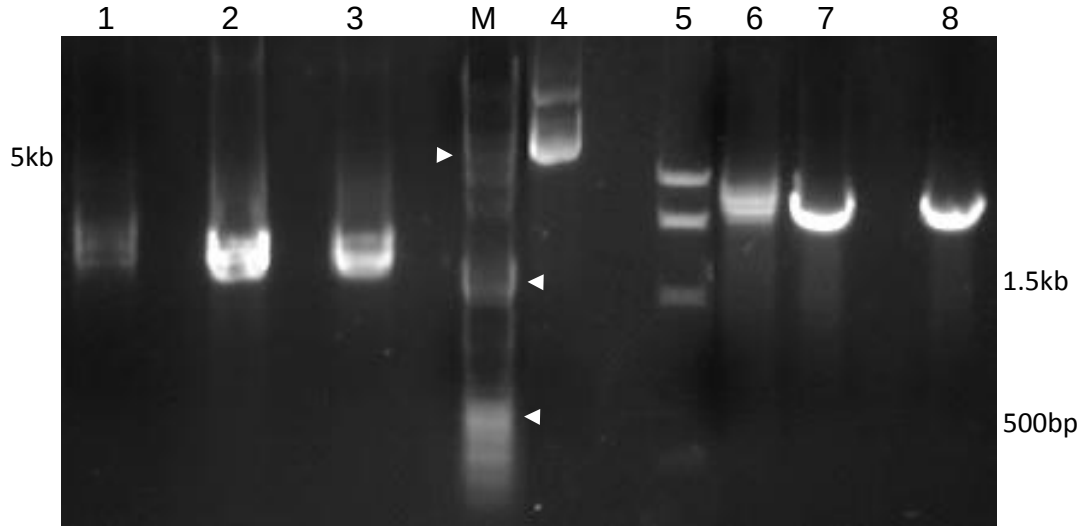

Figure 5.3. PvuI digestion of plasmid DNA from *G. debilis* trans-conjugants containing pNW33N-mob-pxyl-bs2 after one, two, or three sub-cultures at 60 ^{o}C.1) Plasmid DNA extracted after one sub-culture at 60 ^{o}C, undigested; 2) Plasmid DNA extracted after two sub-cultures at 60 ^{o}C, undigested; 3) Plasmid DNA extracted after three sub-cultures at 60 ^{o}C, undigested; 4) pNW33N-mob-pxyl-bs2 reference, undigested; 5) pNW33N-mob-pxyl-bs2 reference, PvuI digested; 6) Plasmid DNA extracted after one sub-culture at 60 ^{o}C, undigested; PvuI digested; 7) Plasmid DNA extracted after two sub-cultures at 60 ^{o}C,PvuI digested; 8) Plasmid DNA extracted after three sub-cultures at 60 ^{o}C, PvuI digested; M, Molecular size marker, 10kb-100bp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

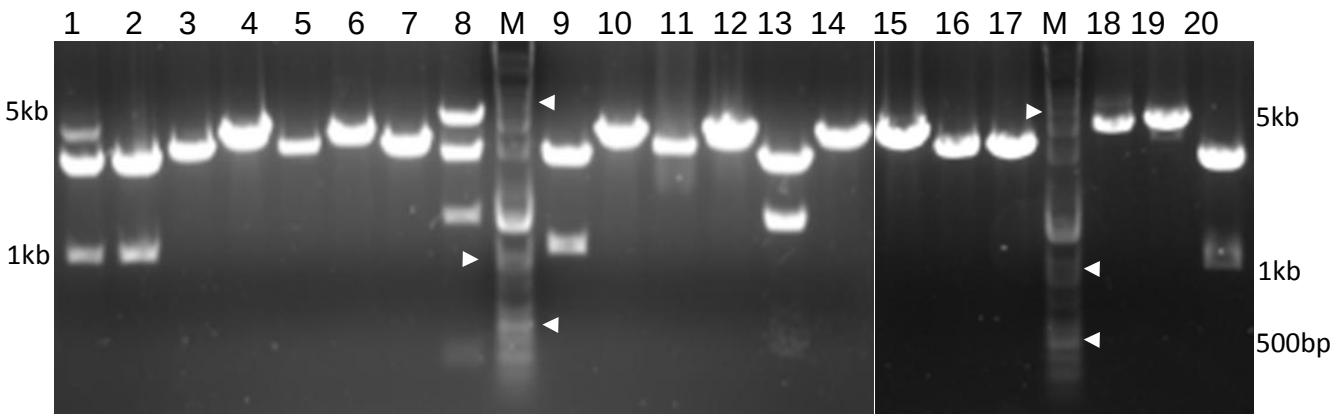

Figure 5.4. PvuI digestion of plasmid DNA extracted from *E. coli* DH5α back transformed with pNW33N-mob-pxyl-bs2 extracted from *G. debilis* trans-conjugants sub-cultured three times at 60 ^{o}C. Lanes 8) PvuI digested pNW33N-mob-pxyl-bs2 DNA, positive control; Lanes 1-7) and 9-20) PvuI digested plasmids extracted from *E. coli* DH5α back transformed with plasmid DNA extracted from *G. debilis* trans-conjugants, Tp60-1 to 7 and Tp60-8 to 19; M, Molecular size marker, 10kb-100bp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp, 100bp. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

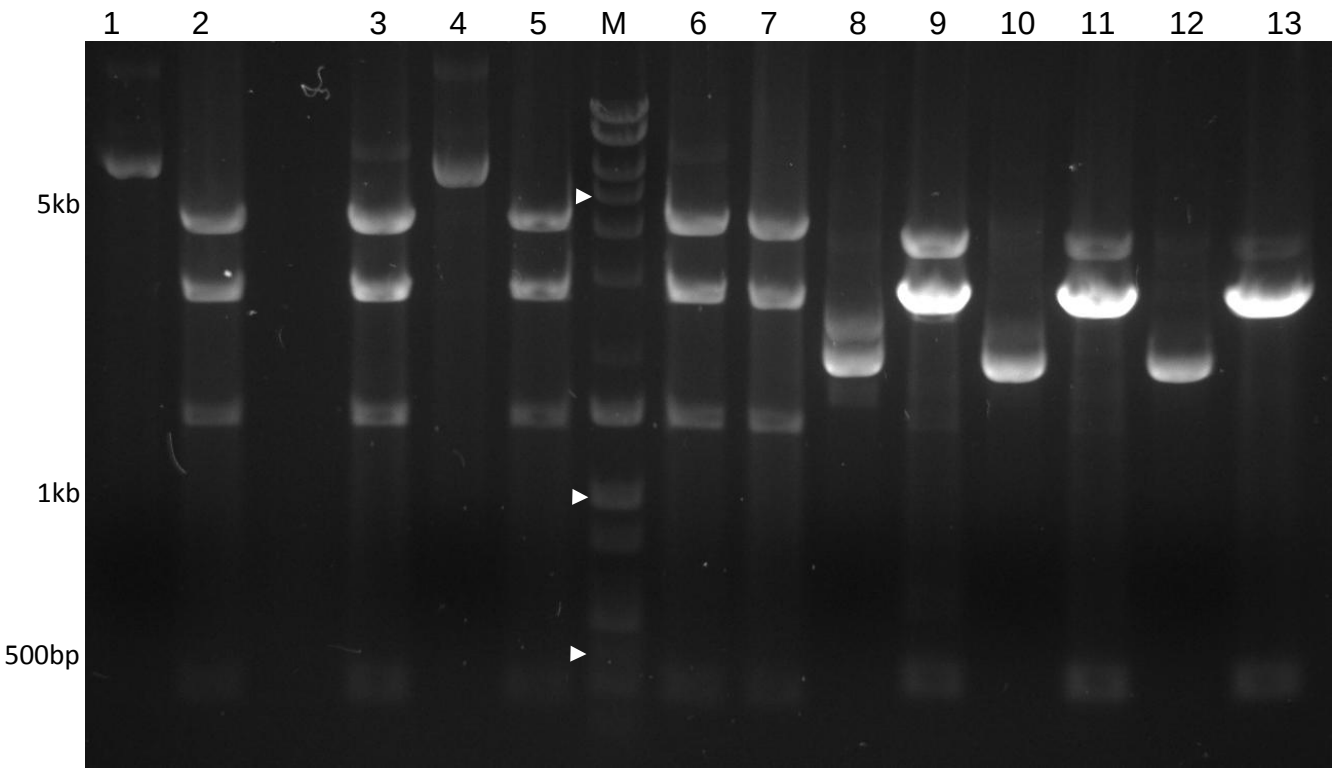

Figure 5.5. PvuI digestion of plasmid DNA from *G. debilis* trans-conjugants containing pNW33N-mob-pxyl-bs2 after one, two, three, or four sub-cultures at 55 ^{o}C without shaking, followed by three consecutive sub-cultures at 60 ^{o}C without shaking.1) Plasmid DNA extracted after one sub-culture at 50 ^{o}C, undigested; 2) Plasmid DNA extracted after one sub-culture at 50 ^{o}C, PvuI digested; 3) Plasmid DNA extracted after one sub-culture at 55^{o}C, PvuIdigested; 4) Plasmid DNA extracted after four sub-cultures at 55 ^{o}C, undigested; 5) Plasmid DNA extracted after two sub-cultures at 55^{o}C, PvuIdigested; 6) Plasmid DNA extracted after three sub-cultures at 55^{o}C, PvuIdigested; 7) Plasmid DNA extracted after four sub-cultures at 55^{o}C, PvuIdigested; 8) Plasmid DNA extracted after four sub-cultures at 55 ^{o}C, followed by one sub-culture at 60 ^{o}C, undigested; 9) Plasmid DNA extracted after four sub-cultures at 55 ^{o}C, followed by one sub-culture at 60 ^{o}C, PvuI digested;10) Plasmid DNA extracted after four sub-cultures at 55 ^{o}C, then two sub-culture at 60 ^{o}C, undigested;11) Plasmid DNA extracted after four sub-cultures at 55 ^{o}C, followed by two sub-culture at 60 ^{o}C, PvuI digested;12) Plasmid DNA extracted after four sub-cultures at 55 ^{o}C, followed by three sub-cultures at 60 ^{o}C, undigested;13) Plasmid DNA extracted after four sub-cultures at 55 ^{o}C, followed by three sub-cultures at 60 ^{o}C, PvuI digested; M, Molecular size marker, 10kb-100bp; 10kb, 8kb, 6kb, 5kb, 4kb, 3kb, 2kb, 1500bp, 1000bp, 800bp, 600bp, 500bp, 400bp, 300bp, 200bp 100bp. The amount of plasmid DNA subjected to PvuI digestions were normalized to 1200ng for all plasmid extracts. Bands for molecular sizes, 5kb, 1kb and 500bp of the ladder are indicated with arrows.

Chapter 6: Summary, Conclusions, and Future Work

6.1 Summary and Conclusions

A transformation system for *Geobacillus debilis*, a facultative anaerobic, thermophilic bacterium that grows in wide range of temperatures up to 60 ^{o}C, was developed. Using the inter-species bi-parental conjugation method, the plasmid pNW33-mob-pxyl-bs2, which possesses the *oriT* site of the RP4 conjugative plasmid, wassuccessfully transferred from *E. coli* S17-1 into *G. debilis*.

Factors such as the cell density (OD_{600}) of the trans-conjugant culture and cell density on the filter paper in the conjugation process were found to affect the outcome of the conjugation. *G. debilis* grown to 0.1 to 0.3 OD_{600} before harvest for conjugation, and were not found to contain spores after18hours in the presence of *E. coli* during the conjugation incubation at 37 ^{o}C. Also, conjugation attempts using concentrated cell suspensions as a droplet on filter paper were unsuccessful, however, spreading the mating mixture as a thin layer of cells on the filter paper was typically successful. Different results with respect to the *in vivo* plasmid stability were obtained with the use of thiamphenicol and spectinomycin. The use of spectinomycin for selection and maintenance ofthe plasmid in sub-cultures resulted in the formation of a rearranged plasmid different from of the starting plasmid, pNW33N-pxyl-bs2-mob. In contrast, if the instability of plasmid due to high temperature occurred at 60^{o}C is disregarded, the use of thiamphenicol for selection and in sub-cultures resulted in stable, non-rearranged plasmids which conveyed the *spc*+ phenotype when back transformed into *E. coli* DH5α.

The thermo-stability of the pNW33-mob-pxyl-bs2 plasmid was investigated using direct confirmation by restriction endonuclease digestion of transformed *G. debilis* plasmids and back transformation of the *G. debilis* trans-conjugant plasmid DNA into *E. coli* DH5α.At 50 ^{o}C and 55 ^{o}C, the *in vivoG. debilis* plasmids are found to be identical to the parental pNW33-mob-pxyl-bs2 plasmid, indicated by restriction endonuclease digestion with PvuI. However severe changes in plasmid size were observed when *G. debilis* trans-conjugants were cultured at 60 ^{o}C. The size reduction of the *in vivo* plasmid at 60 ^{o}C seemed to be a stepwise process involved immediate plasmid with size larger than the final 3kb of the 60 ^{o}C rearranged *in vivo* plasmids.

In summary, it is found that *in vivo* plasmid stability was affected by both the use of different antibiotics for selection of recombinants and the growth temperature of the trans-conjugate.

6.2 Future work

Now that a genetic system for transformation of *G. debilis* has been demonstrated, the conjugation system described in this thesis could be used to develop recombinant strains of *G. debilis* for enhanced production of ethanol and/or other value-added products. However, further work on the nature of the plasmid rearrangements resulting from selection and culturing of*G. debilis* trans-conjugants in the presence of spectinomycin and the size reduction of the *in vivo* plasmid due to elevated temperatures should be conducted. In the presence findings, it is hard to address whether or not that the spectinomycin resistant gene and/or its gene product had any influence to the plasmid instability at 60 ^{o}C, it might be good to avoid using this marker in future construct designs. DNA sequence analyses of the 60 ^{o}C size-reduced plasmids

would reveal the exact size and location of the deletions observed, which may in turn reveal the mechanisms of the instability.

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